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

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Verena Mündler, BSc

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Assoz. Prof. Univ.-Doz. Mag. Julia Walochnik, PhD
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.

*Acanthamoeba*ae 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).

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 (MÉRGERYAN 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 MÉRGERYAN 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.](image)

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 (WEIS-MAN 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.](image)

At the beginning of encystment, vesicles containing fibrillar material are trans-
ported 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 lami-
nar, fibrous layer, the inner endocyst is made of fine fibrils. Interestingly, cellu-
lose 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-specific protein-21 is hydro-

- 5 -
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
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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 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

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

major function of the nucleus is to enclosure 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-
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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 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 predominate conditions. For this reason, acanthamoebae contain a large number of mitochondria enabling the organisms to produce the necessary energy to fulfill 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 synthesize 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 amoebeae. 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).

*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.
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 (HOLD-EN 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).

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, phagocytopsis, proteases. On the other hand there are indirect factors as shown in figure 8 (LORENZO-MORALES et al 2015).
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Diagnosis can be especially difficult due to the un specific 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>
<thead>
<tr>
<th>Acanthamoeba keratitis (AK)</th>
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<tbody>
<tr>
<td>Conjunctivitis</td>
</tr>
<tr>
<td>Corneal ulceration</td>
</tr>
<tr>
<td>Conjunctival hyperaemia</td>
</tr>
<tr>
<td>Lid oedema</td>
</tr>
<tr>
<td>Epithelial defects</td>
</tr>
<tr>
<td>Considerable productions of tears</td>
</tr>
<tr>
<td>Photophobia</td>
</tr>
<tr>
<td>Severe pain</td>
</tr>
<tr>
<td>Stromal abscesses</td>
</tr>
<tr>
<td>Corneal ring stromal infiltrate</td>
</tr>
<tr>
<td>Increased intraocular pressures</td>
</tr>
<tr>
<td>Hypopyon</td>
</tr>
<tr>
<td>Formation of cataract</td>
</tr>
</tbody>
</table>

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

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 rena-
cent 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 reser-
voir (COHEN et al 1987).

1.1.8.2. Cutaneous acanthamoebiasis
This disease particularly occurs in immunosuppressed people and the first de-
scribed 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 ab-
dominal skin (NASSE 1892).

1.1.8.2.1. Disease pattern
Acanthamoebae are the causative agents of nasopharyngeal and cutaneous in-
fec tions. As shown in figure 11 this disease is characterized by skin ulcerations
and nodules. As described by CHAN-
DRASEKAR et al in 1997, in immuno-
competent people cutaneous acan-
thamoebiasis 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

\[ \text{Figure11. Cutaneous acanthamoebiasis in an immunocompromised patient (STEINBERG et al 2002).} \]
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 histopathological aspects this process is mainly characterized by granulomas and parenchymal necrosis (ZAMORA et al 2014).

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

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**Figure 12.** Lesions (white areas) in a human brain due to GAE infection. caused by *Acanthamoeba* spp. (THAMTAM et al 2016).

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

<table>
<thead>
<tr>
<th><strong>Acanthamoeba Granulomatous encephalitis (GAE)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Headache</td>
</tr>
<tr>
<td>Fever</td>
</tr>
<tr>
<td>Sleep disturbances</td>
</tr>
<tr>
<td>Hemiparesis</td>
</tr>
<tr>
<td>Change of mental status</td>
</tr>
<tr>
<td>Visual disturbances</td>
</tr>
<tr>
<td>Ataxia</td>
</tr>
<tr>
<td>Nausea and vomiting</td>
</tr>
<tr>
<td>Hallucinations</td>
</tr>
<tr>
<td>Change in personality</td>
</tr>
<tr>
<td>Photophobia</td>
</tr>
<tr>
<td>Seizures</td>
</tr>
<tr>
<td>Coma</td>
</tr>
</tbody>
</table>
Introduction

symptoms and the lack of awareness among clinicians this often remains undiagnosted (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 moderately 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 underlines the need for more studies to fully understand the epidemiology of this infection. It is still uncertain how many people are asymptomatic while being seropositive. The individuals may harbour and develop disease in case of immunosuppression. This can be observed with the US veterans 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).

![Figure 14. Correlation of rain fall and cases of melioidosis in Darwin, Australia (PARAMESWARAN *et al* 2012).](image)
Introduction

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), chronic lung diseases (12-27% of patients) and chronic 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 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 LIMMATHUROTSAKUL 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 frequently 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).

Interestingly, in Thailand melioidosis is the third most common infectious disease resulting in death (LIMMATHUROTSAKUL 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 (LIMMATHUROTSUKUL 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 re-
lapse. 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 medica-
tion for melioidosis cases in Thailand.

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

<table>
<thead>
<tr>
<th>APPLICATION</th>
<th>AGENT</th>
<th>DOSIS</th>
<th>ROUTE</th>
<th>FREQUENCY</th>
<th>DURATION</th>
<th>VARIATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within 24 h of highly probable exposure</td>
<td>Trimethoprim-sulpha-methoxazole</td>
<td>320:1600 mg</td>
<td>p.o.</td>
<td>12 hourly</td>
<td>3 weeks</td>
<td>Amoxicillin/clavulanic acid if allergy is existent to recommended antibiotic</td>
</tr>
</tbody>
</table>
**Phase 1: Acute & severe infections, induction stage**

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

**Phase 2: Eradication stage**

<table>
<thead>
<tr>
<th>APPLICATION</th>
<th>AGENT</th>
<th>DOSIS</th>
<th>ROUTE</th>
<th>FREQUENCY</th>
<th>DURATION</th>
<th>VARIATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 of, the named ones, after phase 1 or in case of a superficial infection used as phase 1 treatment</td>
<td>Trimethoprim-Sulphamethoxazole</td>
<td>320:1600 mg</td>
<td>p.o.</td>
<td>12 hourly</td>
<td>≥ 3 months</td>
<td>According to antibiotic susceptibility</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>100 mg</td>
<td>p.o.</td>
<td>12 hourly</td>
<td>≥ 3 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amoxicillin/ clavulanic acid</td>
<td>500/125 mg</td>
<td>p.o.</td>
<td>8 hourly</td>
<td>≥ 3 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folic acid</td>
<td>5 mg</td>
<td>p.o.</td>
<td>daily</td>
<td>≥ 3 months</td>
<td>With trimethoprim-sulphamethoxazole</td>
<td></td>
</tr>
</tbody>
</table>
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.

![Predicted global spread of melioidosis](image)

*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 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 18. Different colony morphologies of B. pseudomallei (CHANTRATITA et al 2007).](image-url)
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 (WUTHEIEKANUN 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 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.

In \textit{B. pseudomallei}, five different quorum sensing proteins are found; five LuxR as well as three LuxI homologues. When knock-out 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 \textit{Salmonella enterica serovar Typhimurium} (ATTREE et al 2001) and another one is comparable to \textit{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 \textit{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 downregulation 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-

---

**Figure 22.** Type III secretion system found in several gram-negative bacteria (DIEPOLD *et al* 2014).
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 Th1 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 pneumophila*, 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 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 \textit{B. pseudomallei} in the human body

Since the early 90ies it is well accepted that \textit{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, \textit{B. pseudomallei} had to acquire several routes for intracellular survival (JONES \textit{et al} 1996).

1.3.1.1. Adhesion to host cells

The molecular mechanisms needed for adhesion of \textit{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 \textit{B. pseudomallei} but not as good to the non-pathogenic \textit{B. thailandensis} (KESPICHAYAWAT-TANA \textit{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 \textit{et al} 2001).

\textbf{Figure 25.} Intracellular life of \textit{Burkholderia pseudomallei} with all important factors and genes (ALLWOOD \textit{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 (WINSTANLEY 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.
Introduction

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 bsaZ 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 bsaZ 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 nitric 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 suppressing 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.

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).

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 \textit{B. pseudomallei} and mammalian macrophages has to be proven. One could assume that similar to \textit{L. pneumophila} also \textit{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. \textit{Acanthamoeba} spp. as virtual training grounds

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

Several studies have shown that \textit{L. pneumophila} grown inside of amoebae is highly resistant to unfavourable environmental conditions as fluctuating temperatures, pH and osmolarity (ABU KWAIK \textit{et al} 1997). Furthermore, passaged bacteria are also able to survive biocides and chemical disinfectants. Next to that, \textit{L. pneumophila} is also able to survive in amoebal cysts and this leads to resistance to biochemical and physical agents (BARKER \textit{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 \textit{Acanthamoeba} strains, the trophozoites and cysts have to be transferred to fresh agar plates to the end of getting clonal colonies. However, \textit{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.

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.

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

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5 g bacteriological agar</td>
<td></td>
</tr>
<tr>
<td>475 mL MiliQ H₂O</td>
<td></td>
</tr>
<tr>
<td>5 mL NEFF</td>
<td></td>
</tr>
<tr>
<td>5 mL CaCl₂ x 2 H₂O</td>
<td></td>
</tr>
<tr>
<td>5 mL NaCl</td>
<td></td>
</tr>
<tr>
<td>5 mL KH₂PO₄</td>
<td></td>
</tr>
<tr>
<td>5 mL MgSO₄ x 7 H₂O</td>
<td></td>
</tr>
<tr>
<td>5 mL Na₂HPO₄</td>
<td></td>
</tr>
</tbody>
</table>

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 acanthamoe-bae 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.
endocyst \textit{(end)} more or less star-shaped, ectocyst \textit{(ect)} smooth or slightly wrinkled & at least as thick as the \textit{end}; outer membrane is clearly separated; opercula are not inside of slots; Average diameter of cyst is 18 μm

**Morphological group I**

- **end** not star-shaped or if so, the end of the arms are in slots of the \textit{ect}
  - not more than 5 \textit{end} arms; proliferation at 38°C
    - \textit{Acanthamoeba tubiashi}

- more than 5 \textit{end} arms, no proliferation at 37°C
  - up to 9 arms which all connect with the \textit{ect} on the same level
    - \textit{Acanthamoeba astonyxis}
  - up to 14 arms which connect with the \textit{ect} on different levels
    - \textit{Acanthamoeba comandoni}

**Figure 30.** Identification key for morphological group I.
**Material and Methods**

**End** is usually polyetrical-, star- or egg-shaped; **Ect** is distinct and visible, sometimes as thick as the **end**; Average cyst diameter is less than 18 μm.

**Acanthamoeba lugdunensis**
- **Ect** is keeping its shape and is not in close contact with the **end**.
- In average 7 **end** arms.
- **Ect** and **end** typically polyetrical or spheroidal and are in close contact; arms are broad.

**Acanthamoeba castellanii**
- **End** is more or less egg-shaped and slightly conical.
- **Ect** shows conical or tubular **end** arms, sometimes they are also star- or gearwheel-shaped.

**Acanthamoeba rhysodes**
- **Ect** sometimes is star-shaped and in average 6 armes or opercula are found; proliferation in bacterial cultures 37°C cannot be detected.

**Acanthamoeba griffini**

**Acanthamoeba mauritaniensis**
- Proliferation in bacterial cultures 37°C cannot be detected.

**Acanthamoeba polyphaga**
- **Ect** never shows a net-structure.

**Acanthamoeba divionensis**
- Proliferation in bacterial cultures at 37°C but not at 40°C.

**Acanthamoeba hatchetti**
- **Ect** can be smooth and far apart from **end** which is roundish or egg-shaped; 5 or 6 armes can be visible.

**Acanthamoeba triangularis**
- Proliferation at 40°C.

**Acanthamoeba griffini**

**Figure 31.** Identification key for morphological group II.
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.

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).
Material and Methods

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Approximate location</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1fw (=Rhiz I)</td>
<td>624-642</td>
<td>5'-CAAGTCTGGTGCCAGCAGC-3'</td>
</tr>
<tr>
<td>P1rev (=Rhiz II)</td>
<td>642-624</td>
<td>5'-GCTGCTGGCACCAGACCTTG-3'</td>
</tr>
<tr>
<td>P2fw (=Rhiz 1)</td>
<td>1265-1280</td>
<td>5'-GATCAGATACGGTCGTAGTC-3'</td>
</tr>
<tr>
<td>P2rev (=Rhiz 2)</td>
<td>1280-1265</td>
<td>5'-GACTACGACGGTATCTGATC-3'</td>
</tr>
<tr>
<td>P3fw (=Rhiz 3)</td>
<td>1871-1852</td>
<td>5'-CAGGTCTGTGATGCCCTTCTAG-3'</td>
</tr>
<tr>
<td>P3rev (=Rhiz II)</td>
<td>1852-1871</td>
<td>5'-CTAAGGGCATCACAGACTCTG-3'</td>
</tr>
<tr>
<td>ACfw</td>
<td>1032-1052</td>
<td>5'-TGCCACCCAATACATTAGCAT-3'</td>
</tr>
<tr>
<td>ACrev</td>
<td>1370-1390</td>
<td>5'-ACAAGCTGCTAGGGGAGTCA-3'</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th></th>
<th>Gastb56</th>
<th>Gastb58</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min °C</td>
<td>min °C</td>
</tr>
<tr>
<td>15</td>
<td>95</td>
<td>15</td>
</tr>
<tr>
<td>1</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>2</td>
<td>56</td>
<td>58</td>
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<td>3</td>
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<td>72</td>
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<td>7</td>
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<td>72</td>
</tr>
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<td>∞</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>30 x</td>
<td>30 x</td>
<td></td>
</tr>
<tr>
<td>Rhiz primers</td>
<td>Rhiz primers</td>
<td></td>
</tr>
</tbody>
</table>
Material and Methods

Table 8. PCR programme used for AC primers

<table>
<thead>
<tr>
<th>AC</th>
<th>min</th>
<th>ºC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>95</td>
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<tr>
<td></td>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>∞</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>35 x</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Mastermix</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 μL dH₂O</td>
</tr>
<tr>
<td>5 μL buffer</td>
</tr>
<tr>
<td>5 μL MgCl₂</td>
</tr>
<tr>
<td>5 μL primer forward (10 pmol)</td>
</tr>
<tr>
<td>5 μL primer reverse (10 pmol)</td>
</tr>
<tr>
<td>1 μL dNTPs</td>
</tr>
<tr>
<td>0,25 μL polymerase</td>
</tr>
<tr>
<td>31,25 μL per tube</td>
</tr>
</tbody>
</table>

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.
Material and Methods

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

<table>
<thead>
<tr>
<th>small 1 x TAE agarose gel (10 slots)</th>
<th>big 1 x TAE agarose gel (20 slots)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 g agarose</td>
<td>2 g agarose</td>
</tr>
<tr>
<td>2 μL GelRed™</td>
<td>4 μL GelRed™</td>
</tr>
<tr>
<td>fill up to 50 mL with TAE buffer</td>
<td>fill up to 100 mL with TAE buffer</td>
</tr>
</tbody>
</table>

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.

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.
Material and Methods

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.
Material and Methods

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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABI Mix</td>
<td>1 μL</td>
</tr>
<tr>
<td>PCR Product</td>
<td>1-3 μL</td>
</tr>
<tr>
<td>primer</td>
<td>2 μL</td>
</tr>
<tr>
<td>sequencing buffer (AB Big Day Terminator)</td>
<td>1 μL</td>
</tr>
<tr>
<td>sterile water</td>
<td>fill up to 10 μL</td>
</tr>
</tbody>
</table>
Material and Methods

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.

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).

### Table 12. Programme used for Sequencing PCR.

<table>
<thead>
<tr>
<th>min</th>
<th>°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>96</td>
</tr>
<tr>
<td>10</td>
<td>96</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
</tr>
<tr>
<td>∞</td>
<td>4</td>
</tr>
</tbody>
</table>

40 x

same primers as with PCR reaction

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

<table>
<thead>
<tr>
<th>Buffer Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μL of Natrium-Acetate buffer</td>
</tr>
<tr>
<td>10 μL of DNA sample from Sequencing PCR</td>
</tr>
<tr>
<td>33 μL ice-cold 100% ethanol</td>
</tr>
<tr>
<td>44 μl sample mix</td>
</tr>
</tbody>
</table>
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.

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.

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

<table>
<thead>
<tr>
<th>IUPAC nucleotide code</th>
<th>base</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>A or G</td>
</tr>
<tr>
<td>Y</td>
<td>C or T</td>
</tr>
<tr>
<td>S</td>
<td>G or C</td>
</tr>
<tr>
<td>W</td>
<td>A or T</td>
</tr>
<tr>
<td>K</td>
<td>G or T</td>
</tr>
<tr>
<td>M</td>
<td>A or C</td>
</tr>
</tbody>
</table>
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).

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 processed 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 sample sequences (S17.3cons & S14.3cons) 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 other (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](image)

*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 Chart]

*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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Strain</th>
<th>Morphotype</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>S.1</td>
<td>III</td>
<td>T5</td>
</tr>
<tr>
<td>S2</td>
<td>S.2</td>
<td>II</td>
<td>T5</td>
</tr>
<tr>
<td>S3</td>
<td>S.1</td>
<td>II</td>
<td>T11</td>
</tr>
<tr>
<td>S3</td>
<td>S.2</td>
<td>III</td>
<td>T5</td>
</tr>
<tr>
<td>S3</td>
<td>S.3</td>
<td>II</td>
<td>T11</td>
</tr>
<tr>
<td>S3</td>
<td>S.3.4</td>
<td>II</td>
<td>T11</td>
</tr>
<tr>
<td>S3</td>
<td>S.3.5</td>
<td>I</td>
<td>T11</td>
</tr>
<tr>
<td>S4</td>
<td>S4</td>
<td>III</td>
<td>T5</td>
</tr>
<tr>
<td>S5</td>
<td>S5</td>
<td>III</td>
<td>T5</td>
</tr>
<tr>
<td>S6</td>
<td>S6.1</td>
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<td>T5</td>
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<td>S6</td>
<td>S6.3</td>
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<td>T4/T17</td>
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<td>S6.4</td>
<td>II</td>
<td>T5</td>
</tr>
<tr>
<td>S7</td>
<td>S7.1</td>
<td>III</td>
<td>T5</td>
</tr>
<tr>
<td>S8</td>
<td>S8</td>
<td>III</td>
<td>T5</td>
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<tr>
<td>S9</td>
<td>S9.1</td>
<td>III</td>
<td>T5</td>
</tr>
<tr>
<td>S9</td>
<td>S9.2α</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td>S10</td>
<td>S10</td>
<td>I</td>
<td>T17</td>
</tr>
<tr>
<td>S11</td>
<td>S11</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td>S12</td>
<td>S12</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td>S13</td>
<td>S13.1</td>
<td>III</td>
<td>T5</td>
</tr>
<tr>
<td>S14</td>
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<td>T5</td>
</tr>
<tr>
<td>S14</td>
<td>S14.3</td>
<td>I</td>
<td>T17</td>
</tr>
<tr>
<td>Sample</td>
<td>Strain</td>
<td>Morphotype</td>
<td>Genotype</td>
</tr>
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<td>--------</td>
<td>------------</td>
<td>----------</td>
</tr>
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<td>S15</td>
<td>II</td>
<td>T4</td>
</tr>
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<td>T5</td>
</tr>
<tr>
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<td>S17.3</td>
<td>III</td>
<td>T17</td>
</tr>
<tr>
<td>S18</td>
<td>S18.1α</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td>S19</td>
<td>S19.1</td>
<td>III</td>
<td>T5</td>
</tr>
<tr>
<td></td>
<td>S19.2</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td>S20</td>
<td>S20.1</td>
<td>III</td>
<td>T5</td>
</tr>
<tr>
<td></td>
<td>S20.3</td>
<td>II</td>
<td>T4/T17</td>
</tr>
<tr>
<td>S21</td>
<td>S21</td>
<td>II</td>
<td>T17</td>
</tr>
<tr>
<td>9/C2</td>
<td>9/C2.1α</td>
<td>II</td>
<td>T13</td>
</tr>
<tr>
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<td>9/C2.1β</td>
<td>II</td>
<td>T14</td>
</tr>
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<td></td>
<td>9/C2.2</td>
<td>II</td>
<td>T13</td>
</tr>
<tr>
<td></td>
<td>9/C2.3</td>
<td>II</td>
<td>T4</td>
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<tr>
<td>6/G2</td>
<td>6/G2.2</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td></td>
<td>6/G2.3</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td>9/G1β</td>
<td>9/G1β.1</td>
<td>III</td>
<td>T5</td>
</tr>
<tr>
<td></td>
<td>9/G1β.3α</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td></td>
<td>9/G1β.4α</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td></td>
<td>9/G1β.5α</td>
<td>II</td>
<td>indefinable</td>
</tr>
<tr>
<td></td>
<td>9/G1β.5β</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td>6/B2</td>
<td>6/B2.1</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td></td>
<td>6/B2.2α</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td></td>
<td>6/B2.3</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td>6/C3</td>
<td>6/C3.2</td>
<td>I</td>
<td>indefinable</td>
</tr>
<tr>
<td></td>
<td>6/C3.4</td>
<td>II</td>
<td>indefinable</td>
</tr>
<tr>
<td>8a/3</td>
<td>8a/3.1</td>
<td>I</td>
<td>T13</td>
</tr>
<tr>
<td></td>
<td>8a/3.2</td>
<td>III</td>
<td>T4</td>
</tr>
<tr>
<td></td>
<td>8a/3.6</td>
<td>III</td>
<td>indefinable</td>
</tr>
<tr>
<td>8a/5</td>
<td>8a/5.3</td>
<td>II</td>
<td>indefinable</td>
</tr>
</tbody>
</table>
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](image)

**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.
Results

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Strain</th>
<th>Morphotype</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>BF36</td>
<td>BF36.2</td>
<td>II</td>
<td>T19</td>
</tr>
<tr>
<td></td>
<td>BF36.3</td>
<td>III</td>
<td>T4/T17</td>
</tr>
<tr>
<td>BF40</td>
<td>BF40.3</td>
<td>III</td>
<td>T4</td>
</tr>
<tr>
<td>BF50</td>
<td>BF50.1</td>
<td>III</td>
<td>T15</td>
</tr>
<tr>
<td></td>
<td>BF50.2</td>
<td>III</td>
<td>T16</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Description</th>
<th>Morphotype</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1</td>
<td>K1.1</td>
<td>II</td>
<td>T4/T17</td>
</tr>
<tr>
<td></td>
<td>K1.2</td>
<td>III</td>
<td>T5</td>
</tr>
<tr>
<td>K29</td>
<td>K29.1</td>
<td>III</td>
<td>indefinable</td>
</tr>
<tr>
<td></td>
<td>K29.4α</td>
<td>II</td>
<td>T4/T17</td>
</tr>
<tr>
<td></td>
<td>K29.4β (α=β)</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td>K30</td>
<td>K30.1</td>
<td>III</td>
<td>T5</td>
</tr>
<tr>
<td></td>
<td>K30.2</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td></td>
<td>K30.5α</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td></td>
<td>K30.5β</td>
<td>II</td>
<td>T4</td>
</tr>
</tbody>
</table>
Results

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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Strain</th>
<th>Morphotype</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>E101E</td>
<td>E101E.1</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td>E101E</td>
<td>E101E.2</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td>E101E</td>
<td>E101E.3</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td>E101E</td>
<td>E101E.4</td>
<td>III</td>
<td>T4</td>
</tr>
<tr>
<td>E103B</td>
<td>E103B.1</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td>E103B</td>
<td>E103B.2</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td>E103B</td>
<td>E103B.3</td>
<td>II</td>
<td>T4</td>
</tr>
<tr>
<td>E103B</td>
<td>E103B.4</td>
<td>II</td>
<td>T4</td>
</tr>
</tbody>
</table>
Results

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
Discussion

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 (CLIMATE DATA).

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
Discussion

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),

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 (WALOCNIK 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 save 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 \textit{B. pseudomallei} is in fact rather high. LIMMATHUROTSAKUL \textit{et al} (2010b) demonstrated that there is no even distribution of \textit{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 \textit{B. pseudomallei}. However, this phenomenon had been described previously in other bacterial soil communities (WITHATANUNG \textit{et al} 2016). Overall, the detection of \textit{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 \textit{et al} 2010b).

Soil sampling also provides an insight into the interactions between \textit{B. pseudomallei} and protozoan organisms. According to SANDON \textit{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 (EKE LUND \textit{et al} 2001). Thus, the interactions between \textit{B. pseudomallei} and \textit{Acanthamoeba} spp. mostly take place in the soil surface. As LIMMATHUROTSAKUL \textit{et al} suggested in 2013a, the optimal sampling depth for \textit{B. pseudomallei} is within the upper 30 cm in soil. In the current study, the collection of the soil was performed according to the guidelines of LIMMATHUROTSAKUL \textit{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 bacterial communities in soil and water (HAHN \textit{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 temperature, pH and nutrient compounds the bacterial community, and therefore the food source for \textit{Acanthamoeba} can be influenced. Overall, the interaction of bacteria 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 involved.
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...
Discussion

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

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
<tr>
<td>°C</td>
<td>Celsius</td>
</tr>
<tr>
<td>A549</td>
<td>lung epithelial cell line</td>
</tr>
<tr>
<td>AHL</td>
<td>Acly-homoserine lactones</td>
</tr>
<tr>
<td>AK</td>
<td><em>Acanthamoeba</em> keratitis</td>
</tr>
<tr>
<td>AP</td>
<td>Acanthopodia</td>
</tr>
<tr>
<td>API 20NE</td>
<td>biochemical test kit ©biomerieux</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease, Control and Prevention</td>
</tr>
<tr>
<td>CIP</td>
<td>Climate Information Center</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CpG</td>
<td>DNA-islands with Guanine and Cytosine dominating</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
<tr>
<td>CRU</td>
<td>Climatic Research Institute</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECT</td>
<td>ectocyst</td>
</tr>
<tr>
<td>EN</td>
<td>Encystation</td>
</tr>
<tr>
<td>END</td>
<td>endocyst</td>
</tr>
<tr>
<td>EX</td>
<td>Excystment</td>
</tr>
<tr>
<td>FLA</td>
<td>free living amoeba</td>
</tr>
<tr>
<td>FV</td>
<td>Food vacuole</td>
</tr>
<tr>
<td>fsp</td>
<td>full speed</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte colony stimulating factor</td>
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<tr>
<td>GAE</td>
<td>Granulomatous amoebic encephalitis</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic DNA</td>
</tr>
<tr>
<td>H₂O</td>
<td>water</td>
</tr>
<tr>
<td>HeLa</td>
<td>epithelial cell line</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>i.v.</td>
<td>intra venous</td>
</tr>
<tr>
<td>IDSA</td>
<td>Infectious Diseases Society of America</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleucin</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxidase synthase</td>
</tr>
<tr>
<td>irIR</td>
<td>invasion related locus</td>
</tr>
<tr>
<td>JAK-STAT</td>
<td>signalling pathway</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>potassium dihydrogen phosphate</td>
</tr>
<tr>
<td>L</td>
<td>liter</td>
</tr>
<tr>
<td>LD₅₀</td>
<td>abbreviation for lethal dose 50%</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LuxI</td>
<td>auto-inducer synthase</td>
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<td>LuxR</td>
<td>receptor</td>
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<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>mA</td>
<td>milliampere</td>
</tr>
<tr>
<td>Mbp</td>
<td>Mega base pairs</td>
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<tr>
<td>mg</td>
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<tr>
<td>MgSO₄</td>
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<td>min</td>
<td>minute</td>
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<tr>
<td>MNGC</td>
<td>multinucleated giant cells</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<td>transcription factor</td>
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<td>non nutrient</td>
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<td>NU</td>
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<td>operculum</td>
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<tr>
<td>p.o.</td>
<td>per oral</td>
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<tr>
<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
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</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PP</td>
<td>pseudopodia</td>
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<tr>
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<tr>
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<tr>
<td>SNP</td>
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</tr>
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</tr>
<tr>
<td>spp.</td>
<td>species (plural)</td>
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<td>Tris Acetate EDTA</td>
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<tr>
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<td>toll like receptor</td>
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<td>trophozoite</td>
</tr>
<tr>
<td>TTSS</td>
<td>type three secretion system</td>
</tr>
<tr>
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<td>University of East Anglia</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
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<tr>
<td>WHO</td>
<td>world health organisation</td>
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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
**Glossary**

**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 CD4 TH1 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
Glossary

**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.
Glossary

**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 (Th1):** subset of T cells (CD4) which produce cytokines and are used to activate macrophages
Glossary

**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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Appendix

8. Appendix

8.1. Abstracts

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 on 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.

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