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DIPLOMARBEIT

Amoebae of medical relevance in reptiles

Establishment of a PCR detection system for parasitic amoebae

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1 Abbreviation

AIDS:	acquired immunodeficiency syndrome
AK:	*Acanthamoeba*-Keratitis
bp:	base pair
CNS:	central nervous system
CSF:	cerebrospinal fluid
CT:	computed tomography
DNA:	deoxyribonucleic acid
FISH:	fluorescent in situ hybridization
FLA:	free-living amoebae
GAE:	granulomatous amoebic encephalitis
Ig:	immunoglobulin
IIF:	indirect immunofluorescence
ITS:	internal transcribed spacer
NN:	non-nutrient
PAME:	primary amoebic meningoencephalitis
PCR:	polymerase chain reaction
PK:	penetrating keratoplasty
rDNA:	ribosomal DNA
RNA:	ribonucleic acid
SSU:	small sub-unit

2 Introduction

2.1 History

In the year 1755 a free-living amoeba was described for the first time by a German scientist called August Rösel von Rosenhof. 1822 Jean Baptiste Marcellin, baron Bory De Saint-Vincent mentioned the term “amoeba” (gr.-nlat. change or alteration) for the first time. This term is related to all unicellular organisms with the capability to vary their shape. A few years later, Ehrenberg (1838) established the genus *Amoeba* and suggested that the cell organelles are small vital parts. Schultze (1854) argued that the Rhizopoda body is nothing else than a usual cell found in plants and animals. The class Rhizopoda was established 1835 by Dujardin to combine the Foraminifera and freshwater amoebas. Bronn (1859) included the Rhizopoda with three other classes due to their common morphological characteristics like pseudopods into the class Amorphozoa. A few years later, Haeckel (1862) subdivided the class Radiolaria based on the organisms shell arrangement into two classes namely Radiolaria and Acantharia (Walochnik and Aspöck, 2007).

The Russian physician Fedor Aleksandrovich Lösch identified and described 1875 the agent of the chronic diarrhea, *Entamoeba histolytica*, and caused the disease in an experimental animal (cit. in Lesh 1975).

Nägler (1909) established the class "Limax" amoebae that was subdivided three years later by Alexeieff (1912) into the genera Amoeba, "Limax" amoeba, parasitic amoebas (*Entamoeba*) and *Malpighiella* (Walochnik and Aspöck, 2007)

The existence of parasitic amoebae and non-invasive amoebae demanded for a term that allowed the discrimination between these two groups. This term, which was established for the non-invasive amoebae, is free-living amoebae. Today however, we know that also free-living amoebae can appear as pathogens for humans.

2.1.1 *Acanthamoeba* spp.

In 1930 Sir Aldo Castellani discovered a free- living amoeba in a culture of the fungus *Cryptococcus pararoseus*. This amoeba was placed in the genus *Hartmannella* and was named *Hartmannella castellanii* by Douglas. Volkonsky subdivided the genus *Hartmannella* into three genera (*Hartmannella, Glaeseria* and *Acanthamoeba*) because

he considered the genus *Hartmannella* to be an artificial assemblage of unrelated amoebas.

All amoebas characterized by round, smooth-walled cysts and cylindrical or truncated spindles were placed by Vokonsky into the genus *Hartmannella*, while all amoebae characterized by nuclear division in the cyst were placed in the genus *Glaeseria*. The new established genus *Acanthamoeba* was characterized by amoebae with a pointed mitotic spindle and a typical double-walled cyst with pores.

Singh (1952) stated that classification of amebas by appearance of cysts form and locomotion was of no phylogenetic value for diagnostics. So the genus *Acanthamoeba* was discarded because the shape of the mitotic spindle was poor as a generic character. In 1966, Pussard considered the spindle shape as an unsatisfactory feature for intergeneric differentiation but he regarded the morphology of the cysts as a distinctive character at the genus level. Page studied small free-living amoebae and also several *Acanthamoeba* and *Hartmannella* strains. He concluded 1967 that the spindle shape was an arguable feature for intergeneric differentiation but the structure of the cysts and the presents of acanthapodia were considered sufficiently distinctive. Page concluded that the designations of *Hartmannella* and *Acanthamoeba* because of trophic and cyst characters were justified. After redefinition of the genus *Acanthamoeba*, Page described three species (Page 1967). Nutritional and serological studies led to the differentiation between *Acanthamoeba* and *Hartmannella* (Visvesvara and Balamuth, 1975). Sawyer and Griffin (1975) created the family *Acanthamoebidae* to differentiate between the acanthamoebidae and the hartmannellids in the family Hartmannellidae.

2.1.2 *Balamuthia mandrillaris*

The first *Balamuthia mandrillaris* was isolated from the brain of a pregnant mandrill baboon that died 1986 at the San Diego Wildlife Park (Visvesvara et al., 1993) from meningoencepahlitis. Initially this amoeba was described as a leptomyxid amoeba because of the similarity to soil amoebae included in the family Leptomyxidae. Visvesvara et al. (1993) admitted this amoeba as being different in morphology, physiology and antigenic characteristics from the leptomyxids and established a new genus and species, namely *Balamuthia mandrillaris*. The term *mandrillaris* was chosen to reflect the origin of the type species. In the year 1991 (Anzil et al., 1991) *Balamuthia mandrillaris* was associated for the first time with fatal infections of the central nervous system in humans.

The first isolation of the amoeba *Balamuthia mandrillaris* from environmental soil samples was reported 2003 by Schuster et al. Before, the amoeba was presumed to be free-living, but it had never been isolated from environmental samples.

2.1.3 *Naegleria* spp.

In the year 1909 Kurt Nägler established the term “Limax”-amoebae (meaning slug-like) that was defined by a nucleus surrounded by a bright area, a longish shape and a size not longer than 30 µm lacking larger pseudopods.

In the year 1952, Singh postulated that *Naegleria* belongs to a much more primitive genus than *Hartmannella*, established the genus *Naegleria*. De Jonckheere (2002) reported that Schardinger discovered an amoeba with the ability to transform into a flagellate stage in the year 1899. This amoeba belonged to the Lobosoea and Schardinger called it *Amoeba gruberi*. In the year, 1912 Alexeieff suggested the genus name *Naegleria*.
In the year 1970, Carter separated the pathogenic *Naegleria* from the non-pathogenic *N. gruberi* and called it *Naegleria fowleri* after Malcolm Fowler who first described the disease PAME caused by *N. fowleri* in Australia.

2.2 FLA: Phylogeny

2.2.1 Phylogeny of "amoebae"

In the year 1995, Cavalier-Smith modified the phylum Rhizopoda by adding the classes Chlorarachnea and Sarcomonadea. The authors defined the Lobosea as an own phylum, namely the Amoebozoa. The phylum Rhizaria, containing the Cercozoa (formerly Rhizopoda) and the Radiolaria, has been created in the year 2002 by Cavalier-Smith. A few years later, Cavalier-Smith (2004) established a new system with six kingdoms. In this six-kingdoms system the phylum Rhizopoda, which was renamed to Cercozoa was modified by removing some rhizopods and adding more flagellates. Another achievement of this new system was the reduction of the number of protozoan phyla. The Mycetozoa and the Archamoebae were grouped into a new subphylum, called Conosa within the phylum Amoebozoa beside the subphylum Lobosea.

Nowadays, molecularbiological techniques and morphological approaches entail to frequent changes and new establishment of systematic schemes.

The topology of the ribosomal eukaryotic tree was redrawn with the use of genomic signatures that place the root of all eukaryotic life between two newly uncovered major clades, unikonts (uniciliate) and bikonts (bicilitate). Unikonts, which contain the heterotrophic groups Opisthokonta and Amoebozoa, share a derived three-gene fusion of enzyme encoding genes in the pyrimidine synthesis pathway (Stechmann and Cavalier-Smith 2002), whereas bikonts, which contain the remaining eukaryotic clades, share another derived gene fusion between dihydrofolate reductase and thymidine synthase (Stechmann and Calvalier-Smith, 2002). Following this hypothesis, the root of the eukaryotic tree lies between opithokonts and bikonts, but there are also suggestions that the root could lie within the Amoebozoa (Fahrni et al., 2003). These suggestions based on analysis of actin and SSU rRNA sequences with the result that there is a close relationship between lobose amoebae.

Nikolaev et al. (2006) suggested that the root of Amoebozoa is placed between *Tubulinea*, *Mayorella*, *Platyamoeba steopoda*, *Acanthopodiada & Flabellinea*, *Dermamoeba algensis* and *Conosea* or between *Tubulinea* and all other amoebozoans due to the type of analysis. The taxonomy of the sample, the selected outgroup and the gene as well influences the result. In a phylogenetic tree based on actin (Fahrni et al., 2003) the position of the Amoebozoa is within the opistokonts and they build a sistergroup of the Metazoa and Choanoflagellata.

A dichotomic phylogenetic tree of life is equivocal to a certain extend, because it is widely accepted that recent organisms have acquired an extensive portion of their genome via lateral gene transfer (Walochnik and Aspöck, 2007).

The most extensive consortium paper about a recent scheme based on ultrastructural research and molecular phylogeny studies since 1980 was published by Adl et al. in 2005. Here, formal rank designations were abandoned for a hierarchical system. Six super-groups namely, Amoebozoa, Ophistokonta, Rhizaris, Archaeplastida, Chromalveolata and Excavata have been established within the eukaryotes. The amoebae, formerly all placed in the Rhizopoda, now belong to three different phyla, the Amoebozoa, the Rhizaria and the Excavata. Moreover, many lobose amoeboid taxa, inclusive Entamoebidae and Acanthamoebidae are united within the super-group Amoebozoa. The first rank "Heterolobosea" are placed within the super-group Excavata.

Dacks et al. (2008) suggested, that parasitism is rare in unicellular eukaryotes and occurred several times independently due to the systematic that parasites belong to

different super-groups that also include non-parasitic taxa. Moreover, Dacks et al. (2008) doubt the place of primitive and ancient parasites at the base of the phylogenetic tree. The same evolutionary age of all living eukaryotes is suggested and a divergence or specification of some lineages away from other eukaryotes does not lead to a higher evolutionary age.

Recently, Pawlowski (2009) suggested that this phylogeny based on a single molecular character as the SSU rRNA cannot determine relationships between all amoeboid protists.

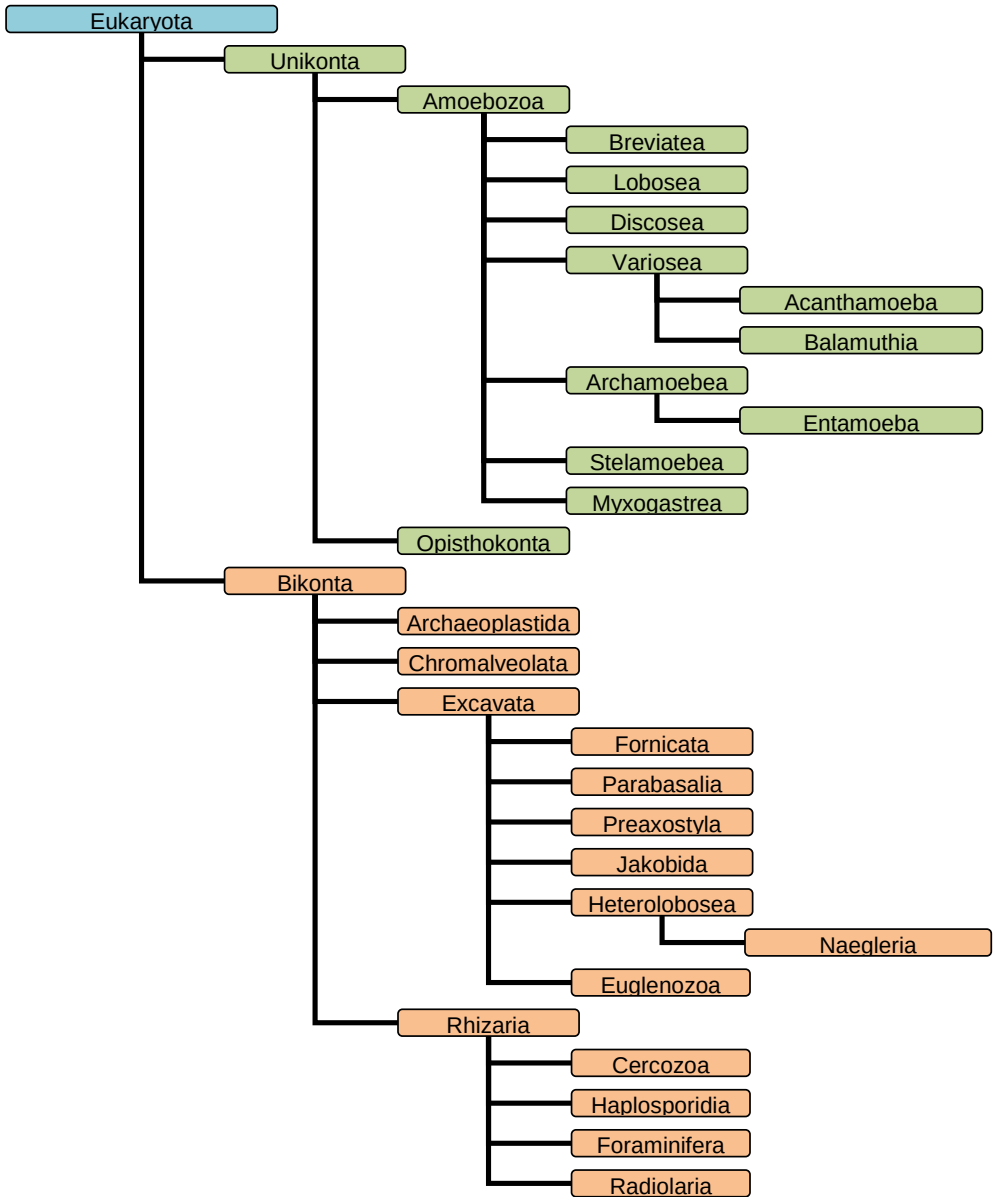


Figure 1: The position of *Acanthamoeba* spp., *Balamuthia* sp., *Entamoeba* spp. and *Naegleria* spp. in the phylogenetic tree (Walochnik and Aspöck, 2007).

2.2.1.1 *Acanthamoeba* spp.

The genus *Acanthamoeba* belongs to the class Variosea, the member of the phylum Amoebozoa. The phylum *Amoebozoa* is the sister phylum of *Opisthokonta* and both belong to the monophylum *Unikonta* as shown in Figure 1 (Walochnik and Aspöck, 2007). Nonetheless, *Acanthamoeba* systematic contains inconsistencies and will probably be further revised.

1977 Pussard and Pons established 18 different species within the genus *Acanthamoeba* including eight new species and subdivided the genus into three groups by their cyst- morphological features. Traditionally, *Acanthamoeba* taxonomy was based on morphological features like size and shape of cysts and trophozoites. Nowadays, *Acanthamoeba* is divided into 15 different genotypic classes which were previously called sequence types (Schuster et al., 2004a). These genotypes (Table 3) are combined in clusters as reported by Booton et al. (2005). One cluster contains the genotypes T3, T4 and T11. Furthermore, two clusters are build by the genotypes T2, T6 and T10, T12, T14. The genotypic classes T7, T8 and T9 isolated until now only from environmental samples belong to the morphological group I of *Acanthamoeba*.

2.2.1.2 Balamuthia mandrillaris

In the year 1993, Vivesvara et al. described the morphological features of *Balamuthia*, based on light and electron microscopy. Like *Leptomyxa* and *Gephyramoeba*, the isolated amoeba had an actively feeding trophic stage containing a direct life cycle and a cyst with a thick wall. Nonetheless, Visvesvara et al. could not assign the isolated amoeba to the genera *Leptomyxa* or *Gephyramoeba* because of elementary morphological, physiological and antigenic differences. The species designation of *B. mandrillaris* reflects the site of discovery.

In the Year 2000, Amaral Zettler et al. showed that *Balamuthia* does not belong to the order Leptomyxida and transferred *B. mandrillaris* to the Acanthamoebidae based on molecular biological studies.

Booton et al. (2003b) showed, by molecular biologically comparison of 7 *B. mandrillaris* sequences with 11 *Acanthamoeba* sequences, that *B. mandrillaris* is a sister genus to *Acanthamoeba*. They found no evidence for the possibility, that *B. mandrillaris* could be

a new species of *Acanthamoeba* so the hypothesis that *Balamuthia* is an independent genus was assured. Nonetheless, the genus *Balamuthia* is a close relative of the monophyletic genus *Acanthamoeba*.

The genus *Balamuthia* belongs together with the genus *Acanthamoeba* and *Phalansterium* to the class Variosea as shown in Figure 1 (Walochnik and Aspöck, 2007).

2.2.1.3 Naegleria fowleri

The classic distinction of *Naegleria* spp. based on physiological features like pathogenicity, thermo tolerance and the ability to transform to flagellates. The current classification of *Naegleria* is based on molecular biology.

Pélandakis et al. (2000) analysed 21 *N. fowleri* strains and eight other species including *N. gruberi* by molecular biology. The phylogenetic tree based on the 5.8S rRNA analysis showed four main clusters. The first cluster (*lovaniensis fowleri* cluster) included all *N. fowleri* strains and *N. lovaniensis* and is the sister group of *N. morganensis*. The second main cluster included *N. gruberi* (except one isolate, which was allocated to the fourth cluster)*, N. australiensis*, *N. clarki*, *N. italiaca* and *N. galeacystis*. *N. jamiesoni* and *N. andersoni* form the third cluster, while the fourth cluster is composed of *N. pussardi* and *N. gruberi*. The authors also distinguished *N. fowleri* from the non-pathogenic *Naegleria* strains by analyzing sequences in the ITS regions. In *N. fowleri*, rearrangements of rDNA in the ITS1 region resulted in numerous repetitions of the motifs.

De Jonckheere (2002) published that the distinction of the two species *N. gruberi* and *N. fowleri* depended only on morphological differences and pathogenicity. Serological, biochemical and molecular techniques corroborated the separation of the pathogenic *N. fowleri* from the non-pathogenic species. *N. aerobia* was another name for the human pathogenic *Naegleria* sp. but this name has been abandoned like the junior synonym *N.*

Zhou et al. (2003) characterized 26 samples of *Naegleria* spp. based on ITS and mitochondrial small subunit rRNA (mtSSU rRNA) sequences. The results of the mtSSU rRNA analysis showed, that there are three genotypes of *N. fowleri,* namely genotype I, II and III. However, these three groups lack sequence differences between each other. The sequence dissimilarities between *N. fowleri* and *N. lovaniensis* are small whereas

the homology between the sequences of *N. australiensis, N. jadini* and *N. gruberi* is very high. These results showed that *N. fowleri* forms a sister group of *N. lovaniensis* and that a cluster was build by *N. australiensis*, *N. gruberi* and *N. jadini.* The analysis of the internal transcribed spacers (ITS) identified two different groups of *N. fowleri*. First, there is the WEA group (the widespread and Euro- American variants) and secondly, there is the SPC group (the South Pacific strains and the French strain). Furthermore, the ITS regions of *N. lovaniensis* and *N. fowleri* showed that the sequences differ in four nucleotides and a deletion of one nucleotide at the end of the ITS region. Among the 22 *N. fowleri* isolates in this study, three ITS genotypes were characterized. Genotype I and II show one long repeat of the sequence ATGGTAAAAAAGGTGAAAACCTTTTTT and genotype III shows two repeats of the same long sequence as genotype I and II and additionally one shortened variant of the long repeat ATGGTAAAAAAGGTG.

Currently, *N. fowleri* is the only species known to be pathogenic for humans and animals, although there are at least 30 species in the genus *Naegleria* (Visvesvara et al., 2005).
The genus *Naegleria* builds together with *Valhkampfia, Paravahlkampfia* and *Acrasis* the class Heterolobosea, a member of the phylum Excavata. The phylum *Excavata* is the sister phylum of *Archaeplatida, Chromalveolata* and Rhizaria and they all are part of the monophylum *Bikonta* as shown in Figure 1 (Walochnik and Aspöck, 2007).

2.2.2 Distribution

2.2.2.1 *Acanthamoeba* spp.

The ubiquitous *Acanthamoeba* spp. occur worldwide and they have been isolated from different habitats like fresh and brackish waters, bottled mineral waters, dialysis machines, cooling towers of electric and nuclear power plants, heating, ventilating and air conditioning units, soil, dust, contact lens paraphernalia, fungal and mammalian cell cultures, ear discharge, pulmonary secretions, swabs obtained from nasopharyngeal mucosa of patients with respiratory complaints as well as of healthy individuals and stool samples (Marciano- Cabral and Cabral, 2003; Visvesvara et al., 2007).
From infected individuals, Acanthamoebae have been isolated from several organs including brain, skin, lungs and cornea.
Moreover*, Acanthamoeba* spp. have been isolated from different animals including reptiles (Walochnik et al., 1999), birds (Visvesvara et al., 2007) and mammals (Bauer et al., 1993; Westmoreland et al., 2004; Lorenzo- Morales et al., 2007).

2.2.2.2 Balamuthia mandrillaris

Balamuthia mandrillaris was presumed to be free-living, but for a very long time it had not been recovered from environmental samples. Principally this amoeba is a soil amoeba and in 2002 Schuster et al. reported the first environmental isolation. They isolated it from soil of a potted plant in the home of a 3-year old child, who had died from amebic encephalitis in northern California. Dunnebacke et al. (2004) reported the second environmental isolation. In this case, a soil sample from an outdoor potted plant located in California was the source of isolation.

2.2.2.3 Naegleria fowleri

The amoeba *Naegleria fowleri* is ubiquitous in nature and has been isolated from different sources like poorly maintained swimming pools, hot springs, hydrotherapy pools, public water supplies, freshwater lakes, thermally polluted waters, soil and even from healthy individuals (by analyzing samples from throats and nasal passages). In contrast to *Acanthamoeba* spp., *Naegleria* spp. have never been isolated from seawater samples. The reason for this is the sensitivity to high levels of osmolarity.

De Jonckheere et al. (2004) studied different *Naegleria* species and their geographical distribution as shown in Table 1. Of 19 described *Naegleria* species 10 are ubiquitous including *N. fowleri*, two species are not and seven species are probably ubiquitous.

Table 1 Ubiquity of *Naegleria* spp. from De Jonckheere (2004)

Species	Georaphic coverage	Ubiquitous
N. fowleri	Europe, North America, Asia, Australia	yes
N. lovaniensis	Europe, North America, Asia, Australia	yes
N. martinezi n. sp 10	Australia	no
N. johanseni n.sp 11	Australia	no
N. australiensis	Europe, North America, Australia	yes
N. italica	Europe, Australia	probably
N. philippinensis	Asia, Australia	probably
N. andersoni	Asia, Africa, Australia, South America	yes
N. jamiesoni	Europe, Asia, Africa	yes
N. sturti	Asia, Australia, Africa	yes
N. pagei	Europe, North America, Africa	yes
N. tihangensis	Europe, Asia, Australia, Africa, North America	yes
N. gruberi	Europe, Asia, North America	yes
N. cateri	Asia, Australia	probably
N. pussardi	Europe, Australia	probably
N. clarki	Australia, Africa	probably
N. americana n. sp. 2	Europe, Australia, North America	yes
N.schusteri n.sp. 4	Asia, North America	probably
N. byersi n. sp. 9	Asia, Australia	probably

2.3 FLA: Physiology and morphology

2.3.1 FLA comparative characteristics

Table 2: Free- living amoebae comparative characteristics (modified from Visvesvara et al. 2007)

	Naegleria fowleri	***Acanthamoeba* spp. (encephalitis)**	***Acanthamoeba* spp. (keratitis)**	***Balamuthia mandrillaris***
Life cycle	Three stages: trophozoite, cyst and flagellate	Two stages: amoeba and cyst	Two stages: amoeba and cyst	Two stages: amoeba and cyst
Distinctive morphological features	Vesicular nucleus; limacine movement of amoebae; flagellated stage; cyst with pores flush at the surface	Vesicular nucleus; finger- like pseudopodia projecting from surface; cyst wall with two layers and with covered pores (opercula)	Vesicular nucleus; finger- like pseudopodia projecting from surface; cyst wall with two layers and with pores	Vesicular nucleus with single or multiple nucleoli, amoeboid and "spider- like" movements in culture; cysts wall with tree layers
In vitro cultivation	Axenic, bacterized and defined media; tissue culture cells; optimal growth at 37°C and above	Axenic, bacterized, and defined media; tissue culture cells; growth at 37°C (CNS isolates) or 30°C (keratitis isolates)	Axenic, bacterized, and defined media; tissiue culture cells; growth at 37°C (CNS isolates) or 30°C (keratitis isolates)	Axenic medium and tissue culture cells; optimal growth at 37°C
Disease	Primary amoebic meningoencephalitis (PAM)	Granulomatous amoebic encephalitits; cutaneous lesions; sinus infections	Amoebic keratitis	Granulomatous amoebic encephalitis; cutaneous lesions; sinus infections
Prodromal period	Days	Weeks to months	Days	Weeks to months
Epidemiology	Humans typically infected while recreating in warm fresh waters	Infection from soil, water, and air; in hospital environment (water taps, hydrotherapy pools, air conditioning cooling towers)	Contaminated contact lenses corneal trauma;	Infection from soil, water, and air
Groups at risk	Children and young adults in good health	Typically, immuno-compromised individuals	Mainly contact-lens wearers; low sectretory IgA may contribute	Immuno-competent (children and elderly) or immuno-compromised individuals;
Disease at presentation	Headache, stiff neck, seizures, coma	Headache, stiff neck, behavioural changes, coma	Intense pain, photophobia, tearing	Headache, nausea, seizures, stiff neck, hydrocephalus; sinus infection; nodule formation in cutaneous infection
Clinical course	Fulminant disease; death within 1- 2 weeks without treatment	Idolent subacute course; acute stage fatal in weeks	Penetration of amoebae into cornea; stromal ring due to PMN infiltrate	Indolent subacute course; once in acute stage, fatal in weeks

Laboratory diagnostic methods	Amoebae present in CSF; no cyst seen in brain tissue; elevated PMNs in CSF	Cysts seen in brain tissue; IFA, IIF and PCR	Corneal scrapings or biopsy; confocal microscopy	Elevated CSF protein and pleoctic lymphocatosis, normal glucose; cyst seen in brain tissue; IFA, IIF and PCR
Neuroimaging (CT and/ or MRI)	Unremarkable; not helpful diagnostically	Presence of space- occupying or ring-enhancing lesions	Not relevant	Presence of space-occupying or ring-enhancing lesions
Humoral reaction	Typically weak but titre rises with length of infection	Usually strong; protective value uncertain	IgA at corneal surface may be protective	Usually strong; protective value uncertain
Prevention	Public health monitoring of warm, fresh-water recreational sites for amoebae	Widespread in soil and water; in hospital setting, monitoring of water supply, ventilators, air conditioning units	Use of sterile lens solution; maintaining clean lens case; avoiding swimming with contact lenses	Found in soil; preventive measures not feasible
Antimicrobial therapy	Intrathecal amphotericin B, miconazole	Pentamidine, azole compounds, flucytosine, sulfadiazine	PHMB, chlorhexidine, Brolene	Pentamidine, azithromycin, fluconazole, flucytosine

PAME (primary amoebic meningoencephalitis): a disease of the central nervous system due to the free-living amoeba *N. fowleri*

CNS (central nervous system)

CSF (cerebrospinal fluid): a clear fluid within the subarachnoid space and ventricular system as well as in the inside and surrounding of the brain.

2.3.2 *Acanthamoeba* spp.

2.3.2.1 Life cycle

In the life cycle of *Acanthamoeba* spp. are two different stages. On the one hand, there is a vegetative or trophozoite stage (Figure 2) in which the amoeba divides by binary fission and feeds actively on bacteria and detritus present in the environment. On the other hand, *Acanthamoeba* spp. round up and encyst to a dormant but resistant cyst (Figure 2 and Figure 3b) under inappropriate environmental conditions e. g. desiccation, environmental stress or when food becomes scarce. If better environmental conditions are given, *Acanthamoeba* changes to the trophozoite stage by excystation. Both, the trophozoites and the cysts, can enter the body of the host (Figure 2 Nr. 4) and cause disease in different parts of the body depending on the entry site (Marciano- Cabral and Cabral, 2003; Visvesvara et al., 2007).

The life cycle of *Acanthamoeba* spp., as in *Balamuthia mandrillaris* and *Naegleria fowleri*, depends on environmental conditions.

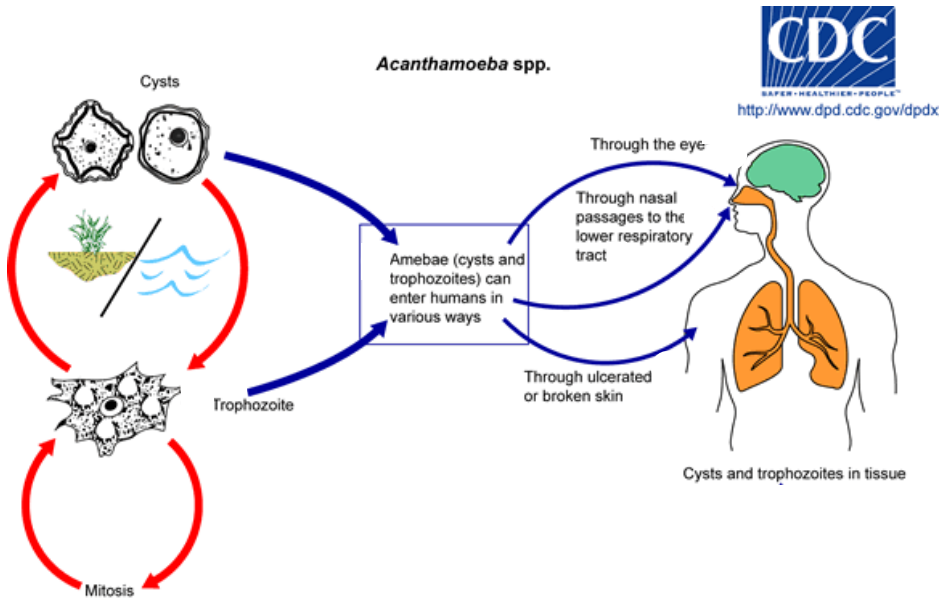


Figure 2: *Acanthamoeba* spp. life cycle: The infective trophozoites replicate by mitosis and encyst under adverse environmental conditions. Both, trophozoites and cysts enter the body (eye, nasal passages, and skin) and cause diseases at different sites (skin, brain and eye).

(Modified after: http://www.dpd.cdc.gov/dpdx/HTML/Frames/A-F/FreeLivingAmebic/body_FreeLivingAmebic_acanthamoeba.htm)

2.3.2.2 Trophozoites

Depending on the species or genotypes, the size of the trophozoites (Figure 3 a) ranges from 15 to 50 µm in diameter. Unique and characteristic features of the trophozoites are the acanthopodia, that arise from the surface of the amoeba-body. Acanthopodia are characteristically fine, tapering, thorn-like pseudopods, which play an important role in adhesion to surfaces, capturing prey and cellular movements. The slow and sluggish locomotion of *Acanthamoeba* depends on the formation of hyaline pseudopods.

The uninucleated trophozoites show a large, densely staining nucleus in a central position surrounded by a finely granular cytoplasm, containing numerous mitochondria, ribosomes, food vacuoles and a contractile vacuole to control the water content of the cell. To take up food *Acanthamoeba* spp. generally use different mechanisms for example by forming pseudopods and subsequent phagocytosis or by formation of food cups and ingestion of particulate matter. These food cups are temporarily formed structures on the surface of the amoeba and act as the unit for ingesting bacteria, yeast or cells. *Acanthamoeba* spp. feed preferably not on encapsulated or pigmented bacteria like *Escherichia coli* or *Enterobacter aerogenes*, because the mucoid capsule of the

bacteria inhibits phagocytosis by amoebas, and because of the toxicity of bacterial pigments (Visvesvara et al., 2007).

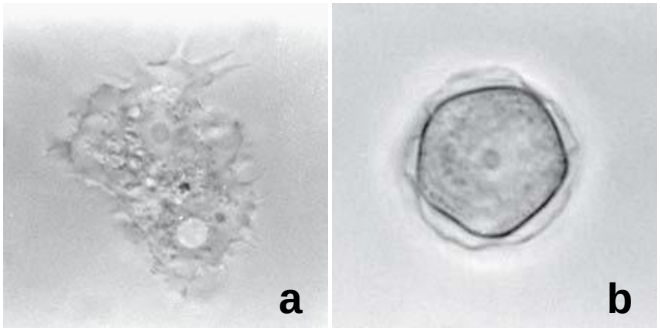


Figure 3 *Acanthamoeba* spp.; a) trophozoite; b) cyst (Neumeister et al., 2009)

2.3.2.3 Cysts

Pussard and Pons (1977) divided the genus *Acanthamoeba* into three gross groups (Table 3) based on the morphological characteristics of the cysts. At that time the morphology of the cysts was the most appropriate criterion.

Group I: (Figure 4 a)
The individuals in group I are the largest, concerning trophozoites and cysts as well. Typically the size of the cysts is larger than 18 µm and the shape is given by a rounded outer wall which is clearly separated from the inner wall. The shaped structure of the inner wall results from joints between the inner and the outer wall without immersed opercula.

Group II: (Figure 4 b)
The cysts have a variable shape and are usually smaller than 18 µm. The mostly wrinkled ectocysts can be thick or thin and may be separated from the endocyst or not. The shape of the endocyst can be triangular, polygonal, oval or round. Clear radiations are normally not visible and usually the operculum is immersed into the ectocyst.

Group III: (Figure 4 c)
The cysts with poorly separated walls are less than 18 µm in diameter. The very often ripped ectocyst is rather thin and the shape of the endocyst is usually round but sometimes rectangular or slightly triangular.

Table 3: Classification of *Acanthamoeba* species according to Pussard and Pons (1977), modified according to Marciano-Cabral and Cabral (2003).

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

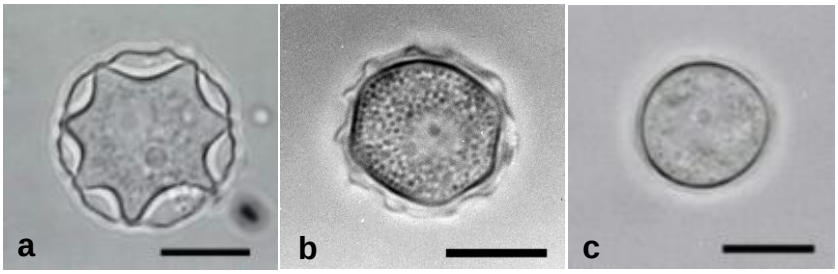


Figure 4: *Acanthamoeba* cysts representing morphological group I (a), II (b) and III (c)

(Walochnik and Aspöck, 2005) scale bar= 10µm

Generally cyst formation occurs under inappropriate environmental conditions, desiccation, food deprivation and changes in pH and temperature. Like the encysted amoebas are resistant to chlorine, biocides and antibiotics and they can survive low temperature (0°C to 2°C). To reliably eliminate *Acanthamoeba* cysts autoclavation is necessary. *Acanthamoeba* spp. excyst under suitable environmental conditions by emerging from the cyst.

2.3.3 *Balamuthia mandrillaris*

2.3.3.1 Life cycle

The life cycle (20-50 h) of *Balamuthia mandrillaris* (Figure 5) includes two stages, the motile actively feeding trophozoite and the dormant but resistant cyst. However, the amoeba replicates by binary fission, which includes mitosis without remaining nuclear membrane. Trophic amoebae as well as cysts can be inhaled into the lower respiratory

tract or brought in through skin lesions of the host although the trophozoites are the infective stage.

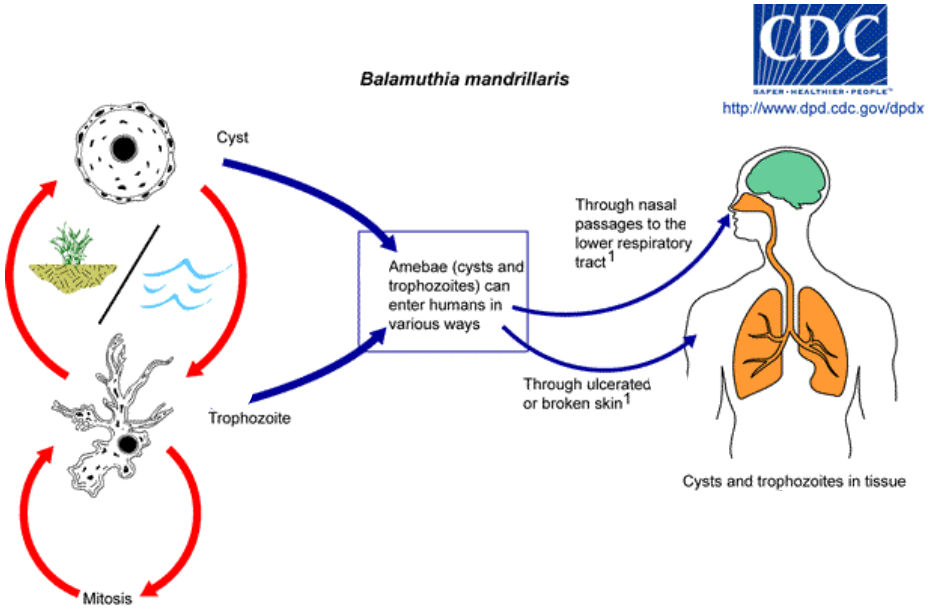


Figure 5: *B. mandrillaris* life cycle: the infective trophozoites replicate by mitosis and encyst under adverse environmental conditions. Both, trophozoites and cysts enter the body (skin nasal and passages) and cause disseminated diseases or GAE.

(Modified after: http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/FreeLivingAmebic_il.htm)

2.3.3.2 Trophozoites

The actively feeding trophozoite of *Balamuthia mandrillaris* as shown in Figure 6 a, b is pleomorphic with a size from 12 to 60 µm (mean of 15 µm). Usually the trophic amoebae are uninucleate; however, binucleate forms have been described. The nucleus contains a centrally located, large nucleolus. Especially in infected tissues, amoebae with two or three nucleolar bodies have been found (Visvesvara et al., 2007). According to Visvesvara et al. (1993) the pseudopodia of *Balamuthia mandrillaris* either can be broad to allow slowly moves or finger-like for spider-like movements.

Dunnebacke (2007) reported that *B. mandrillaris* feeds on mammalian cells in culture by protruding the pseudopodia into the cells which results in clear vesicles in the cell cytoplasm. This mode of feeding is enabled by the growth of finger-like structures, which are capable to snap together within one second to trap small bits of cytoplasm. The sizes of the vesicles are about the size of the pseudopodia tips. It is not clear if the vesicles

result from a digestive enzyme or if the pseudopodia tip removes particles from the cytoplasm of the target cell.
Furthermore, the whole amoeba can invade cells for the uptake of nourishment. For this invasion, an enzymatic release is necessary but it is not clear how the cell can survive for a few days after the amoeba entered the cell, moved around and finally left the host cell. This technique is more common for feeding on mammalian cells than the engulfment of the whole cell or a part of it by the amoebae.

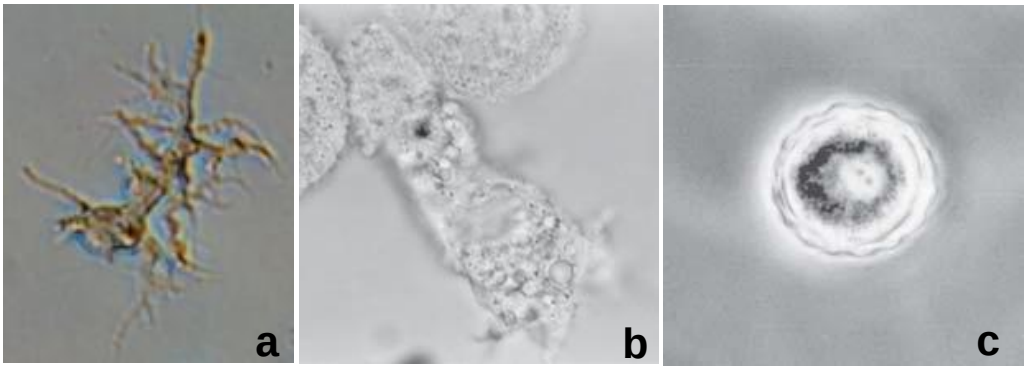


Figure 6 *Balamuthia mandrillaris.* a, b) trophozoite; c) cyst

(Figure 6 a: orig., figure 6 b and c: Neumeister et al., 2009)

2.3.3.3 Cysts

The cysts of *Balamuthia mandrillaris* (Figure 6c) are uninucleate but frequently with two nucleoli with a size from 12 to 30 µm (mean of 15 µm in diameter) and more or less spherical. In light microscopy the cysts appear to be round, or oval in shape and double-walled with a round inner wall and a wavy and irregular outer wall without pores.
According to Visvesvara et al. (1993) the cyst wall has three layers, it is composed of the thin and irregular outer ectocyst, the thick inner layer called endocyst and finally the middle amorphous fibrillar mesocyst.

2.3.4 *Naegleria fowleri*

2.3.4.1 Life cycle

Naegleria fowleri has three live-cycle stages (Figure 7), the resistant cyst, the actively feeding trophozoite that replicates by promitosis with an intact nuclear membrane and a transient flagellate stage. Infection with *N. fowleri* occurs mainly during swimming. *N. fowleri* penetrates the nasal mucosa and moves via the nervus olfactorius to the brain. The life-cycle depends on temperature, salt concentrations and nutrients in the

environment of the amoeba. Usually *N. fowleri* is much more sensitive to environmental conditions, for example pH or desiccation than other amoebae (Schuster and Visvesvara, 2004; Visvesvara et al., 2007).

In its natural habitat as well as in the laboratory the amoeba feeds actively on bacteria (*Escherichia coli, Enterobacter aerogenes* or *Klebsiella pneumoniae*).

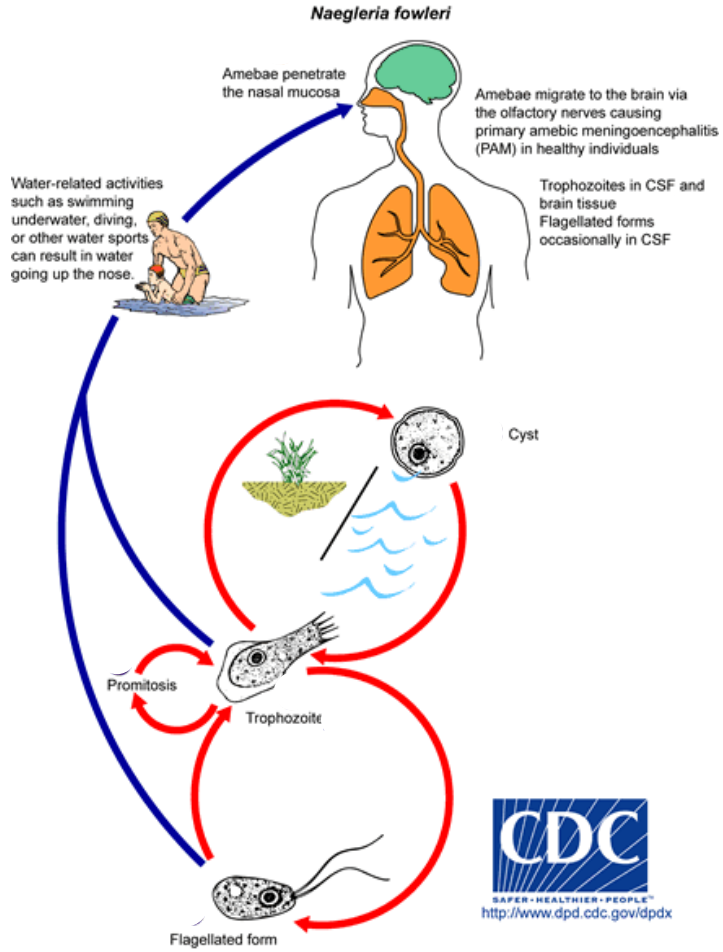


Figure 7: *N. fowleri* life cycle shows three stages (cyst, trophozoite and flagellate). Infection occurs during water related activities due to trophozoites that enter the body trough the nasal mucosa and cause PAME.

(Modified after: http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/FreeLivingAmebic_il.htm)

2.3.4.2 Trophozoites

The actively feeding trophozoite (Figure 8 a) measures from 10 to 25 µm and is the infective form of this amoeba. *N. fowleri* usually has one or two ectoplasmic pseudopods and shows a rapid slug-like pattern of locomotion ("Limax" amoebae"). Furthermore, this amoeba has a single nucleus with a centrally placed nucleolus that can be stained with Calcofluor-white, Acridinorange, Giemsa staining or with HE-staining in case of histological samples. The ectoplasm and the endoplasm are clearly distinguished within

the cytoplasm. The cytoplasm contains protoplasmic filaments and contractile vacuoles. The posterior end of the amoeba is called uroid.

In vitro, *Naegleria* spp. can feed on Gram-negative bacteria and reproduce by binary fission. The trophic amoeba destroys prokaryotic and eukaryotic target cells by releasing two pore-forming polypeptides, the so called naegleriapores (Herbst et al., 2002).

Furthermore, this thermophilic amoeba tolerates temperatures up to 45°C and proliferates during the warmer months of the year when the ambient temperature is higher than 30°C. However, temperature tolerance is not the only prerequisite for pathogenicity, because other thermophilic amoebae like *N. lovaniensis* show no pathogenicity (Visvesvara et al., 2007).

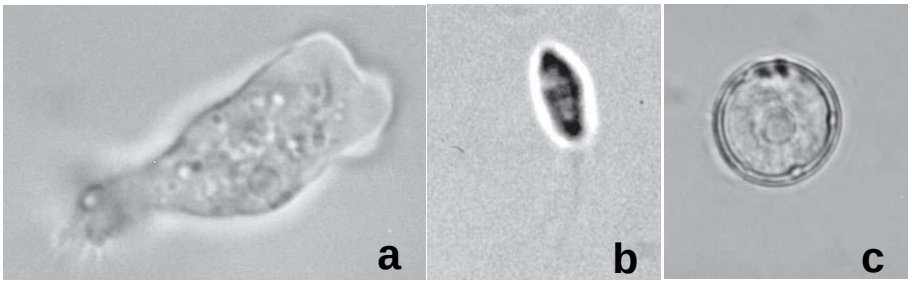


Figure 8 Naegleria a) trophozoite; b) flagellate; c) cyst (Neumeister et al., 2009)

2.3.4.3 Flagellate

The flagellated stage of *N. fowleri* (Figure 8) develops from the trophic amoeba and can reverse to the trophic stage within an hour or less. Triggered by contact with water or the changing ionic concentration of the milieu, the trophozoite transforms (over 30-60 min) into a flagellated stage with a size from 10 to 16 µm that does not feed or divide but helps to disperse the amoebae in their natural soil or water habitats. Furthermore, the flagellate stage has a single nucleus with a prominent nucleolus (Schuster and Visvesvara, 2004).

De Jonckheere (2002) reported that there are several *Naegleria* species without the flagellated stage or with the ability to divide in the flagellated stage..

2.3.4.4 Cysts

The hallmarks of *N. fowleri* cysts (Figure 8 c) are the size and a smooth surface. Furthermore, the cysts have a round shape and pores. *N. fowleri* cysts contain a single nucleus with a prominent nucleolus and the perinuclear deposit can be absent or faint in the cyst as well as in the amoeba (Schuster and Visvesvara, 2004).

Usually, the trophic amoeba transforms into the cyst under adverse environmental conditions, like reduced food amount or bad growth conditions.

2.4 FLA: Medical relevance

2.4.1 *Acanthamoeba* spp.

Infections with *Acanthamoeba* spp. are rare but several cases have been reported from all over the word.

Until now, about 2000 cases of AK were diagnosed worldwide. Especially in industrial countries increase of AK cases associated with contact lenses have been observed. Majority of patients were from the USA and Europe, whereas the most affected country is Great Britain. Also in Austria, a few cases of AK have been reported (Walochnik and Aspöck, 2002).

The number of GAE cases caused by *Acanthamoeba* spp. is about 150. European cases have been reported by a few authors. A case of detecting *Acanthamoeba* spp. in CSF, during a routine examination was reported by Petry et al. (2006). In that case, a 64 year-old woman from Germany presented with headache and nausea but without neurological symptoms. Computer tomographical findings were air-filled cavities in the brain, called pneumatocele but no meningitis or encephalitis has been diagnosed. CSF examination led to the detection of amebic trophozoites and microscopically investigation resulted in the identification of *Acanthamoeba* spp. A subsequent performed PCR characterized the amoebae as *Acanthamoeba* spp. genotype T4. The woman left the hospital after treatment with parenteral fluconazole, oral sulfadiazine, metronidazole and rifampin without neurological aftereffects.

In Belgium, a 66-year-old hepatitis C infected woman died in hospital, after presenting with status epilepticus and subsequent seizures. Autopsy showed *Acanthamoeba* spp. trophozoites and cysts in necrotic lesions of the brain and meninges. Sequencing revealed that these acanthamoebas had genotype T4 (Meersseman et al., 2007). Apart from these cases from Germany and Belgium, cases of GAE in Austria were published by Aichelburg et al. (2008) and Lackner et al. (submitted publication). A 25-year-old HIV negative native Indian, living in Austria, presented with neurological and respiratory syndromes as well as skin lesions. Clinical diagnosis showed that the young man had a co-infection with *Mycobacterium tuberculosis* and *Acanthamoeba* genotype T2 (and not genotype T4, which is the typical GAE-causing genotype) (Walochnik et al. 2008). Treatment with miltefosine and amikacin led to a positive result and the patient was released from hospital. Lackner et al. (submitted publication) reported a case of GAE

due to *Acanthamoeba* genotype T5 in a 17-year-old HIV-negative male in Austria. The patient had fever, meningitis, decreased consciousness and a history of a surgically treated sinusitis. It is assumed, that the patient became infected nosocomially because of a leaking pipe in the ceiling above the care unit. The patient survived the GAE after treatment with rifampicin, cotrioxazole and fluconazoe, but remained with a neurological syndrome.

2.4.1.1 *Acanthamoeba* keratitis

The upward trend of using soft contact lenses led to an increase of *Acanthamoeba* keratitis in the 1980s, a disease that was first described in the early 1970s (Hammersmith, 2006).

Acanthamoeba keratitis is a painful progressive corneal disease that generally affects immunocompetent individuals that do not develop a protective immunity so that a reinfection with *Acanthamoeba* spp. is possible. The symptoms of AK include redness, photophobia, tearing and lid edema. Although symptoms can vary remarkably the most characteristic one is a ring forming stromal infiltration caused by the invasion of inflammatory cells. (Claerhout et al., 2004)

Different reports of *Acanthamoeba* keratitis have shown that this disease can be caused by several *Acanthamoeba* species and genotypes but it has been emphasised that genotype T4 is the causative of almost all AK cases (Booton et al., 2005). Different *Acanthameoba* spp. genotypes T3, T4, T5, T6 and T11 are associated with AK (Walochnik et al., 2000, Seal et al., 2003, Spanakos et al., 2006).

The most important factors for *Acanthamoeba* keratitis are contact lens wear and poor contact lens hygiene. Many contact lens wearers use home-made saline. But not only the home-made saline is a risk for *Acanthamoeba* keratitis, it has been shown that most commercially available contact lens solutions are ineffective against *Acanthamoeba* spp. (Hiti et al., 2005)

Very popular contact lenses are silicone hydrogel lenses made of balafilcon A and lotrafilcon A which were found to be easy to adhere to for *Acanthamoeba* spp. trophozoites (Hammersmith, 2006).

Other important predisposing factors are corneal traumata due to injury by a foreign body. A subsequent exposure to contaminated water can result in an invasion of the amoebas through small lesions in the cornea, which can then lead to *Acanthamoeba* keratitis. In the beginning of the infection, the location of the acanthamoebae is limited to

the corneal epithelium, but during progress of the disease, the amoebae invade the underlying stroma resulting in an inflammation of the eye.

Diagnosis

Unfortunately, there are a lot of cases in which this disease has been diagnosed mistakenly as herpes simplex keratitis or keratitis caused by fungi or by *Pseudomonas aeruginosa*. Generally, *Acanthamoeba* keratitis should be considered in every case that a patient with chronic corneal ulcers does not response to antibiotic therapy. Trophozoites and cysts can be detected from corneal material by microscopy and isolated from *Acanthamoeba* keratitis patients by using corneal scraps or biopsy and culture on non-nutrient agar plates coated with *E. coli* as a food source (Shi et al., 2009). The agar plates should be incubated at 28°C to 35°C but not higher because some *Acanthamoeba* strains do not grow at temperatures higher than 35°C. The incubated plates should be checked every day for amoebae.

An alternative standard technique for detection of *Acanthamoeba* spp. in clinical samples is PCR with subsequent designation of the genotype (Schroeder et al., 2001).

Treatment

Treatment of AK is rather complex. The standard treatment includes a combination of polyhexamethylene biguanide PHMB (0.02%), chlorhexidine (0.02%), imidazol (1% solution), propamidin isethionate in combination and not as a monotherapy. It is necessary to apply the medication every hour around the clock for the first days of therapy. Treatment has to bee applied for several weeks to months. For some reasons an antibacterial, antiviral, antifungal or corticosteroid treatment can improve the patient's state in the beginning of therapy but usually subsequently leads to a worsening of the disease.

If an *Acanthamoeba* infection of the eye is not treated in an early stage of the disease, the trophozoites invade deeper layers of the cornea which then requires a therapy for several months up to one year or longer.

An alternate approach is debridement. In some cases a debridement is successful in removing infectious organisms if the epithelium alone is involved in the infection so that a subsequent medication can be improved.

Unfortunately, *Acanthamoeba* cysts are very resistant and can provoke a recurrence of AK, so a constant monitoring of the patient is essential for a complete healing.

Thebpatiphat et al. (2007) reported on amniotic membrane transplantation (AMT) to treat three cases of *Acanthamoeba* keratitis. With AMT technique it was possible to heal

epithelial defects and reduce the inflammation in two cases of painful and nonhealing AK (Thebpatiphat et al., 2007). AMT is an effective method to reconstruct the ocular surface by transplantation of amniotic membrane obtained from donors undergoing caesarean section (Sangwan et al., 2007).

In case of a nonhealing, unresponsive AK a penetrating keratoplasty (PK) can be accomplished. For postoperative treatment 0.02% chlorhexidine eye drops and 0.5% metronidazole should be applied four times a day for three weeks up to one month. In addition, the treatment with corticosteroids in cases of severe inflammation of the eye after PK and intravenous treatment with hydrocortisone can be effective. The development of endophthalimits after PK can lead to enucleation (Shi et al., 2009).
Treatment of AK within 18 days of onset of the first symptoms may avoid penetrating keratoplasty and results in a better outcome (Claerhout et al., 2004)

2.4.1.2 Granulomatous amoebic encephalitis (GAE)

Granulomatous amoebic encephalitis is an opportunistic, slowly progressive disease that affects immunocompromised hosts at any time of the year without seasonal pattern. Infection occurs by inhalation of dust containing cysts of acanthamoebae through the nasal passages and lungs. Another route of infection is by introducing the amoebae through skin lesions. *Acanthamoeba* spp. enters the CNS by hematogenous spread from a primary site in the lungs or skin. The vast majority of GAE cases have been caused by genotype T4, however genotypes T1, T10, T12 can cause GAE if they invade the central nervous system (CNS) as reported by Booton et al. (2005). GAE occurs mainly in individuals with underlying diseases like HIV/ AIDS, diabetes, tuberculosis or patients who had organ transplantation. Other predisposing factors for this disease include alcoholism, steroid treatment or drug abuse (Visvesvara et al., 2007).
However, Singhal et al. (2001) reported on 3 cases of GAE in immunocompetent children without any of the predisposing factors.
The characteristic symptoms of this disease are headache, fever, behavioral changes, hemiparesis, lethargy, stiff neck, aphasia, ataxia, vomiting, nausea, cranial nerve palsies, increased intracranial pressure, seizures and ultimately death. Death occurs due to hemorrhaging necrotic lesions with meningeal irritation and encephalitis. Another common symptom is facial asymmetry that results from facial palsy with numbness. The most affected areas of GAE are the basal ganglia, midbrain, brainstem and the cerebral hemispheres, which are often edematous, with extensive hemorrhagic necrosis involving

the parietal, temporal and occipital lobes. Chronic granulomatous encephalitis is the result of typical lesions located in the CNS parenchyma. Although the incubation period is unknown, for the establishment of clinical signs several weeks or months may be necessary (Visvesvara et al., 2007).

Visvesvara and Stehr-Green (1990) reported that not only humans can be infected by *Acanthamoeba* spp. These amoebas also cause infections in animals like gorillas, monkeys, dogs, ovines, bovines, horses, kangaroos, birds, reptiles, amphibians and fishes.

Diagnosis

Diagnosis of GAE includes techniques like PCR from liquor samples, immunofluorescence staining from tissue samples (Walochnik et al., 2007), and inoculating tissue on NN agar plates for the culture of amoebae (Seijo Martinez et al., 2000, Walochnik et al., 2007). Observations of *Acanthamoeba* spp. trophozoites can be difficult in wet preparations of CSF due to the fact that the trophic amoebas resemble macrophages, so they can remain undetected in CSF samples.

Treatment

Currently there is no drug of choice for GAE treatment. Different drugs have been used with varying success for treatment of GAE. For example a human immunodeficiency virus (HIV) positive male with GAE was treated successfully with fluconazole and sulfadiazine and the excision of lesions as reported by Seijo Martinez et al. (2000). Other potentially effective drugs against *Acanthamoeba* are streptomycin, trimethoprim-sulfamethoxazole, miltefosine (oral, topical), amikacin (intrathecal, intravenous) and flucytosine (Walochnik et al., 2008).

2.4.1.3 Cutaneous Acanthamoebosis

It is not clear if the skin lesions represent a primary focus of infection. Maybe they are the result of an *Acanthamoeba* spp. infection which is hematogenously disseminated from other sites of the body such as the respiratory tract or the CNS.

Cutaneous infections caused by acanthamoebae generally occur in immunocompromised patients (AIDS or organ transplant patients). This disease is manifested in the beginning by the presence of firm papulonodules that drain purulent

material and develops later into non-healing idurated ulcerations and hard erythematous nodules or skin ulcers.

In general, the histological samples from the skin lesions caused by *Acanthamoeba* spp. show foci of necrosis surrounded by inflammatory cells, vasculitis, trophozoites and cysts of this amoeba. The histological appearance of the skin lesion is similar to those occurring by infection with fungi, viruses, mycobacteria or inflammation caused by a foreign body.

Diagnosis

Cultivation of *Acanthamoeba* spp. from cysts and trophozoites containing tissue samples from a biopsy is possible.

Tissue material from cutaneous *Acanthamoeba* lesions can be inoculated on non-nutrient (NN) agar plates coated with *E. coli* or *Enterobacter aerogenes* as a food source as well as in amoeba growth medium filled in sterile flasks. Furthermore, the tissue samples can also be inoculated onto mammalian cell cultures. The eventuality of a secondary infection should be considered. For that reason antibiotic drugs should always be added to amoebae cultures from biopsy samples. Biopsy samples usually are stained with hematoxylin and eosin (H&E), calcofluor, or immunofluorescence staining. Molecular biological techniques for *Acanthamoeba* spp. detection in skin lesions are polymerase chain reaction (Walochnik and Aspöck, 2005).

Treatment

There is no treatment for cutaneous Acanthamoebosis. Different drugs were given to patients, like intravenous pentamidine and itraconazole with ketoconazole and chlorhexidine. Rosenberg and Morgan (2001) reported on an AIDS patient with an ulcer of the nasal septum and nodules on the lower extremity. This patient has been successful treated with itraconazole, azithromycin, 5- flucytosine and rifampin. Another successful treatment with drugs was reptorted by Walia et al. (2007). A woman with a lung transplant and subsequently drug based immunosuppression developed a cutaneous acanthamoebosis which was successfully treated with intravenous amphotericin B lipid complex (ABLC) and intravenous voriconazole for a few weeks.

2.4.2 *Balamuthia mandrillaris*

GAE due to *B. mandrillaris* is a rare disease in humans. Worldwide, about 80 cases from different countries have been reported. The majority of these cases occurred on the

American continent. About 50 cases were reported from the United States, 45 from South America and Mexico. 50% of the cases reported from the United States involved people of Hispanic ancestry, though only 12% of the inhabitants are Hispanic Americans. Moreover, 73% of GAE cases in the United States occurred in California (Schuster et al., 2004b). GAE also occurred on other continents like Australia (eight cases) and Asia (two case). Until now, two cases of amoebic encephalitis due to *B. mandrillaris* in Europe were published. One case of *Balamuthia* GAE in a child from the Czech Republic was reported by Kodet et al. (1998). Another case occurred in Portugal, where an 8-year-old boy died because of *B. mandrillaris* infection (Tavares et al., 2006). Until now, no case of GAE due to *B. mandrillaris* has been reported from Austria.

***Balamuthia* GAE**

Balamuthia mandrillaris can, like acanthamoebas cause GAE (granulomatous amoebic encephalitis) in humans and animals including primates like gorillas, baboons, gibbons, monkeys (Visvesvara et al., 1990; Rideout et al., 1997; Canfield et al., 1997) horses (Kinde et al., 1998) and dogs (Foreman et al., 2004; Finnin et al., 2007). GAE due to *Balamuthia mandrillaris* concerns immunocompromised hosts as HIV/ AIDS patients and intravenous drug users but also immunocompetent individuals can develop a GAE. People of any age but especially young children and older individuals are at risk to develop a GAE (Visvesvara et al., 2007). Interestingly, approximately 44% of GAE cases due to *B. mandrillaris* have occurred in patients of Hispanic ethnicity, whereas the Hispanic population in the Unites States averages 12%. Reasons for this are probably genetic, environmental or social factors (Bakardjiev et al., 2003).

An infection by *B. mandrillaris* similarly to *Acanthamoeba* spp. does not depend on seasonal changes, the disease occurs at any time of the year. *B. mandrillaris* enters the body via a skin lesion or through the respiratory tract by inhalation of wind-blown soil particles carrying cysts. In both cases, the amoebas that emerge from the cysts cause a type of granulomatous encephalitis by hematogenously transport to the CNS where the amoebas access the brain by penetrating the blood-brain barrier (Deetz et al., 2003; Maciver, 2007). The chronic, subacute phase of granulomatous amoebic encephalitis develops in a time period between 2 weeks and 2 years. Main symptoms of Balamuthia GAE are headache, fever, stiff neck, nausea, vomiting, skin lesions, hydrocephalus, otitis media, a confused state and haemorrhagic necrotizing lesions in the brain. In this case, death occurs within days.

Skin infections by *B. mandrillaris*

GAE is not the only disease caused by *B. mandrillaris*, individuals with an infection by this amoeba can also develop skin infections, similar to that caused by *Acanthamoeba*. The skin lesions are painless, with a size of several centimetres and a thickness of a few millimetres. Usually, most patients have only one lesion in the face, trunk, hands and feet, but more lesions are possible. A skin lesion due to *B. mandrillaris* leads to GAE in a time period from one month to two years, but not necessarily. One unique case of *Balamuthia* skin lesions has been described in Peru. However, the patient showed no neurological symptoms although the amoeba was isolated by a skin biopsy and he recovered from the disease without any medical treatment (Visvesvara et al., 2007).

Diagnosis

B. *mandrillaris* can be detected in CSF preferably by indirect immunofluorescent antibody test. Generally, it is difficult to differentiate between *B. mandrillaris* and *Acanthamoeba* spp. in tissue from biopsies by light microscopy because of their similar morphology. Clinical samples should be analyzed as soon as possible and freezing may cause destruction of trophozoites but not of the cysts within the samples (Visvesvara et al., 2007).

Treatment

The prognosis for patients with a *B. mandrillaris* infection is poor because of the problematic diagnosis and the lack of effective medical agents.

Unfortunately, there is no standard treatment for *Balamuthia* GAE. Patients, who survived *Balamuthia* GAE, were treated with combinations of petamidine isethionate, clarithromycin, fluconazole, sulfadiazine and flucytosine (Deetz et al., 2003). In addition, in case of cutaneous infections the surgical removal of large skin lesion can support the healing process. A case of GAE due to *B. mandrillaris* was successfully treated with treatment with a combination of dexamethazone, azathioprine, rifampicin, ketoconazole, metronidazole, ceftriaxone and co- trimoxazole, while treatment with dexamethazone, fluconazole and flucytosine has been ineffective (Bodi et al., 2008).

Dunnebacke (2007) reported on the acquisition of nourishment from mammal cells by *B. mandrillaris*. Therefore, the amoebas can invade into the target cells and this could possibly be an opportunity to mask and protect the amoebas. Generally, the protection by masking against antimicrobial agents and immune cells of an infected host makes the GAE treatment ineffective.

2.4.3 *Naegleria fowleri*

About 200 cases of PAME due to *N. fowleri* have been reported worldwide. Many cases occurred in the United States, but also cases from Australia and New Zealand were published (Schuster and Visvesvara, 2004). A case of PAME in two children from Arizona was reported by Marciano- Cabral et al. (2003). In this report, 19 samples from domestic water sources form the children's home were collected and investigated for the occurrence of *N. fowleri*. 17 of 19 samples turned out to be *N*. *fowleri* positive and the source of infection seemed to be the bathtub where the kids routinely played.

Until now, no case from Austria has been reported, although a few cases occurred in Europe. In the year 1968, 16 cases of *N. fowleri* infection in Bohemia have been published by Cerva and Novak (1968). From 1970 to 1972, four cases of PAME occurred in children with a history of swimming in the surrounding area of Antwerp/ Belgium (cit. in De Jonckheere, 2002). Moreover, a nine year old boy was infected by *N. fowleri* while playing in a swimming hole connected to the Po River in Italy (Cogo et al., 2004).

PAME

N. fowleri is the only species of the genus *Naegleria* that has been isolated from humans, though it was possible to isolate more than 30 species of this genus from the environment (Marciano- Cabral and Cabral, 2007).

Naegleria fowleri is the causative of PAME (primary amoebic meningoencephalitis), an acute and rapidly fatal central nervous system disease that involves healthy children with a history of swimming and diving in freshwater lakes or not adequately chlorinated swimming pools. Due to the fact that *N. fowleri* infects healthy people it is not regarded as an opportunistic amoeba in difference to *Acanthamoeba* and *Balamuthia*. The path of infection is via the olfactory neuroepithelium, the amoeba migrates to the cribriform plate where it enters the central nervous system (CNS). This results in a rapid destruction of the olfactory bulbs, widespread meningitis and hemorrhagic, necrotizing encephalitis without a hematogenous spread of the amoebae. The most battered regions of the brain are the frontal and olfactory lobes due to their proximity to the cribriform plate.

The period of incubation between exposure and clinical manifestation varies from one day to one week. Frequent symptoms are headache, fever, nausea, vomiting and a stiff neck. A progressive neurological deterioration, coma and death occur in 95% of cases within a few days caused by brain herniation that increases the intra-cranial pressure (Visvesvara et al., 2007).

Young and healthy persons are most affected by PAME, maybe due to their behaviour while swimming or to a higher porosity of the cribriform plate.
Shrestha et al. (2003) reported one of the few cases of PAME in a chronically ill person. This person lived in Nepal, had no history of swimming or of exposure to free- standing water, so it is unclear how the patient was infected with *N. fowleri*.

Diagnosis

Only trophozoites and flagellates can be detected in clinical samples by microscopic investigation of cerebrospinal fluid (CSF) which can be hemorrhagic and cloudy with a colour from grey to yellow-white. *N. fowleri* forms no cysts in the tissue in contrast to *Acanthamoeba* spp. and *B*. *mandrillaris*. Furthermore, a slight increase of erythrocytes and leukocytes can be observed in the CSF. Microscopically *Naegleria* spp. can be detected by staining with Calcofluor-white, Acridine orange or Giemsa staining. In case of histological diagnosis HE staining is performed. *N. fowleri* can be detected in biopsy material from the brain, especially from the lobus olfactorius by using molecular biological techniques like PCR or ELISA (Walochnik and Aspöck, 2005; Neumeister et al., 2009). The gold standard for the diagnosis of PAME is the microscopical investigation of CSF.

Treatment

Due to the high sensitivity of *Naegleria* spp. to the antifungal drug amphotericin B (AmB), this is the drug of choice for PAME treatment (Shrestha et al., 2003). However, AmB has a lot of adverse effects like renal toxicity, anaemia, fever, nausea or headache. Other effective agents are miconazole, rifampin, azithromycin, clotrimazole, intraconazole, fluconazole and ketoconazole. The dosage of these drugs depends on the amount of amoebae, the virulence of the respective strain as well as the health state of the patient (Visvesvara et al., 2007).

2.4.4 Free-living amoebae in animals

Several authors reported the presence of free-living amoebas in samples from animals. It is important to differentiate between the simple presence of amoebae by chance or the appearance of amoebae due to a true colonization. Madrigal Sesma et al. (1988) suggested that a true colonization bases on the appearance of vegetative forms of amoebae, in various organs. Furthermore, amoebic parasitism is difficult to prove even with the casual detection of trophozoites. However, apparently a correlation between

reptile host taxon and dispersion of cysts and consequently the ability for colonizing the intestinal tract of the host is not influenced by phylogeny (Hassl and Benyr, 2003). Furthermore, Hassl et al. (2000) suggested, that pseudo parasitism due to uptake and dispersion of unchanged cysts is not given in reptiles because of the disability to isolate cysts of the same amoeba species from faeces samples and the terrarium.

The authors Frank and Bosch (1972) reported the detection of amoebae of the genera *Acanthamoeba* and *Naegleria* in tissue and gut samples from reptiles. Furthermore, amoebas were isolated by swabbing the cloaca of asymptomatic animals. The majority of the investigated animals were co-infected with *Acanthamoeba* spp. and *Entamoeba*, predominantly *Entamoeba* type E2.

Amoebic infections due to *Acanthamoeba* spp. in animals have been the topic of several publications during the past years. Lorenzo- Morales et al. (2007) screened for acanthamoebas in wild squirrels from the Canary Islands and Morocco and were able to isolate *Acanthamoeba* spp. from 14 samples. Furthermore, Visvesvara et al. (2007) reported the detection of *Acanthamoeba* spp. in liver tissue samples of a dead keel-billed toucan (*Ramphastos sulfuratus*) in a zoo in Texas. Lethal *Acanthamoeba* spp. infection occurred in a SIV-infected rhesus macaque in the USA as reported by Westmoreland et al. (2004) or in a greyhound published by Bauer et al. (1993). Similar to cases in humans trophozoites were located in the brain and lungs. Walochnik et al. (1999) reported the isolation of *Acanthamoeba castellanii*, *Echinamoeba* sp., *N. gruberi* and *Hartmannella vermiformis* from necrotic tissue of a *Basiliscus plumifrons*.

The appearance of FLA in cold- blooded animals was reported by Madrigal Sesma et al. (1988). For this study, al samples of 150 reptiles and amphibians were analyzed for the presence of protozoa. Encountered amoebae were *Acanthamoeba* spp., *Echinamoeba* sp., *Hartmannella* spp., *N. gruberi* and *Vahlkampfia* sp. Another study on free-living amoebae in reptiles was published by Hassl and Benyr. (2003). The authors analyzed faeces samples of captive reptiles and amphibians and found *Acanthamoeba* spp and *Naegleria* spp. However, there was no information on the animals' health status. Symptoms of *Acanthamoeba* spp. infections include skin lesions, neurologic and respiratory disease and the gut. However, it is also possible that infected animals are free of symptoms.

Moreover, there have been several reports on *B. mandrillaris* infections in animals. GAE due to *B. mandrillaris* in a horse was reported by Kinde et al. (1998) and two cases of infections in dogs by Foreman et al. (2004) and Finnin et al. (2006). Furthermore, there are several publications on fatal infections with these amoebas in primates like a mandrill (*Papio sphinx*) in San Diego by Visvesvara et al. (1990), an orang utan (*Pongo pygmaeus*) by Canfield et al. (1997) or different primates from the San Diego Zoo and San Diego Wild Animal Park by Rideout et al. (1997). Typical symptoms of GAE in animals are depression, lethargy, head pain, vomiting, seizures, circling and head tilt to the left or to the right, incapable of any movement or dragging an extremity, coma and death.

Primary amebic meningoencephalitis (PAME) in animals due to *N. fowleri* was reported by several authors. Lozano-Alarcón et al. (1997) published a case of PAME in a South American Tapir (*Tapirus terrestris*) in a zoo in Phoenix, Arizona (USA). Fatal *N. fowleri* infections in cows were reported by Daft et al. (2005) and by Visvesvara et al. (2005). The perished cows lived in California (USA) and the disease occurred during the summer months of 1998 and 1999. Typical symptoms of primary amoebic meningoencephalitis due to *N. fowleri* in animals are anorexia, facial paralysis, circling, ataxia, convulsions, a head tilt to the left or to the right and the disability to stand without assistance. The South American tapir and the Holstein cattle died because of the amoebic infection in spite of medical treatment.

2.5 Entamoeba invadens

2.5.1 History

In 1875 the Russian physician Fedor Aleksandrovich Lösch identified and described *Entamoeba histolytica* as an agent of chronic diarrhoea and produced the disease in an experimental animal (Lösch 1875 in Lesh, 1975).
In the year 1925 Chatton postulated the family Entamoebidae to encompass the genera *Entamoeba, Endamoeba* (a parasite of invertebrates), *Endolimax*, *Dientamoeba* and *Iodamoeba* (Silberman et al., 1999).

2.5.2 Phylogeny

The genus *Entamoeba* belongs to the class *Archamoebae*, which is a member of the phylum *Amoebozoa*. The phylum *Amoebozoa* is the sister phylum of the *Opisthokonta*

and both are part of the monophylum *Unikonta* as shown in Figure 1 (Walochnik and Aspöck, 2007).

The genus *Entamoeba* has been divided into different groups of species based on cyst morphology. Cysts can contain one, four or eight nuclei, and there are also groups that do not form cysts (Clark, 2000). Microscopy is a possibility for *Entamoeba* differentiation based on the size of cysts and trophozoites. In addition, the host species, the number of nuclei and the existence of chromatoid bars are significant considerations for species determination.

Bosch et al. (1972) delimitated four different *Entamoeba* types isolated from gut and tissue samples of reptiles based on their morphological characteristics.
Entamoeba type E1: the size of the cyst is 15 µm - 30 µm and the pseudopod's size compared to the size of the trophozoite is in a ratio of less than a fourth. Furthermore, the endoplasm contains vacuoles of different sizes and the trophozoites of E1 feed on bacteria in culture.
Entamoeba type E2: the cysts size of E2 is 15 µm - 30 µm, and the pseudopod's size is in a ratio of less or more than a fourth to the trophozoites size. The endoplasm contains predominantly small vacuoles and type E2 feeds on bacteria in culture.
Entamoeba type E3: the cyst size is 10 µm - 20 µm and the pseudopods size is in a ratio of less than a fourth to the size of the trophozoites. In culture, this *Entamoeba* type feeds on rice starch and the endoplasm contains predominantly small vacuoles. Bosch et al. suggested that this type is probably *E. invadens*.
Entamoeba type E4: Type E4 has a cyst size from 20 µm - 30 µm and compared to the trophozoite's size, the pseudopod's size is in a ratio of more or less than a fourth. This *Entamoeba* type feeds from bacteria and rice starch and contains small vacuoles in the endoplasm.

One of the most important morphological features for *Entamoeba* taxonomy is the number of nuclei in mature cysts, which varies between one, four and eight nuclei. The interference of the last common ancestor of all *Entamoeba* species is difficult to find. The taxa with eight nuclei per cyst have a basal position followed by species with a single nucleus in the cyst and species without encystment, the prior of which diverges into species that include four-nucleated Entamoebae. The assumption of a cyst building ancestor for every node in the clade of *Entamoeba* leads to the possibility that *E. gingivalis* lost its ability to encyst. Interestingly, there are only two pathogenic *Entamoeba*

species (*E. histolytica* and *E. invadens*) and these belong to separate clades within the genus. This phylogenetic tree postulated by Silberman et al. (1999) shows that host switching of the host seems to have been common event in the lineage of *Entamoeba* because there is no evidence for co-evolution of *Entamoeba* species and their hosts.
Based on tRNA gene analysis, the two parasitic amoebas in reptiles *Entamoeba invadens* and *Entamoeba terrapinae* are not closely related to each other or to *E. histolytica, E. moshkovskii* and *E. dispar* (Tawari et al., 2008).

2.5.3 Biology

The anaerobic *E. invadens* is considered a commensal to most species of the chelonians if they are herbivorous. In that case, the amoebae live at least in a symbiotic relationship with the chelonians without any pathogenicity. In herbivorous turtles the amoebae build cysts by taking up nutrients from ingested plants and *E. invadens* can complete its life cycle without any pathogenicity to the gastro-intestinal tract. However, the amoebae cannot complete its life cycle in the intestine of a carnivorous reptile without specific nutrients. In that case the entamoebas are forced to invade the mucosa of the host and cause infection.

2.5.4 Life cycle

Entamoeba invadens shows a direct life cycle (Figure 9) with the cyst as the infective stage. The host has to ingest the quadri-nucleate cysts with faecally contaminated food or water. When the cysts reach the large intestine they start to transform into motile trophozoites and reproduce themselves by binary fission. On the one hand, trophic amoebae can invade the mucosa of the host's intestine and on the other hand, they can live in the lumen without harming the mucosa. In that case, *E. invadens* does not cause disease. Lesions caused by *Entamoeba* trophozoites have been found in the whole gastro-intestinal tract, from the stomach to the colon.
Usually, trophozoites can be found in diarrheal faecal specimens while the cysts can be observed in formed stool

Figure 9 shows the life cycle of *E. histolytica*, which is the same as the one of *E. invadens*, only the host differs.

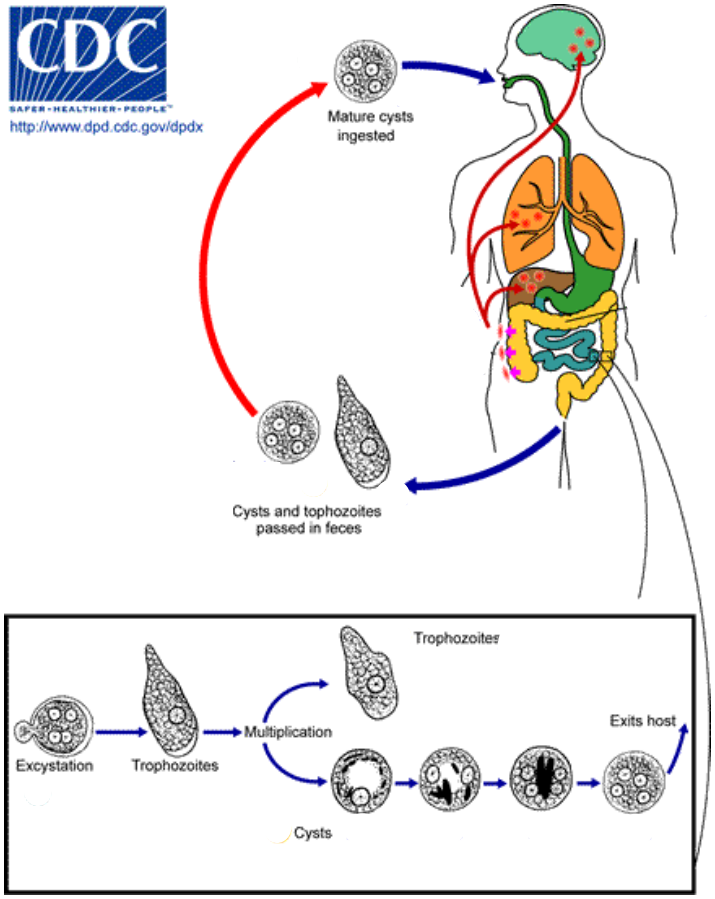


Figure 9: Life cycle of *E. histolytica*: trophozoites and cysts are passed by faecally contaminated water, food or by hand. The amoebae excyst in the small intestine and migrate to the large intestine where it multiply and produce new cysts. Furthermore trophozoites can enter the blood stream and can be transported to different sites of the body (liver, brain and lungs). Trophozoites and cysts are passed with faeces to new hosts.

(Modified from: http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Amebiasis_il.htm)

2.5.5 Physiology and morphology

2.5.5.1 Trophozoites

Morphological features of the tissue invasive *E. invadens* trophozoites are one single large ring-like nucleus with a small central karyosome and a size from 10-40 µm in diameter. One hallmark of the Entamoebae are chromatoid bars which are aggregates of ribosomes. The DNA content in these chromatoid bars is high and this is why they stain so well.

The triggers for encystment are still unknown. Trophozoites can not survive for a longer time in the environment. Byers et al. (2005) reported on the inhibition of encystation in *E.*

invadens by short-chained fatty acids produced by endogenous bacteria within the colon of infected animals.

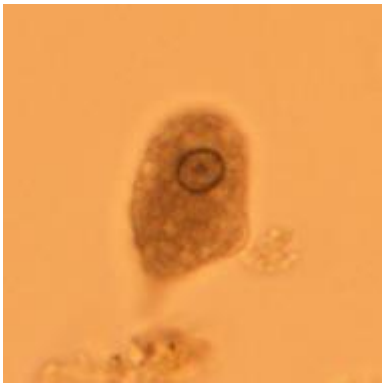


Figure 10: *E. histolytica* trophozoite

From: Walochnik and Aspöck, 2008. The Wilhelm Foissner Festschrift, Denisia 23, 2008. Page 321

2.5.5.2 Cysts

Stress is a trigger for *E. invadens* trophozoites to undergo a process of differentiation, called encystment to form a dormant cyst. The main "task" of the cyst is to transmit the organism from one infected individual to another and to assure the survival of the amoebae under harsh environmental conditions.

The infective cysts of *Entamoeba invadens* measure between 11-20 µm and have a pronounced chitinious cell wall. Furthermore, the cysts contain chromatoid bodies and the numbers of nuclei vary between 2 to 4 (mature state). The number of nuclei in the cysts is an important feature to differentiate *E. invadens* cysts from other *Entamoeba* cysts, which contain eight nuclei and are frequently found in reptile faeces. Nonetheless, cysts are resistant against harsh conditions in the environment and the viability of these resisting stages may be weeks to months.

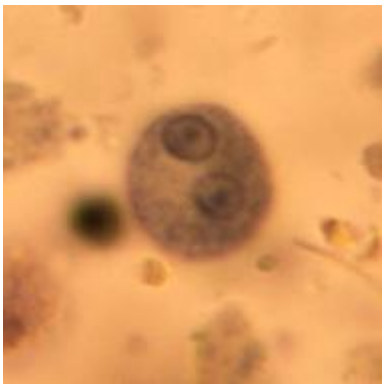


Figure 11: *E. histolytica* cyst

From: Walochnik and Aspöck, 2008. The Wilhelm Foissner Festschrift, Denisia 23, 2008. Page 321

2.5.6 Medical relevance for animals

Entamoeba invadens can cause enteritis, colitis and death in many reptile species. This *Entamoeba* is the most pathogenic and infectious amoeba species in reptiles. Some reptiles like many crocodile and turtle species act as carriers and are not as susceptible as snakes and lizards, which show the highest mortality.
Trophic *Entamoeba invadens* damage the colon of the host by invading the epithelium which results in increased thickening of the mucosa. However, this may also result in perforation of the intestinal mucosa and a secondary bacterial infection. Furthermore, haematogenous spread of *Entamoebae* to the liver, peritoneum and other organs of the host is possible if they enter the mesenteric circulation. This causes abscesses, thromboses and blood or mucus containing faeces. Amoebosis in reptiles does not always lead to clinical signs, so it is possible that animals may sudden be found dead. Clinical signs vary between no abnormalities to anorexia, dehydration, bloody faeces and general debility of the infected animals (Miller, 2007).

Meerovitch (1960) reported on the optimal growth temperature for *E. invadens* in snakes. This experiment showed that *E. invadens* cannot establish infection at high temperatures (34°C to 37°C). Optimal temperature was (20°C to 30°C).

Unfortunately, there are only a few reports on amoebic infections in chelonians, for example MacNeill et al. (2002) reported more than 40 dead hatchling wood turtles due to *Entamoeba* sp. Ozaki et al. (2000) published a case of amoebosis in flat- shelled spider tortoises (*Acinixys planicauda*) in Japan. Both authors described symptoms like anorexia, depressions, lethargy, inactivity and periocular swelling. However, the animals died in spite of medical treatments.

Cases of amoebosis in snakes due to *E. invadens* have been reported by several authors. Jakob et al. (1995) published the results of investigated tissue samples from the intestine of 51 snakes from which 22 turned out to be positive for *E. invadens*. However, the snakes showed typical symptoms like an inflamed intestinal wall.
A case of four dead ball pythons (*Python regius*) in Japan has been reported by Kojimoto et al. (2001). The snakes were dissected and tissues samples from lung, heart, liver, stomach, large and small intestine were investigated. As almost all organs of the snakes showed a protozoan infection, this was a case of disseminated infection. An

immunofluorescence assay with an antibody against *E. histolytica* was performed and in tissue samples detected cysts showed an immuno-positive reaction.
Richter et al. (2008) investigated tissue samples from 182 snakes by in-situ hybridization to detect *Entamoeba* spp. and *E. invadens*. Positive signals with the probe for *E. invadens* were detected in the colonic walls of nine snakes which also showed positive signals with the *Entamoeba* spp. probe. Interestingly, eight of these nine *E. invadens* positive snakes were giant snakes. Just in one case symptoms like bloody vomitus and diarrhoea were observed, whereas the other snakes apparently seemed to be healthy. Necropsy demonstrated that all nine snakes had severe diphtheroid colitis and in five of them additionally necrotizing hepatitis was observed. However, trophozoites together with inflammatory reaction and tissue necrosis were detected in the liver, lungs, heart, kidneys and spleen when suggests haematogenous spread in case of multiple organ infections.

Bosch and Deichsel (1972) investigated gut and tissue samples from 71 reptiles for amoebas and isolated 114 culture types of the genera *Acanthamoeba* and *Entamoeba*. Morphological analysis delimitated four *Entamoeba* types namely E1, E2, E3 and E4. *Entamoeba* type 1 was isolated from tissue samples and swabs from snakes and chelonians which suffered from amoebosis as well as from asymptomatic animals. *Entamoeba* type 2 caused disease more frequently than type E1 in different reptiles. Furthermore, E3 (probably *E. invadens*) was mainly isolated from tissue samples and sometimes from the gut of lizards, snakes and chelonians. *Entamoeba* type E4 was isolated from lizards, snakes and chelonians which died either of amoebosis or acted as carriers. However, animals which suffered from acute amoebosis appeared to be infected more often with *Entamoeba* type E1 and E3 than with E2 and E4.

Diagnosis of *E. invadens* infection

Generally, the detection of an *E. invadens* infection in reptiles is not easy due to the possibility that the infected animal lacks clinical signs. Also, a low burden of parasites in carrier animals like crocodiles and some turtle species makes it hard to find amoebae in the investigated material.
Infections of *E. invadens* can be diagnosed by detecting trophozoites and cysts in a saline faecal smear from fresh stool containing blood or mucus or by faecal flotation. The slowly moving trophozoites can be easily observed by light microscopy.

Exposed to the environment, trophozoites can only survive for a short period. This fact is very important for the detection of trophozoites in faecal samples because if the samples are older than several hours it is hard to find remaining trophozoites.
Unfortunately, it is quite difficult to differentiate *E. invadens* cysts from other non-pathogenic *Entamoeba* spp. cysts as e.g. *E. barreti* and *E. ranarum.*

Treatment

Animals infected with *E. invadens* have been treated based on human treatment regimens with metronidazole in different dosages ranging from 275 mg/ kg orally once (Denver et al., 1999), 100 mg/ kg orally (Kojimoto et al., 2001) to 20 mg/kg orally daily for 5 days (Mac Neill et al., 2002). However, metronidazole shows activity against *Entamoeba* trophozoites but does not affect cysts. The treatment with metronidazole at high dosages leads to side effects like neurological signs (Miller, 2007), hepatotoxicity (Denver et al., 1999) or several physical abnormalities and death of four animals in a study on pharmacokinetics of metronidazole after a single intracoelomic injection in turtles by Innis et al. (2007). However, these turtles died 2-5 months after the end of the study and histopathologic evaluation showed bacterial and parasitic disease. It is not clear if these pathological changes were side effects of this study. Denver et al. (1999) reported on the safe treatment of black rat snakes with metronidazole, iodoquinol and diloxanide furoate in three different dosages.

2.6 Aims:

Amoebae in animals of a zoological collection are of twofold medical relevance. Firstly, transmission of amoebas between animals, secondly the potential risk of infection of humans, keepers and visitors, coming into close contact with these animals.
To our knowledge, this is the first study on the occurrence of FLA and *E. invadens* in reptiles of a zoological collection based on culture and PCR.

E. invadens is an anaerobic protozoon, that might cause a severe amoebosis and lead to death in different reptile species within a short period of time. To our knowledge, previously published studies on *E. invadens* in reptiles related to sick or dead animals. Moreover, the rate of infection with *Entamoeba invadens* especially in asymptomatic reptiles is largely unknown.

3 Material and Methods

3.1 Animals

The majority of the 54 reptiles investigated in this study are animals confiscated at airports or were abandoned and for that reason under quarantine at the zoo in Schönbrunn. The housing and terrariums are located in the basement and in the exhibition of the amphibian and reptile house, the elephant house and rain forest house.

The snakes in the zoo were kept in a room together with a few lizards in the basement of the reptile and amphibian house. The glass terrariums contained small tanks with water and parts of trees but no soil or sand. Most of the snakes were housed separately but in a few cases, two snakes were kept in one terrarium. Furthermore, the snakes cohabit in the terrariums changed from one sample drawing to the other. Moreover, some snakes were part of the exhibition in the reptile and amphibian house or rain forest house in terrariums with soil, water, wood and plants.

In the chelonian room in Schönbrunn, the animals were kept separated from other reptiles in crates filled with sand and sometimes also with water and plants. Marine and freshwater species were kept in water filled plastic tanks. Furthermore, a few chelonians lived together in each crate or tank. To our knowledge, there was no central water supply with a connection to each tank or crate. Heat lamps were installed above the crates for terrestrial species.
Up to the time of sample drawing the giant turtles (*Geochelone elephantopus* A and B) lived in the elephant house in a concreted part of the elephants enclosure separated by a lattice door. This enclosure at the elephant house was a temporary solution while the dwelling of the *Geochelone elephantopus* was under construction. In this temporary enclosure was no water or sand but some straw.

The lizard terrariums were placed in a separate room in the basement of the reptiles and amphibian house and some terrariums were located at the snake room. The terrariums in the separated room were filled with sand and wood. Furthermore, manly several lizards were housed together in one enclosure but a few were housed alone. In addition, these terrariums had no connection to a central water supply.

3.2 Sample drawing

Although 22 mouth and cloaca swaps were collected from 11 snakes, 40 mouth and cloaca swabs and one stool sample from 20 turtles and 32 swabs from 16 lizards. Furthermore two stool samples from fodder animals (mice and rats) were collected. Four tissue samples of a snake which had to be euthanized and two stool samples of a dead snake including soil of the terrarium were collected.

The mouth and cloaca of the reptiles were swabbed by using sterilized cotton tipped applicators moisturized with NaCl solution and sterilized gloves. The samples were transported to the lab in sterile 15 ml BD Falcon tubes.

3.3 Culture

3.3.1 Aerobic amoebas

The aerobic amoebas were isolated and cultivated on NN agar plates, in presence of living bacteria as a food source. The bacterium of choice was *Escherichia coli*. After adding 50 µl of a 48h old *E. coli* culture on a NN agar plate, the bacteria were evenly spread on the agar plate. Subsequently a cross was drawn with a swab on the NN agar plate and the cotton tipped applicator was left in the middle of the plate. The agar plates were sealed with parafilm and incubated at room temperature. *Balamuthia mandrillaris* will not grow with bacteria as a food source but has been grown from mammals cells in tissue culture.

Acanthamoeba* spp. and *Naegleria fowleri

These amoebas were cultured on NN agar plates coated with *E. coli* as a food source and incubated at room temperature.

Balamuthia mandrillaris

B. mandrillaris were cultured on Vero cells in tissue culture. The Amoebas were cultivated in PC1^{TM} medium incubated at 34°C.

Vero cells were cultured in PC1^{TM} medium + CO_2 independent medium.
500 ml PC1- medium (Bio Whittaker, PC-1^{TM} Medium, Lonza) was mixed with supplement, 5 ml L-glutamine (1%) and 5 ml AA (1%) and 500 ml CO_2 Independent medium (1x, GIBCO) mixed with 5 ml L- glutamine (1%) and 5 ml AA (1%). Finally PC1 medium and CO_2 Independent medium were mixed and stored at 4°C.

Tubes with cells were thawed at 37°C in the water bath and then transferred without centrifugation. Subsequently PC1+ CO_2 medium was added and incubated at 37°C. After a few hours the cells were microscopically controlled and the medium has to be changed to wash the DMSO out of the cells.

Cell cultures were subcultured every third day. To split the cells the medium was aspirated and then 5-7 ml trypsin (0.25%) was added so that the surface of the flask completely covered. After 2-3 minutes the cell film changed its colour to white. The trypsin has to be removed and 12-15 ml sterile PC1+ CO_2 was added. After shaking the flasks the medium with the detached cells were split to new culture flasks and 20-30 ml fresh medium (PC1+ CO_2) were added.

The freshly subcultured cells with PC1+ CO_2 medium were transferred to sterile tubes and centrifuged for 10 min. at 18 g/ min. Subsequently the supernatant was discarded and the pellet was carefully resuspended with freezing medium. 1 ml of this suspension was moved to sterile NUNC tubes and 10% DMSO was added. Finally the tubes were stored in liquid nitrogen.

3.3.2 Anaerobic amoebae:

Isolation of *E. invadens*

A cotton tipped applicator and antibiotics (1%; Antibiotic-Antimycotic 100X, Invitrogen/ GIBCO; 10,000 U penicillin, 10,000 µg streptomycin and 25 µg amphotericin B/ ml) were added to a culture flask filled with TYS- I 33 medium for isolation of the anaerobic *E. invadens*. Subsequently the cultures were incubated at room temperature.
For primary isolation of *E. invadens* from swaps was the LE- medium (Diphasic medium, Clark and Diamond, 2002). The cotton tipped applicator and antibiotics (1%) were added to the LE- medium and incubated at 26°C. As the isolation of amoebae out of clinical samples is known to be difficult due to bacterial contamination two different procedures were performed in parallel.

Cultivation in axenic culture

The *Entamoeba invadens* was cultivated in 25 ml culture flasks (Falcon), filled with 25 ml TYS- I 33 medium (Diamond et al. 1978). Every 2- 3 days the nutrients in the medium were used up by the amoebas so that the medium has to bee changed.

Preparation of TYS- I 33 medium

TYS- I 33 (Diamond et al. 1978) is the standard medium for *Entamoeba* spp. cultivation. To prepare TYS- I 33 medium 20.0 g Trypticase Peptone, 10.0 g yeast extract, 2.0 g NaCl, 1.0 g L- cystein. HCl, 0.2 g Ascorbinacid, 22.8 mg Fe- Ammonium Citrat, 10.0 g glucosis, 1.0 g K_2HPO_4, 0.6 g potassium phosphate were solved in 870 ml distilled water (pH 6.8) 100 ml bovine serum (inactivated) and 30 ml special 107 vitamin mix was added. Subsequently, the medium was sterilized by filtration and stored at 4°C.

Preparation of LE Medium (Diphasic medium)

The LE Medium (Clark and Diamond, 2002) is a xenic medium for *Entamoeba* spp. isolation from environmental samples. To prepare the Locks's solution 8.0 g sodium chloride (NaCl); 0.2 g CaCl, 0.2 g KCl; 0.01 g $MgCl_2$; 2.0 g sodium phosphate, dibasic (Na_2HPO_4); 0.4 g sodium bicarbonate and 0.3 g potassium phosphate, monobasic were solved in 1,000 ml distilled water and autoclaved for 15 min. at 121°C.

The egg slant was prepared by breaking three eggs (=150 ml) into a glass and subsequently blended with 41.66 ml Locke's solution. This egg mixture was filtered through gauze and afterward filled with a pipette into glass tubes (five ml in each glass tube). To build a bigger surface the tubes were placed diagonal into a water bath at 100°C for 10 min. and afterwards stored at room temperature to cool down. Finally the egg slant was overlaid with 6 ml of Locke's solution and autoclaved 15 min. at 121°C. The LE- medium was stored at -20°C.

3.3.3 Nematode cultivation

The aerobic nematodes were cultured on non nutrient agar plates coated with bacteria *(E. coli)* as a food source and incubated at room temperature.

3.4 Molecular biology

3.4.1 Primer design

The establishment of a PCR detection system for *Balamuthia mandrillaris* and *Entamoeba invadens* was an important part of this project. Target gene was the high conserved 18s rRNA, and 5,8s rRNA gene to assure a high primer specificity. For this reason sequences from *B. mandrillaris* and *E. invadens* were downloaded from the GenBank Database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nuccore&itool=toolbar) and subsequently converted into FASTA files. To compare the gene sequences from

Gene- Bank- Database these files were aligned with the programs CLUSTALX (Thompson et al., 1997) and GENEDOC (Nicholas et al., 1997)

After locating an appropriate sequence as primer binding site it was important to design a primer with a well-balanced AT and CG ratio and with a length of about 20 base pairs. The reason for choosing a 20 bp primer is that the primer melting temperature can be nearly calculated by the AT/ CG ratio and the attachment of the primer to the primer binding site is easier.

Melting temperature = 4x (G+C) + 2x (T+A)

Primer design: *Balamuthia mandrillaris*

To design a primer with high specificity and sensitivity for *Balamuthia mandrillaris* four sequences from this amoeba were downloaded from GenBank Database and compared with the programs CLUSTALX and GENEDOC to different *Balamuthia mandrillaris* sequences and *Acanthamoeba* spp.

The binding sites for the forward primer Bal18F (5-GTCCGCCGAAATAGGTGAGC-3') and the reverse primer Bal18rev (5'-GCCTTTTGGGCAGCATGTG-3') are located in the 18s rRNA gene and built an amplicon with the length of ~ 470bp. To be sure that the primer have a high specificity for the amoeba of choice, the short primer sequences were compared by blasting the primer to the sequences of other organisms at the NCBI website (http://www.ncbi.nlm.nih.gov/blast). Finally the primers were ordered at MWG AG Biotech (http://www.mwg-biotech.com/).

Primer design: *Entamoeba invadens*

Primer in the 18s rRNA gene:

To design primers for *E. invadens* in the 18s rRNA gene, it was necessary to compare sequences from different *Entamoeba* species. For this reason five different Entamoebae sequences (Table 4) were downloaded at the GenBank database and aligned with the programs CLUSTALX (Thompson et al., 1997) and GENEDOC (Nicholas et al., 1997).

Table 4: Species and GenBank numbers of the respective sequences

species	GenBank number
Entamoeba invadens	AY769863 (gi53988537)
Entamoeba nuttalli	AB282657 (gi145698413)
Entamoeba moshkovskii	EF204916 (gi124244238)
Entamoeba dispar	EF204917 (gi124244239)
Entamoeba histolytica	EF204915 (gi124244237)

The primer binding sites for the forward primer (Inv18sf 5'-GCCACAATA GATGAACCTGG-3') and the reverse primer (Inv18sr 5'- CTATGCCGACAT CCAATAGTC-3') are located in a high conserved region of the 18s rRNA gene. Both primers got a length from about 20 bp and a balanced GC/ AT ratio. To be sure that the primers are high specific and only for the amplification of *E. invadens* DNA, it was important that the primer sequences were blasted with other organisms and amoebae at the NCBI website (http://www.ncbi.nlm.nih.gov/blast). Finally the unmodified oligos were ordered at MWG AG Biotech (http://www.mwg-biotech.com/).

Primer design: *E. invadens*:

Primer in the 5,8s rRNA gene:

The primer binding sites for the forward primer (Inv5, 8f: 5' GAATTGTACCAGGAA AAGTAG 3') and the reverse primer (Inv5, 8r: 3' GAGTCATCAAGAAATTCTCAG 5') are located at a high conserved region in the 5,8s rRNA gene to exclude primer binding to other species of Entamoebae. To establish these primers it was necessary to compare different *Entamoeba* spp. sequences (*E. moshkovskii, E. dispar, E. histolytica* and *E. invadens*) which were aligned with the programs CLUSTALX (Thompson et al., 1997) and GENEDOC (Nicholas et al., 1997).

Table 5 species and GenBank number of the sequences

species	GenBank number
Entamoeba moshkovskii	X89635 (gi908849)
Entamoeba dispar	Y12251 (gi1929041)
Entamoeba histolytica	Y12249 (gi1929042)
Entamoeba invadens	Y12250 (gi1929043)

After checking the AT/ GC ratio, calculating the melting temperature and blasting the primer sequence at the NCBI Website the primers were ordered at MWG AG Biotech (http://www.mwg-biotech.com/).

3.4.2 DNA isolation

DNA isolation from swabs

The DNA isolation from mouth and cloaca swabs was performed by using the QIAamp DNA- Mini Kit (QIAGEN, Vienna, Austria) according to the manual. Briefly, before starting the DNA isolation procedure the cotton tipped applicators were shaken out in 200 µl sodium chloride (NaCl) solution and subsequently discarded. After centrifugation of the samples were transferred from the 15 ml BD falcon tubes which were used for the sterile transport of the samples to the lab, to 1.5 ml microcentrifuge tubes.
Following the manual the DNA was isolated. To elute the DNA 200 µl AE buffer were dropped to the membrane of the column, incubated for 5 min. at RT and centrifuged for 1 min. at 5500 g. The isolated DNA was stored at -20°C.

DNA isolation from stool samples

The DNA isolation from stool samples was performed according to the QIAamp DNA stool Mini Kit manual. 180 to 220 mg cooled stool were weighted in a 2 ml microcentrifuge tube. After lysing and washing, the DNA was eluted in 200 µl AE buffer.
The DNA was stored at -20 °C.

DNA isolation from cultured cells in liquid media

Amoebae were harvested by shaking the culture flask hard to detach the trophozoites from the bottom of the flask. Subsequently, the whole suspension was transferred into a sterile 50 ml tube and centrifuged for 8 min. at 420 g. Afterwards the supernatant was discarded and the pellet was stored at -20°C to break up the cell wall of the amoebae.
Finally the DNA isolation was done by using the QIAamp DNA Mini Kit according to the manual, the same procedure as for the DNA isolation from swabs.

DNA isolation from agar plates

Agar plates abundantly covered with trophozoites were harvested by wiping off the surface with cotton tipped applicators. Subsequently the cotton tipped applicators were swung in a microcentrifuge tube filled with 1ml NaCl solution to detach the trophozoites from the applicator. After harvesting 2-3 Agar plates, depending on the density of

amoebae the suspension was centrifuged for one min. at 1700 g and finally stored at -20°C for 2-3 hours to break up the cells.
The DNA isolation was done by using the QIAamp DNA Mini Kit according to the same procedure as for DNA isolation from swabs.

DNA isolation from nematodes

Harvesting of the nematodes from agar plates was performed by adding 3-5 ml NaCl solution to the plate and shaking it until the nematodes detach from the surface. Subsequently the nematodes containing NaCl solution was transferred to sterile tubes and centrifuged at 1700 g for 3 min. The pellet was resuspended in 1 ml NaCl and stored for 2-3 hours at -20°C to break up the cell walls of the worms. DNA isolation was performed similar to the DNA isolation from swabs by using the QIAamp DNA Mini Kit.

DNA isolation from tissue samples

Isolation of DNA from tissue samples was performed by using the QIAamp DNA Mini Kit according to the manual (Tissue protocol). After cutting the tissue to small pieces, lysating and washing, the DNA was eluted in 200 µl AE buffer.
The isolated DNA was stored at -20°C.

3.4.3 Primer evaluation

3.4.3.1 *Balamuthia mandrillaris* Primer specificity testing

***Balamuthia mandrillaris* primer**

The primer pair Bal18F/ Bal18rev was tested with undiluted *B. mandrillaris* DNA isolated from liquid culture. For this PCR 1 µl, 3 µl, 5 µl and 10 µl were filled into clean 0.2 ml PCR tubes, 31.25 µl Master Mix were added and finally the mixture was filled up to 50 µl with distilled water.
The PCR was performed by using a standard PCR program called GastB with different annealing temperatures 52°C, 54°C and 56°C: 15 min. 95°C, followed by 30 cycles of 1 min. 95°C, 2 min. 52°C/ 54°C or 56°C, 3 min. 72°C and the elongation step with 7 min. at 72°C.

***E. invadens* primer**

First, the two primer pairs Inv18sf/ Inv18sr and Inv 5,8f/ Inv 5,8r were tested with 1: 10 diluted and undiluted *E. invadens* DNA isolated from liquid culture.

4 Eppendorf tubes with 1 µl and 3 µl undiluted DNA were mixed with 31.25 µl master mix (one master mix contained the primer Inv 18sr/ Inv 18sf and one contained Inv5,8f/ Inv5,8r) in a clean microcentrifuge tube an filled up to 50 µl with distilled water. In the same way the PCR with 1:10 diluted *E. invadens* DNA was performed. For polymerase chain reaction a standard program was chosen: GastB52°C: 15 min. 95°C, followed by 30 cycles of 1 min. 95°C, 2 min. 52°C, 3 min. 72°C and the elongation step was 7 min. at 72°C.

3.4.3.2 Primer sensitivity testing

Counting of trophozoites from liquid culture:

Necessarily the trophozoites in liquid culture had to be counted out for dilution series and primer test by using a Fuchs Rosenthal counting chamber as shown in Figure 12.

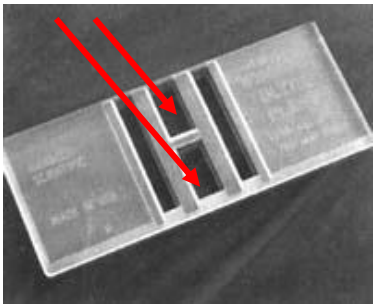


Figure 12 Fuchs- Rosenthal counting chamber
http://www.aname.es/microscopia/ems/magnifier/images/63512_10.jpg

To calculate how many ml from liquid culture are necessary to harvest 1 million trophozoites the culture flask with the axenic culture has been shaken very hard to be sure that the trophozoites detach from the bottom of the flask. Subsequently one drop of the suspension was transferred to the counting chamber, covered with a cover glass and subsequently the trophozoites are counted with a tally counter by screening diagonal four squares (each square contains 16 smaller squares as shown in Figure 13). This method was used two times for each sample.

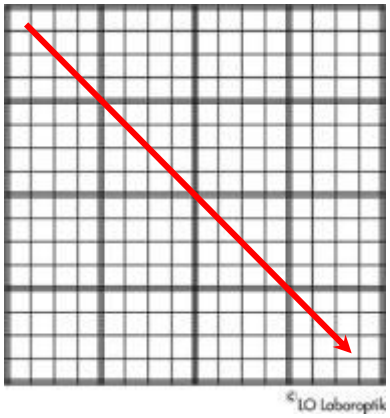


Figure 13 Details of the Fuchs- Rosenthal counting chamber
(http://www.zaehlkammer.de/gfx/fuchs_rosenthal.jpg)

After counting the trophozoites in about 5 drops of liquid culture the mean value was calculated and divided through ten. Afterwards the result was multiplied with 16 and subsequently divided through three. This result was multiplied with 1,000 and the resulted number is the number of trophozoites in 1 ml liquid culture.

Dilution series of trophozoites from liquid culture

For primer testing it was necessary to make a dilution series of trophozoites to determine the sensitivity of the designed primer.

After the calculation of the required quantity to have 10^6 trophozoites this amount of liquid from axenic culture was transferred to a 50 ml tube and centrifuged for 8 minutes at 2500 g. After discarding the supernatant and resuspending the pellet in 1ml distilled water 100 µl of the suspension was mixed with 900 µl distilled water in a clean 1.5 ml Eppendorf tube. The resulted mixture contained 10^5 trophozoites. To produce suspensions with 10^4, 10^3, 10^2, 10 and 1 trophozoites this procedure has been continued.

Finally the DNA was isolated by using the QIAGEN QIAamp DNA Mini Kit.

Primer sensitivity test:

Subsequently after designing the primers it was necessary to test the primer pairs in a PCR performance. Each primer pair which was designed for *B. mandrillaris* and *E. invadens* was used to amplify a part of the 18s rRNA or 5,8s rRNA gene of these both amoebae.

For PCR performance all primer were diluted in a 1:10 ratio. The master mix for each sample was prepared by mixing 5 µl PCR buffer (Solis BIODYNE, $MgCl_2$ free), 5µl $MgCl_2$

(25mMol), 11 µl distilled water, 1 µl dNTP (20mM of each, Solis BIODYNE), 5 µl primer I (1:10 dilution, 1µM), 5 µl primer II (1:10 dilution, 1µM) and 0.25 µl (1.25 U) polymerase (Hot Fire DNA Polymerase I, 5U/ µl, Solis BIODYNE) in a clean 1.5 ml Eppendorf tube.

***B. mandrillaris* primer**

To test the sensitivity of this primer pair PCR with two dilution series of *B. mandrillaris* DNA was performed. For these two PCRs 1 µl, 5 µl and 10 µl *B. mandrillaris* DNA from 10^6 to 1 trophozoite per ml was pipetted into a clean 0.2 ml PCR tube, mixed with 31.25 µl master mix and filled up to 50 µl with distilled water.

The PCR was performed by using a standard PCR program called GastB with different annealing temperatures 52°C, 54°C and 56°C: 15 min. 95°C, followed by 30 cycles of 1 min. 95°C, 2 min. 52°C/ 54°C or 56°C, 3 min. 72°C and the elongation step with 7 min. at 72°C.

***E. invadens* primer: Inv 18sf/ Inv 18sr**

The primer sensitivity was tested with two different dilution series of *E. invadens* from axenic culture.

From each dilution step (10^6 to 1 trophozoite per ml) three different DNA amounts were tested with the designed primers.

31.25µl of the master mix were filled into three clean 0.2 ml PCR tubes. Subsequently 1 µl, 8.75 µl and 18.75 µl DNA were added to the master mix and filled up with distilled water to 50 µl.

Afterwards the samples were centrifuged for 10 sec. at 420 g and finally placed in a cycler for the PCR.

The PCR was performed by using a standard PCR program GastB 52°C: 15 min. 95°C, followed by 30 cycles of 1 min. 95°C, 2 min. 52°C, 3 min. 72°C and the elongation step was 7 min. at 72°C.

***E. invadens* primer: Inv 5,8f/ Inv 5,8r**

The primer test was performed exactly the same way as the primer test for Inv18sf/ Inv18sr to show the primer sensitivity.

3.4.4 Nested PCR establishment

Necessarily a nested PCR for *Acanthamoeba* spp. detection was established because the detection of these amoebae did not work by using the standard PCR program for called JDP (Schroeder et al. 2001): 15 min. 95°C; 45 cycles: 1 min. 95°C, 1 min. 60°C, 2 min. 72°C; and finally the elongation step 7 min. 72°C.

For establishment of the nested PCR a standard PCR program was performed with different temperatures and number of cycles in varying combinations for detecting acanthamoebae in tissue samples of a snake.

The first step of the nested PCR was always performed by using the primer pair JDP1 (5'-GGCCCAGATCGTTTACCGTGAA-3')/ Rhiz3r (5'-CTAAGGGCATCACAGACCTG-3') by performing a standard PCR program GastB in different temperatures (54°C or 56°C) and cycle number (20 or 30): 15 minutes 95°C, followed by 20/ 30 cycles of 1 minute 95°C, 2 minutes 54°C/ 56°C, 3 minutes 72°C and the elongation step was 7 minutes at 72°C.

The primer pair for the second step was Rhiz2for (5'-GATCAGATACCGTCGTAGTC-3') and JDP2 (5'- TCTCACAAGCTGCTAGGGAGTCA-3'). The PCR was performed by using the standard PCR program GastB in different temperatures (54°C or 56°C) and cycle number (20 or 30): 15 minutes 95°C, followed by 20/ 30 cycles of 1 minute 95°C, 2 minutes 54°C/ 56°C, 3 minutes 72°C and the elongation step was 7 minutes at 72°C.

The first nested PCR was performed with 20 cycles in the first step at 54°C. The following step was performed one time with 20 cycles at 54°C and one time with 30 cycles at the same temperature. Gel electrophoresis demonstrated that this temperature was not high enough for specific primer binding. Furthermore, performing this PCR assay with 30 cycles led to much more unspecific signals compared to the assay with 20 cycles in step two as demonstrated in Figure 25.

The second nested PCR assay was performed with 20 cycles in the first step at 56°C and the second step was performed once with 20 cycles and one time with 30 cycles at 56°C. The higher temperature led to decrement of unspecific signals due to a higher specificity of primer binding but performing this nested PCR with 20 cycles in both steps resulted in faint signals on the agarose gel. Using 30 cycles in the second step demonstrated stronger signals. Furthermore, signals from shorter amplicons (*Acanthamoeba* spp. genotype T5) were detectable under UV exposure.

Finally, the nested PCR assay was performed with increases cycle numbers but with equal temperatures. The first step was performed with 30 cycles at 56°C and the following step once with 30 cycles and once with 20 cycles.

This nested PCR establishment showed that the best combination in both steps is the PCR program GastB 56°C with 30 cycles.

3.4.5 Nematodes PCR

The PCR for the identification of isolated nematodes was performed by using the standard PCR program GastB 52°C: 15 minutes 95°C, followed by 30 cycles of 1 minute 95°C, 2 minutes 52°C, 3 minutes 72°C and the elongation step was 7 minutes at 72°C. Therefore, 3 different primer pairs were tested in one PCR performance. The first pair was Rhiz1r (5' GCT GCT GGC ACC AGA CTT G 3') and SSU1 (5' CGA CTG GTT GAT CCT GCC AGT AG 3'), the second Wurm18sf (5' ATC GCA GTG GCT TGA ACC G 3') and Wurm5,8rev (5' CAT CGA TAC GCG AAT CGG C 3') and the third pair was Rhiz1 (5' CAA GTC TGG TGC CAG CAG C 3') and Rhiz2r (5' GAC TAC GAC GGT ATC TGA TC 3').

3.4.6 Gelelectrophoresis

To prepare a 2% agarose gel with 150 cm², 1.4 g agarose (Sigma) was mixed with 70 ml 1x TAE buffer (0.04 M Tris- acetat, 0.001 M EDTA). To prepare a 2% agarose gel (150 cm²) with deeper slots 2 g agarose and 100 ml 1x TAE buffer were mixed and boiled on a magnetic stirrer. After the mixture cooled down 7 µl ethidium bromide were added and filled into a horizontal Electrophoresis system (BIO- RAD Wide Mini- Sub Cell GT).
2% agarose gels with 49 cm² and 56cm² were made by boiling a mixture of 1 g agarose with 50 ml 1x TAE on a magnetic stirrer and adding 5 µl ethidium bromide. The mixture for the 49 cm² gel was filled into a horizontal gel electrophoresis system from BIO RAD (Mini sub cell GT) and the 56 cm² gel to an electrophoresis system from PEQLAB (PerfectBlue Gelsystem Mini S).

The reason for choosing different sizes of agarose gels was the number of samples. On 150 cm² gels it is possible to apply 15 to 20 samples compared to the 49cm²/ 56 cm² gels where only 6 to 10 samples can be applied.

If more than one band on the agarose gel is expected, a gel with a high density should be chosen. Because of the higher density the DNA bands on the gel are clearly

disconnected from each other so that the cutting out of the bands is simpler and the DNA yield is higher. These are very important facts for the sample sequencing.

Depending to the depth of the slots in the agarose gel, the PCR product was mixed in an Eppendorf tube with 10x loading buffer.
Subsequently the sample loading buffer mix was filled to the slots and 1x TAE buffer was refilled in the gel electrophoresis system, so that the gel is completely covered with TAE buffer.
If a 150 cm² gel (2%) was used the applied voltage was 96 V and 65 V for a 49 cm²/ 56 cm² gel. The electrophoresis was performed until the buffer reached the edge of the agarose gel.
To know the length of the amplicon 17 µl Step Marker (Step Ladder 50 bp/ Sigma-Aldrich) was pipette into the first slot of the gel and if a slot was unused at the end of a 150 cm² gel a second slot was filled with Step Marker. With this method it was easier to calculate the amplicon length.

Purification of bands from agarose gels

To purify cut out samples from agarose gels the GE Healthcare illustra GFX PCR DNA and Gel Band Purification Kit was used.
The purification with the GE illustra Kit was performed according to the instruction manual. After the agarose was completely dissolved the DNA was eluted into an Eppendorf tube by adding 10 – 50 µl Elution buffer (depending on the size of the sample amount in the gel slice).The DNA was stored at -20°C.

3.4.7 Sequencing

Sequencing PCR

To perform sequencing PCR only one primer for each sample was used. To prepare the PCR 1 µl (1:10 dilution, 1µM) primer and 2 µl Big Dye (Big Dye Terminator v1.1 cycle sequencing Kit, AB Applied Biosystems, Austria) were mixed in a 0.2 ml PCR soft tube (Biozym scientific GmbH, Austria). Afterwards the mixture was refilled to 10 µl volume by adding 4 or 6 µl distilled water and centrifuged for a few seconds at 420g.
The sequencing PCR program starts with 96°C for 30 sec. and continues with 30 cycles of 96°C for 10 seconds, 50°C for 5 sec. and 60°C for 4 min.

Purification of the sequencing PCR product

1 µl NaAc and 33 µl 100% Ethanol were filled to a 500 µl Eppendorf tube and mixed with the PCR product from the sequencing PCR. These tubes were stored for 17 min. on ice and subsequently centrifuged at 4°C (13,800 g/ 30 min.). The supernatant was discarded, 70 µl 70% Ethanol were added to the pellet without resuspending and centrifuged for 10 min. at 13,800 g. Afterwards the supernatant was carefully removed and the sample was stored for 5 min. at room temperature with opened lid to dry. 20 µl HI-DI^{TM}–420Formamide (Applied biosystems, Austria) were added and stored for another 5 min. with opened lid at room temperature. Next this mixture was boiled for 5 min. at 95°C in a water bath (water filled 1, 5 ml Eppendorf tubes in an Eppendorf Thermomixer) and finally stored for 5 min. on ice.

The lids of the Eppendorf tubes were cut off and the samples were automatically sequenced by using an ABI PRISM 310^{R} Sequencer (PE Applied Biosystems, Langen, Germany)

DNA alignments

To identify the DNA which was amplified during the PCR performance, the sample sequences were compared to sequences downloaded at GenBank database by using the computer programs GENEDOC (Nicholas et al. 1997) and CLUSTALX (Thompson et al. 1997).

4 Results

4.1 Culture

4.1.1 Snakes

4.1.1.1 Snakes: first sample drawing (6th of November 2007)

Table 6: results culture snakes first sample drawing (06.11.2007)

Sample		*Acanthamoeba* spp.	*B. mandrillaris*	*N. fowleri*	*E. invadens*	Other microorganisms
FPu M	**mouth**	-	-	-	-	Ciliata
FPu K	**cloaca**	-	-	-	-	
FPo M	**mouth**	-	-	-	-	
FPo K	**cloaca**	-	-	-	-	
La M	**mouth**	-	-	-	-	*Echinamoeba*
La K	**cloaca**	-	-	-	-	
TP M	**mouth**	-	-	-	-	*Echinamoeba*
TP K	**cloaca**	-	-	-	-	
Ana M	**mouth**	-	-	-	-	
Ana K	**cloaca**	+	-	-	-	
KP M	**mouth**	-	-	-	-	*Vannella*
KP K	**cloaca**	+	-	-	-	
Bk M	**mouth**	-	-	-	-	*Echinamoeba*
Bk K	**cloaca**	-	-	-	-	*Echinamoeba*
BZ M	**mouth**	-	-	-	-	
BZ K	**cloaca**	-	-	-	-	
AG M	**mouth**	-	-	-	-	
AG K	**cloaca**	-	-	-	-	

Aerobic culture:

The isolation of *Acanthamoeba* spp. from mouth and cloaca swabs was successful in 2 of 9 (22%) snakes (*Anaconda* cloaca and *Python regius* cloaca) as illustrated in Table 6. Furthermore, no trophozoites or cysts of *B. mandrillaris* and *N. fowleri* were detected on Agar plates. Anyway, some other microorganisms were detected in aerobic cultures of mouth and cloaca swabs like Ciliata (FPu= *Python sebae* "lower"), in three of nine snakes (33%) *Echinamoeba* (La= *Langaha madagascariensis*; TP= *Python morulus* and Bk= *Boa constrictor* "small") and in one snake (11%) *Vannella* (KP= *Python regius*).

Table 6 shows, that six of nine (66%) snakes were amoeba positive in the first sample drawing.

Anaerobic culture:

Our attempts to isolate *E. invadens* trophozoites from mouth and cloaca swabs by culture were without success although this amoeba was detected by PCR.

4.1.1.2 Snakes: second sample drawing (13th of December 2007)

Table 7: results culture snakes second sample drawing (13.12.2007)

Sample		*Acanthamoeba* spp.	*B. mandrillaris*	*N. fowleri*	*E. invadens*	Other microorganisms
KP M	**mouth**	-	-	-	-	
KP K	**cloaca**	-	-	-	-	
FP2 M	**mouth**	-	-	-	-	
FP2 K	**cloaca**	-	-	-	-	
Ana Gr. M	**mouth**	-	-	-	-	
Ana Gr. K	**cloaca**	-	-	-	-	
FP1 M	**mouth**	-	-	-	-	
FP1 K	**cloaca**	-	-	-	-	
ASN M	**mouth**	-	-	-	-	
ASN K	**cloaca**	-	-	-	-	
BZ M	**mouth**	-	-	-	-	*Echinamoeba*
BZ K	**cloaca**	+	-	-	-	
Bk M	**mouth**	-	-	-	-	*Echinamoeba*
Bk K	**cloaca**	-	-	-	-	*Echinamoeba*
AG M	**mouth**	-	-	-	-	
AG K	**cloaca**	-	-	-	-	

Aerobic culture:

In the second sample drawing one sample from mouth and cloaca swabs was positive for *Acanthamoeba* spp. (*Boa* "accordion disease"; cloaca swab) as shown in Table 7. In 16 collected swabs, no *B. mandrillaris* and *N. fowleri* were detected on agar plates. However, three swabs turned out to be positive for *Echinamoeba*.

Anaerobic amoebae:

All samples were negative for *E. invadens* in culture.

4.1.1.3 Summary snakes:

As illustrated in Figure 14 only three samples were positive for *Acanthamoeba* spp. (*Anaconda* cloaca; *Python regius* cloaca and *Boa* accordion disease cloaca). In two sample drawings from snakes all snakes turned out to be negative for *B. mandrillaris*, *N. fowleri* and *E. invadens* in culture. However, it was possible to isolate other microorganisms in seven of 17 (41%) snakes (Table 6 and Table 7)

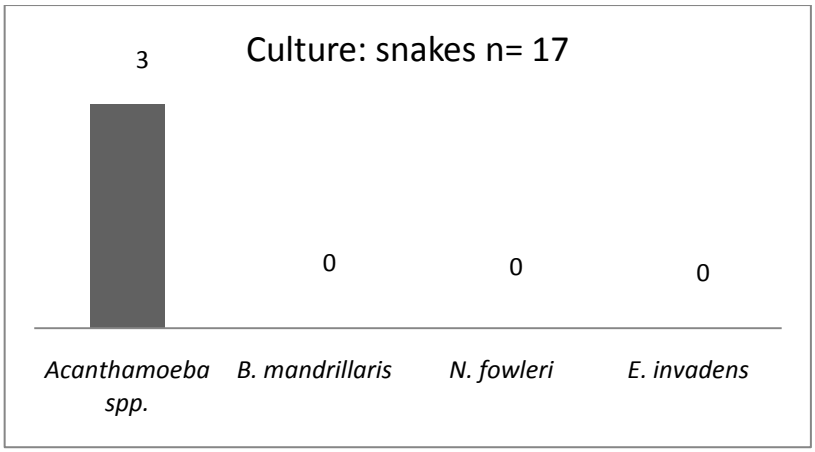


Figure 14: results summary culture snakes

4.1.1.4 *Boa* accordion disease: tissue samples

Aerobic culture:

The tissue samples from the intestine of the *Boa* "accordion disease" turned out to be positive for *Acanthamoeba* spp. Moreover, no *B. mandrillaris* and *N. fowleri* were isolated from tissue samples (brain, liver and intestine) in aerobic culture.

Anaerobic culture:

All samples turned out to be negative for *E. invadens*.

4.1.2 Chelonians

4.1.2.1 Chelonians: first sample drawing (6th of February 2008)

Table 8: culture results chelonians first sample drawing (06.02.2008)

Sample		*Acanthamoeba* spp.	*B. mandrillaris*	*N. fowleri*	*E. invadens*	Other microorganisms
1 M	mouth	-	-	-	-	
1 K	cloaca	+	-	-	-	

2 M	**mouth**	-	-	-	-	
2 K	**cloaca**	-	-	-	-	*Echinamoeba* Valkampfiidae
2 Kot	**faeces**	-	-	-	-	
3 M	**mouth**	-	-	-	-	
3 K	**cloaca**	-	-	-	-	Valkampfiidae
4 M	**mouth**	+	-	-	-	
4 K	**cloaca**	-	-	-	-	*Echinamoeba*
5 M	**mouth**	-	-	-	-	
5 K	**cloaca**	-	-	-	-	
6 M	**mouth**	-	-	-	-	
6 K	**cloaca**	-	-	-	-	
7 M	**mouth**	-	-	-	-	*Echinamoeba*
7 K	**cloaca**	-	-	-	-	Valkampfiidae
8 M	**mouth**	-	-	-	-	*Echinamoeba*
8 K	**cloaca**	-	-	-	-	*Echinamoeba*
9 M	**mouth**	-	-	-	-	
9 K	**cloaca**	+	-	-	-	
10 M	**mouth**	+	-	-	-	
10 K	**cloaca**	+	-	-	-	

Aerobic culture:

Twenty mouth and cloaca swabs and one faeces sample from 10 chelonians were analyzed. Altogether, four of 10 (40%) chelonians (1: *Trachemys scripta elegans*; 4: *Testudo kleinmanni*; 9: *Geochelone elephantopus* A and 10: *Geochelone elephantopus* B) were *Acanthamoeba* spp. positive in culture. *B. mandrillaris* and *N. fowleri* were not detected as illustrated in Table 8.

Aerobic cultures from 5 of 10 chelonians (50%) were positive for other microorganisms like *Echinamoeba* (2: *Astrochyles radiata*; 4: *Testudo kleinmanni;* 7: *Heosemys*, dam; 8: *Phrynops hilarii*). Furthermore, 3 of 10 (30%) animals were positive for *Valkampfiidae* in culture (2: *Astrochyles radiata*; 3: *Heosemys*; 7: *Heosemys*, dam). In summary, 8 of 10 (80%) chelonians were amoeba- positive in aerobic culture as shown in Table 8.

Anaerobic culture:

All samples were *E. invadens*-negative in culture as shown in Table 8.

4.1.2.2 Chelonians: second sample drawing (7th of May 2008)

Table 9: culture results chelonians second sample drawing (07.05.2008)

Sample		*Acanthamoeba* spp.	*B. mandrillaris*	*N. fowleri*	*E. invadens*	Other microorganisms
1 M	**mouth**	+	-	-	-	
1 K	**cloaca**	-	-	-	-	
2 M	**mouth**	-	-	-	-	*Echinamoeba*
2 K	**cloaca**	-	-	-	-	
3 M	**mouth**	-	-	-	-	
3 K	**cloaca**	-	-	-	-	*Echinamoeba*
4 M	**mouth**	-	-	-	-	Valkampfiidae
4 K	**cloaca**	-	-	-	-	*Echinamoeba*
5 M	**mouth**	-	-	-	-	*Echinamoeba*
5 K	**cloaca**	-	-	-	-	
6 M	**mouth**	-	-	-	-	
6 K	**cloaca**	-	-	-	-	
7 M	**mouth**	-	-	-	-	
7 K	**cloaca**	-	-	-	-	*Echinamoeba*
8 M	**mouth**	-	-	-	-	*Hartmannella* Valkampfiidae
8 K	**cloaca**	-	-	-	-	*Hartmannella* Valkampfiidae
R M	**mouth**	-	-	-	-	
R K	**cloaca**	-	-	-	-	
G M	**mouth**	+	-	-	-	
G K	**cloaca**	-	-	-	-	

Aerobic culture:

Two of 10 chelonians (20%) were *Acanthamoeba* spp.-positive (1M= *Trachemys scripta elegans* and GM= *Geochelone elephantopus* B). However, all cultures were negative for *B. mandrillaris* and *N. fowleri* as shown in Table 9.

Furthermore, 5 of 10 (50%) chelonians were positive for *Echinamoeba* (2: *Astrochyles radiata*; 3: *Heosemys*; 4: *Testudo kleinmannii;* 5: *Heosemys*, juvenile; 7: *Heosemys*, dam). 2 of 10 (20%) chelonians were positive for Valkampfiidae (4: *Testudo kleinmannii;* 8: *Phrynops hilarii*) One of 10 (10%) chelonian turned out to be *Hartmannella*- positive in plate culture (8: *Phrynops hilarii*).

In summary, 8 of 10 (80%) chelonians from the second sample drawing were amoeba positive in culture as shown in Table 9.

Anaerobic culture:

No *E. invadens* were detected in liquid culture as demonstrated in Table 9.

4.1.2.3 Summary chelonians

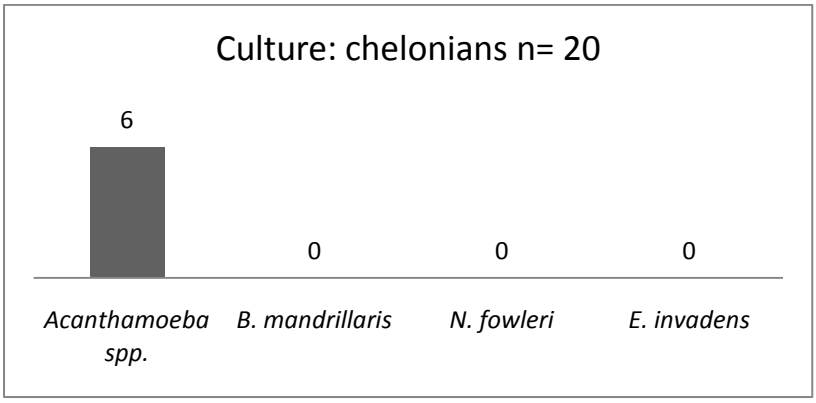


Figure 15 results summary culture chelonian

Aerobic culture:

All in all, 20 chelonians were analyzed for *Acanthamoeba* spp., *B. mandrillaris* and *N. fowleri*. Six of 20 chelonians (30%) were positive for *Acanthamoeba* spp. (1: *Trachemys scripta elegans;* 4: *Testudo kleinmanni*; 9: *Geochelone elephantopus* A and 10: *Geochelone elephantopus* B), but no other amoebas were detected as shown in Table 10.

Anaerobic culture:

It was not possible to detect *E. invadens* in culture as shown in Figure 15 although these amoebas have been detected by PCR performance.

Culture results: comparison of the first and second sample drawing from chelonian:

A direct comparison of chelonians culture results from both sample drawing is not possible due to the fact that it is unsure if exactly the same animal has been swabbed two times.

In the first sample drawing 4 of 10 (40%) of the swabbed chelonians were *Acanthamoeba* spp.-positive and 2 of 10 (20%) in the second. All animals were negative for *B*. *mandrillaris*, *N. fowleri* and *E. invadens* in both sample drawings.

4.1.3 Lizards

In the first sample drawing mouth and cloaca swabs from nine lizards were collected.

4.1.3.1 Lizards: first sample drawing (8th of May 2008)

Table 10: culture results lizards first sample drawing (08.05.2008)

Sample		*Acanthamoeba* spp.	*B. mandrillaris*	*N. fowleri*	*E. invadens*	*Other microorganisms*
I M	**mouth**	-	-	-	-	
I K	**cloaca**	-	-	-	-	
II M	**mouth**	-	-	-	-	
II K	**cloaca**	-	-	-	-	*Echinamoeba*
III M	**mouth**	-	-	-	-	
III K	**cloaca**	-	-	-	-	
IV M	**mouth**	-	-	-	-	
IV K	**cloaca**	-	-	-	-	
V M	**mouth**	-	-	-	-	
V K	**cloaca**	-	-	-	-	Valkampfiidae
VI M	**mouth**	-	-	-	-	
VI K	**cloaca**	+	-	-	-	
VII M	**mouth**	-	-	-	-	
VII K	**cloaca**	-	-	-	-	*Echinamoeba*
VIII M	**mouth**	-	-	-	-	
VIII K	**cloaca**	-	-	-	-	
IX M	**mouth**	-	-	-	-	
IX K	**cloaca**	-	-	-	-	*Hartmannella*

Aerobic culture:

One of nine (11%) lizards (VI= *Crotaphytus collaris*) was positive for *Acanthamoeba* spp. in culture. No *B. mandrillaris, N. fowleri* or *E. invadens* were detected in these samples. Altogether, four of nine (44%) lizards were amoeba positive, but not for the amoebas of choice as shown in Table 10. However, other amoebae, as *Echinamoeba* (II: *Corucia zebrata* and VII: *Tiliqua gerrardii*), Valkampfiidae (V: *Varanus exanthematicus*) and *Hartmannella* (IX: *Pogona* spp.) were detected.

Anaerobic culture:

The detection of *E. invadens* was without success from culture as shown in Table 10 although this amoeba was detected by PCR.

4.1.3.2 Lizards: second sample drawing (19th of August 2008)

16 mouth and cloaca swabs were collected from eight lizards in the second sample drawing.

Table 11: culture lizards second sample drawing (19.08.2008)

Sample		*Acanthamoeba* spp.	*B. mandrillaris*	*N. fowleri*	*E. invadens*	*Other microorganisms*
I M	**mouth**	-	-	-	-	*Echinamoeba*
I K	**cloaca**	-	-	-	-	
II M	**mouth**	-	-	-	-	
II K	**cloaca**	+	-	-	-	
IV M	**mouth**	-	-	-	-	Valkampfiidae
IV K	**cloaca**	-	-	-	-	*Echinamoeba*
V M	**mouth**	-	-	-	-	Valkampfiidae
V K	**cloaca**	-	-	-	-	*Echinamoeba* Valkampfiidae
VI M	**mouth**	-	-	-	-	
VI K	**cloaca**	-	-	-	-	Valkampfiidae
VII M	**mouth**	-	-	-	-	*Echinamoeba* Valkampfiidae
VII K	**cloaca**	-	-	-	-	
VIII M	**mouth**	-	-	-	-	
VIII K	**cloaca**	-	-	-	-	Valkampfiidae
IX M	**mouth**	-	-	-	-	
IX K	**cloaca**	-	-	-	-	Valkampfiidae

Aerobic culture:

One of eight (12%) lizards was *Acanthamoeba* spp.-positive in culture. *B. mandrillaris* and *N. fowleri* were not detected. However, seven of eight lizards (87%) were amoeba-positive, but not for the amoebas of choice. The detected organisms were *Echinamoeba*

(I: *Chamaeleon calyptratus*; IV: *Cyclura cornuta*; V: *Varanus exanthematicus* and VII: *Tiliqua gerrardii*) and *Valkampfiidae* (IV: *Cyclura cornuta*; V: *Varanus exanthematicus*; VI: *Crotaphytus collaris*; VII: *Tiliqua gerrardii*; VIII: *Dipsosaurus dorsalis* and IX: *Pogona* spp.) as demonstrated in Table 11.

Anaerobic culture:

As demonstrated in Table 11 no *E. invadens* were detected.

4.1.3.3 Summary lizards

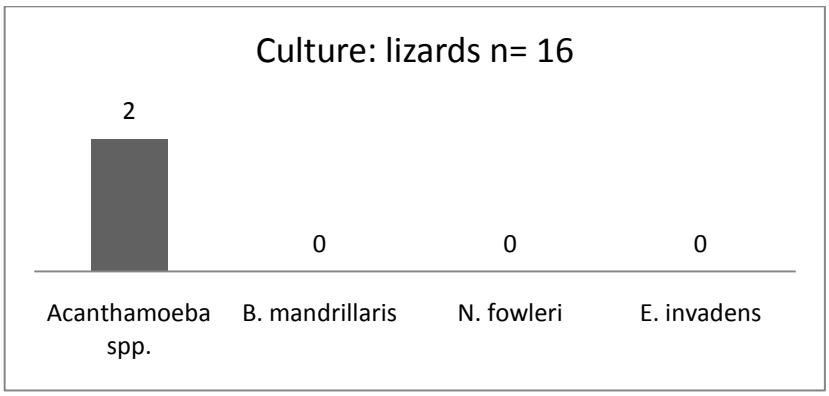


Figure 16 results summary culture lizards

Aerobic culture:

All in all two of 16 (12%) lizards (II= *Corucia zebrata* and VI = *Crotaphytus collaris)* from both sample drawings were positive for *Acanthamoeba* spp. in culture. No *B. mandrillaris* and no *N. fowleri* were detected in culture as shown in Figure 16.

Anaerobic culture:

All lizards were *E. invadens* negative in culture as demonstrated in Figure 16, although this amoeba was detected by PCR.

Culture results: comparison of the first and second sample drawing from lizards

With the excuse of one lizard, *Chamaeleon calyptratus* (I) it is unclear if exactly the same individual has been swabbed in both sample drawings. Therefore, a direct comparison of culture results from both sample drawing is not possible for the other lizards in this study. No aerobic or anaerobic amoeba of choice was detected in both sample drawings from *Chamaeleon calyptratus* (I) swabs as shown in Table 10 and Table 11.

4.2 Molecular biology

4.2.1 Primers

Balamuthia mandrillaris primer:

Due to its high conservation a region with the length of 473 bp in the middle of the 18S rRNA gene of *B. mandrillaris* was chosen for the primer design. This part of the 18S rRNA gene is appropriate to be a primer binding site for the designed primer pair.
The forward primer starts at nucleotide number 677 and ends at 694 and the reverse primer starts at nucleotide number 1132 and ends at 1150.
The amplification of the 18S rRNA gene between these primers produces an amplicon with a length of 473 bp during the polymerase chain reaction.

Forward primer:

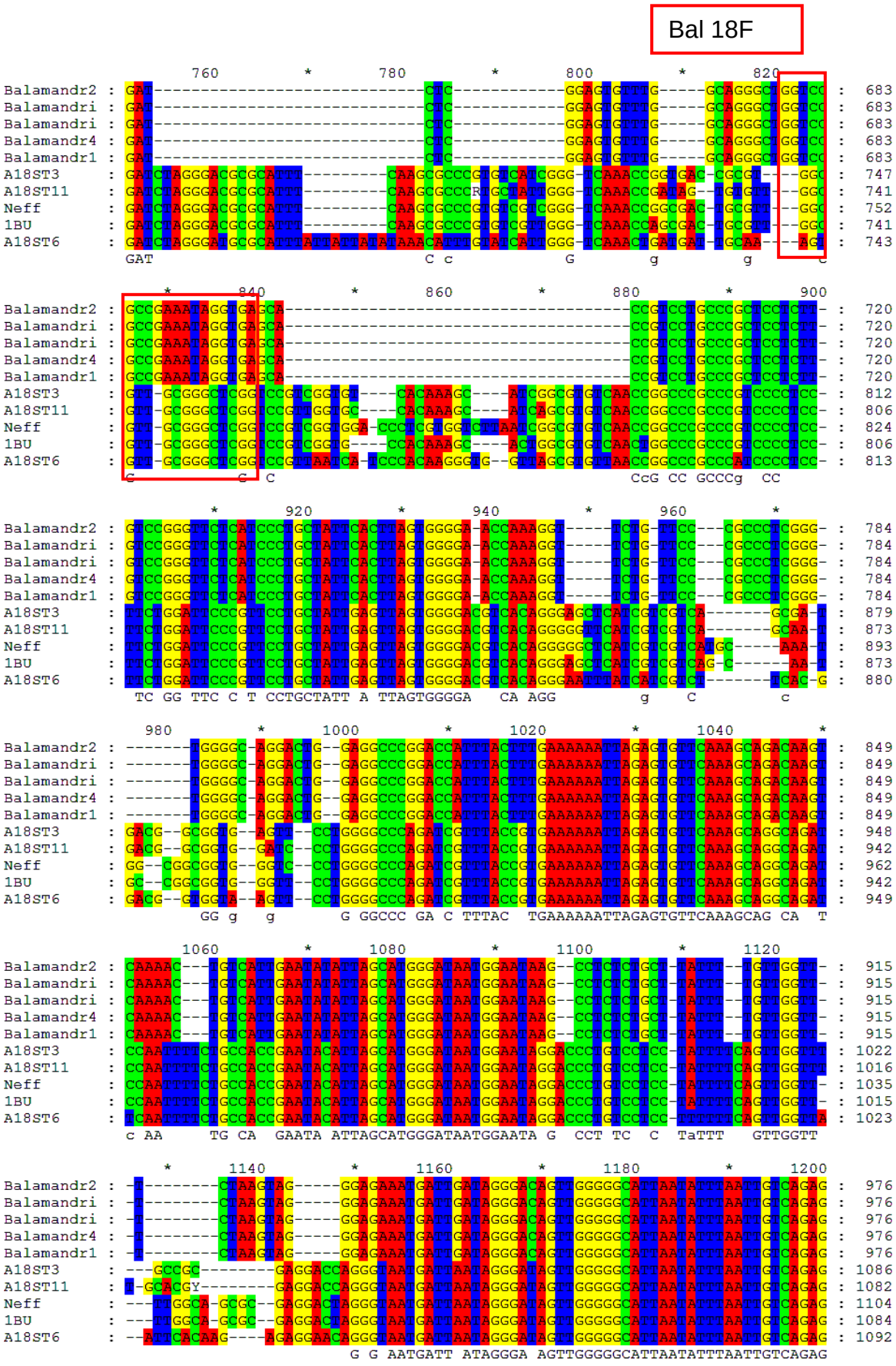
The forward primer to detect *Balamuthia mandrillaris* is located in the 18S rRNA gene and consists of 20 nucleotides. The AT/ GC ratio amounts 40/60% and the melting temperature of the primer is 64°C.

→ Bal18F (5’ GTCCGCCGAAATAGGTGAGC 3’)

Reverse primer:

The 19 nucleotide consisting reverse primer for *B. mandrillaris* has a AT/ GC ratio of 42/57% and the melting temperature is 60°C.
This primer is also located in the 18S rRNA gene.

→Bal18rev (3’ GCCTTTTGGGCAGCATGTG 5’)


Bal 18F

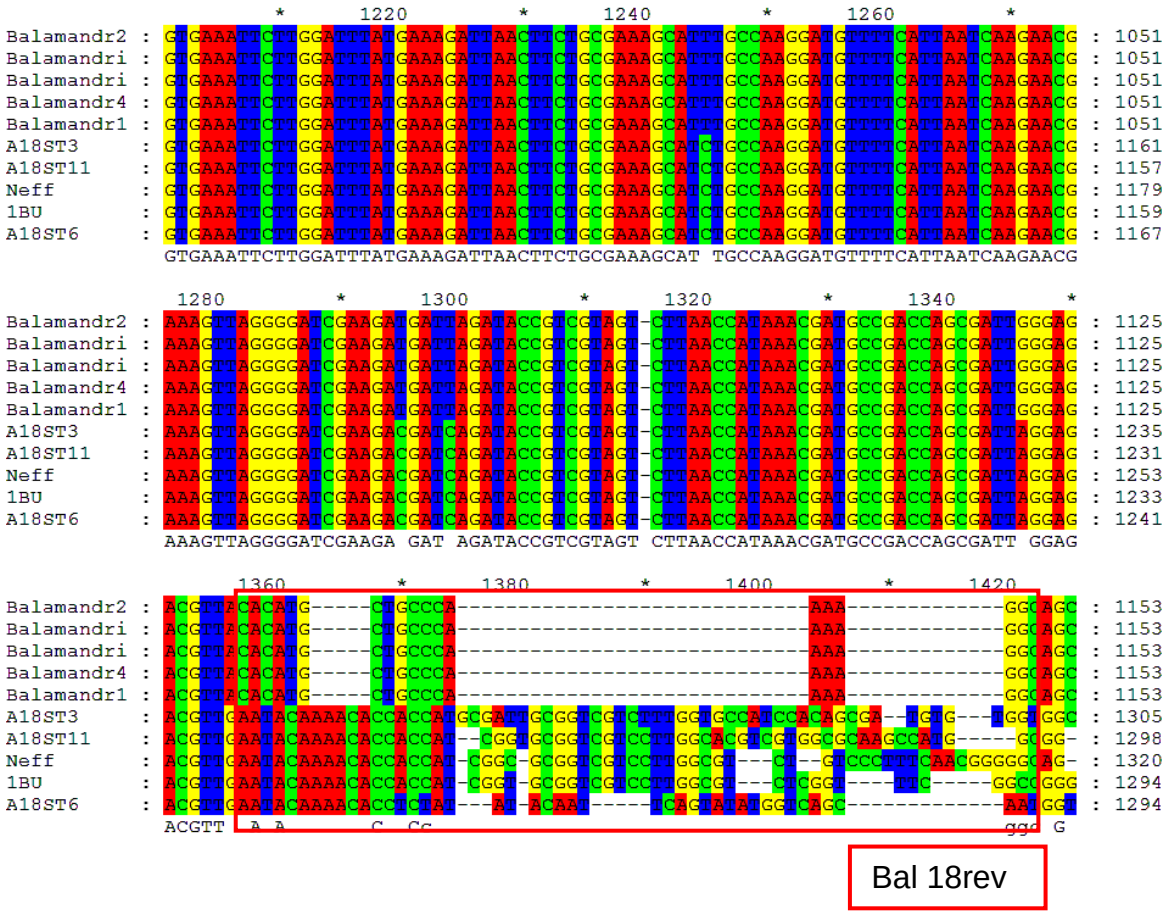


Figure 17: *Balamuthia mandrillaris* and *Acanthamoeba* spp. alignment for primer establishment

Entamoeba invadens primer in the 18S rRNA gene:

Forward primer Inv18sf:

To be sure that the *E. invadens* specific primer does not detect other Entamoebae and to discriminate between *Entamoeba invadens* and other *Entamoeba* species the alignment was made with different Entamoeba species sequences as shown below in Figure 18.

The forward primer Inv18sf contains 20 nucleotides with an AT/ GC ratio of 50/ 50% and a melting temperature of 60°C. This primer is located in a high conserved region of the 18s rRNA gene where the primer starts at nucleotide number 252 and ends at nucleotide number 271.

→ Inv18sf (5' GCCACAATAGATGAACCTGG 3')

Reverse primer Inv18sr:

The localization of the reverse primer is also in the 18S rRNA gene and consists 21 nucleotides. It has an AT/ GC ratio of 52/47% and a melting temperature of 62°C. The primer starts at nucleotide number 669 and ends at 689 in the gen on which primer design was based.

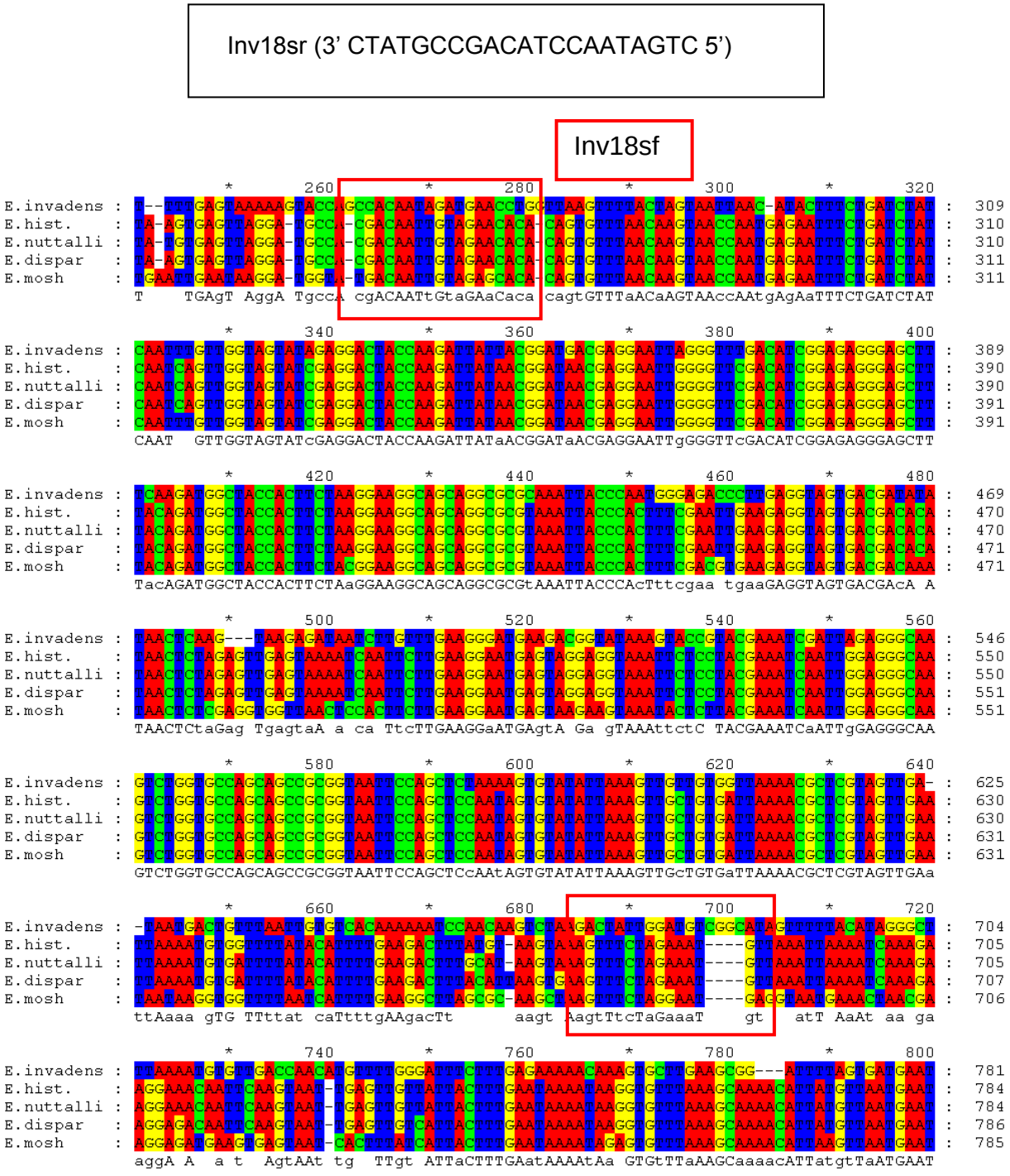


Figure 18: *Entamoeba* spp. alignment for *E. invadens* 18s rRNA primer establishment

E. invadens primer in the 5,8S rRNA gene:

As there is only one sequence of *E. invadens* 5,8S rRNA in the GenBank database, so it was difficult to design a primer in this region. To differentiate between *E. invadens* and other species of *Entamoeba* an alignment with different *Entamoeba* species (*E. moshkovskii* X89635, *E. dispar* Y12251 and *E. histolytica* Y12249) was performed.
The alignment in Figure 19 shows the sequence of *E. moshkovskii* at the top, followed by the sequence of *E. dispar* and *E. histolytica*. Finally, at the bottom of the alignment the sequence of *E. invadens* is located.

Forward primer Inv5,8f:
The primer to detect *E. invadens* by using gene amplification of the 5,8S rRNA gene has 21 base pairs (starts at nucleotide 12 and ends at nucleotide 32) and an AT/ GC ratio of 61/38%. Furthermore the melting temperature is calculated by using the formula
4x (G+C) + 2x (T+A) to 58°C.

→ Inv5.8f (5’ GAATTGTACCAGGAAAAGTAG 3’)

Reverse primer Inv5,8r:
This primer is also located in the 5,8S rRNA gene of *E. invadens*, has 21 nucleotides (starts at nucleotide number 312 and ends at 332), a GC/ AT ratio of 38/ 61% and a melting temperature of 58°C.

→Inv5.8r (3’ GAGTCATCAAGAAATTCTCAG 5’)

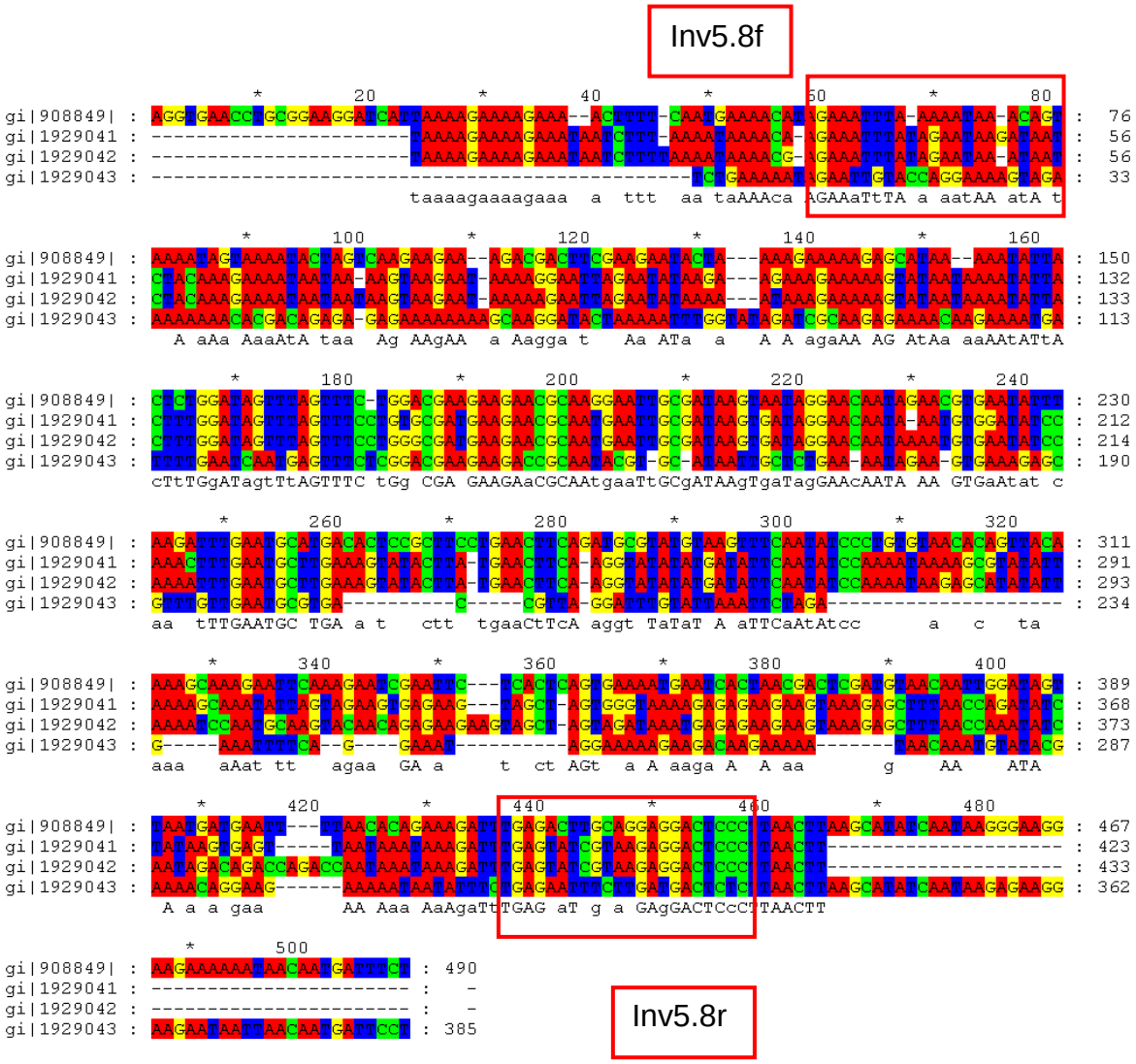


Figure 19: *Entamoeba* spp. Alignment for *E. invadens* 5,8s rRNA primer establishment

4.2.1.1 Primer specificity testing

***B. mandrillaris* primer**

The specificity of *B. mandrillaris* primer pair Bal18F/ Bal18rev was demonstrated by amplifying undiluted *B. mandrillaris* DNA isolated from Vero- cell culture by using the standard PCR program GastB56°C. Independent from the applied DNA amount the primer pair produced an amplicon with the size of 473bp as expected for these amoebas (Figure 20)

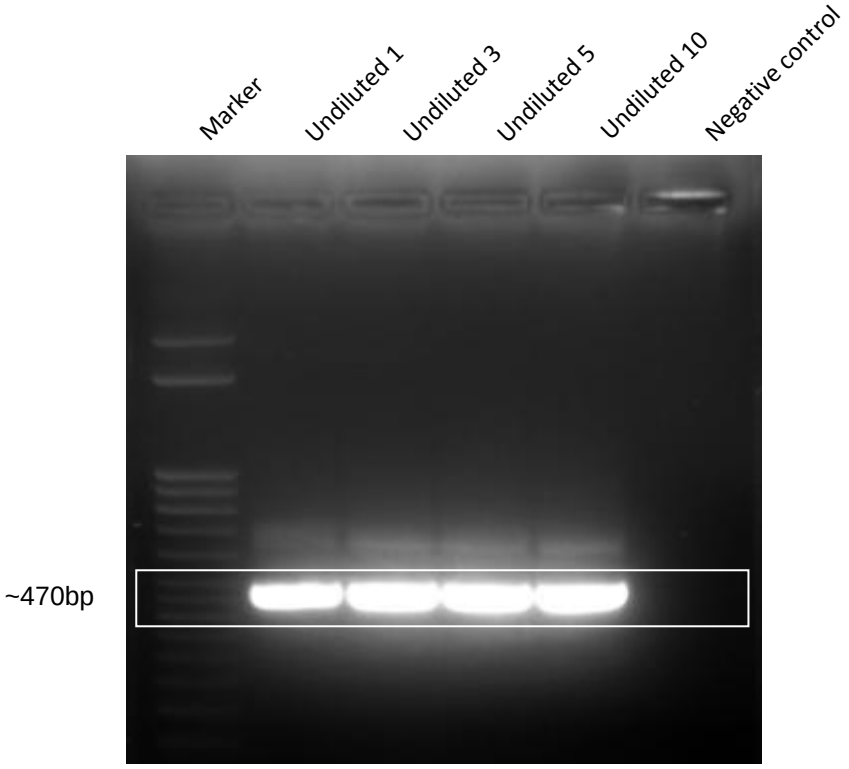


Figure 20: *B. mandrillaris* 18s rRNA primer specificity testing

E. invadens primer

As shown by agarose gelelectrophoresis the primers Inv18sf, Inv18sr, Inv5,8f and Inv5,8r are able to amplify *E. invadens* DNA. Both primer pairs, the 5,8S rRNA as well as the 18S rRNA primer show high sensitivity for polymerase chain reaction performance, but anyway in this study only the 18S rRNA primers were used for gene amplification.

The titration PCR was performed with different *E. invadens* DNA concentrations (undiluted and 1:10 diluted DNA, each time 1µl and 3µl) from cell culture for both primer pairs and demonstrated that these primers perform in lower concentrations as well as in higher.

Figure 21 shows a picture of an agarose gel under UV exposure with the step marker and eight PCR products applied on the gel.

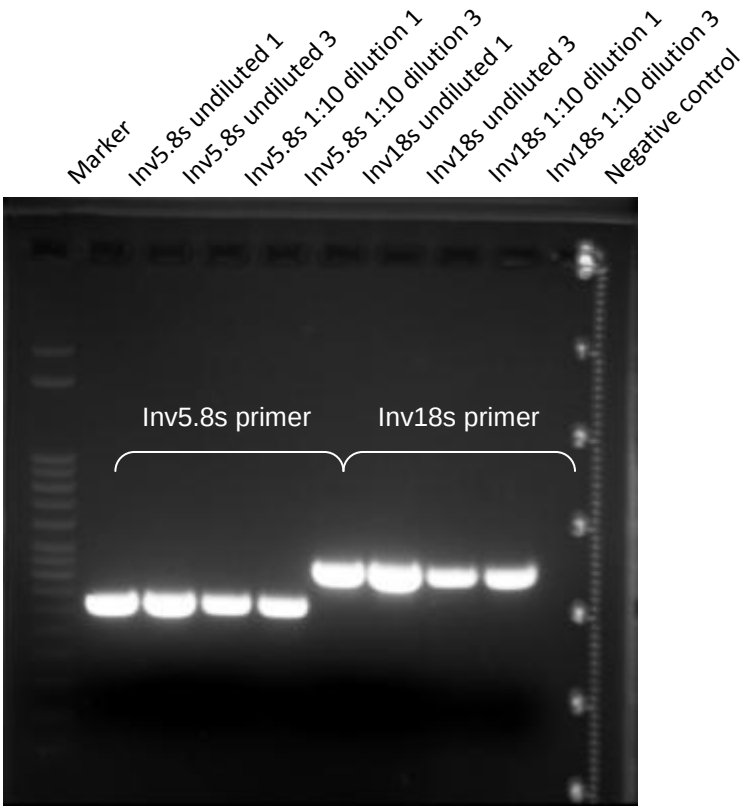


Figure 21: PCR Products of undiluted and 1:10 diluted *E. invadens* DNA amplified with 5,8s rRNA and 18s rRNA primer pairs, 27µl DNA, 2µl PCR loading buffer and 17µl step marker

4.2.1.2 Primer sensitivity testing

***Balamuthia mandrillaris* primer Bal18F/ Bal18rev**

The sensitivity of *B. mandrillaris* PCR using the primer pair Bal18F/ Bal18rev was demonstrated by amplifying *B. mandrillaris* DNA isolated from Vero- cell culture.

It has been found out, that the detection limit of this primer pair are 0.30 amoebae in 1 ml (arrow in Figure 22).

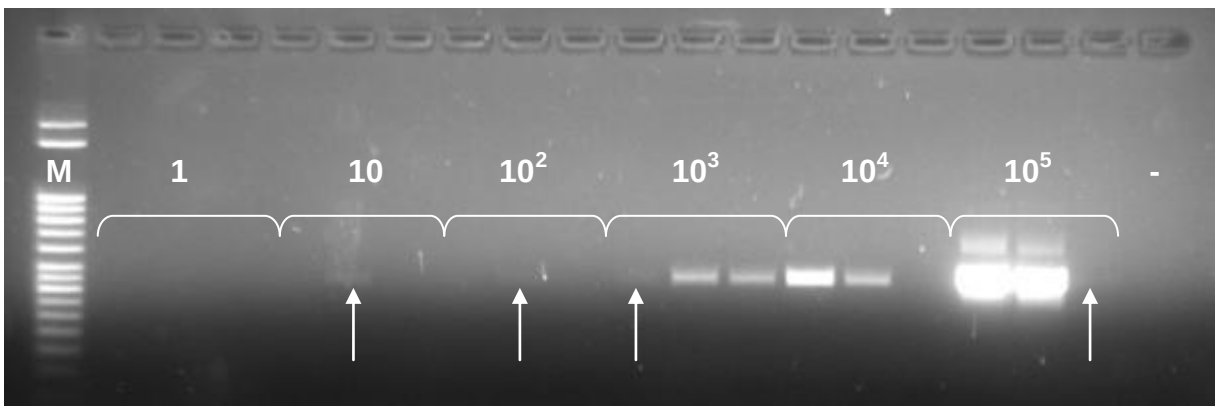


Figure 22: *B. mandrillaris* Primer sensitivity testing Bal18F/ Bal18rev

E. invadens primer Inv18sf/ Inv18sr

The sensitivity of *E. invadens* primer pair Inv18sf/ Inv18sf was demonstrated by amplifying *E. invadens* DNA from axenic culture by using the standard PCR program GastB52°C. In this PCR performance amplicons with a size of 437 bp were produced by applying a high amount of amoebae as well as a lower per ml. As shown in Figure 23 the detection limit of this primer pair are 0.05 amoebas in 1 ml axenic culture.

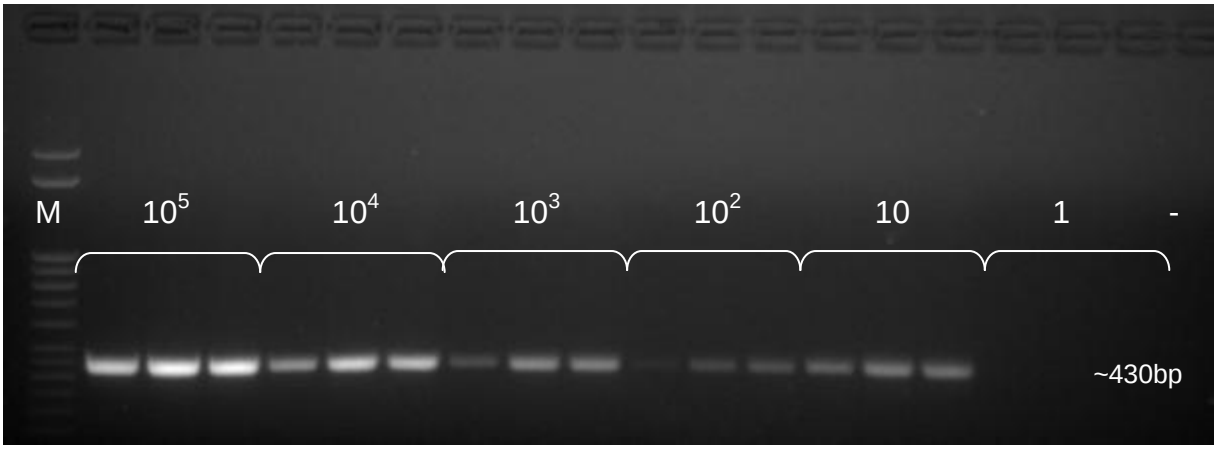


Figure 23: *E. invadens* primer sensitivity testing Inv18sf/ Inv18sr

E. invadens primer Inv5.8f/ Inv5.8r

The sensitivity of the primer pair Inv5.8f/ Inv5.8r was demonstrated by amplification of *E. invadens* DNA from axenic culture. As demonstrated in Figure 24 the sensitivity of this primer pair is not a high as of the primer pair Inv18sf/ Inv18sr. Unfortunately, the detection limit of the primer in the 5.8S rRNA are only 93.75 amoebas per ml so the primer of choice for analyzing the samples were Inv18sf/ Inv18sr due to the higher sensitivity.

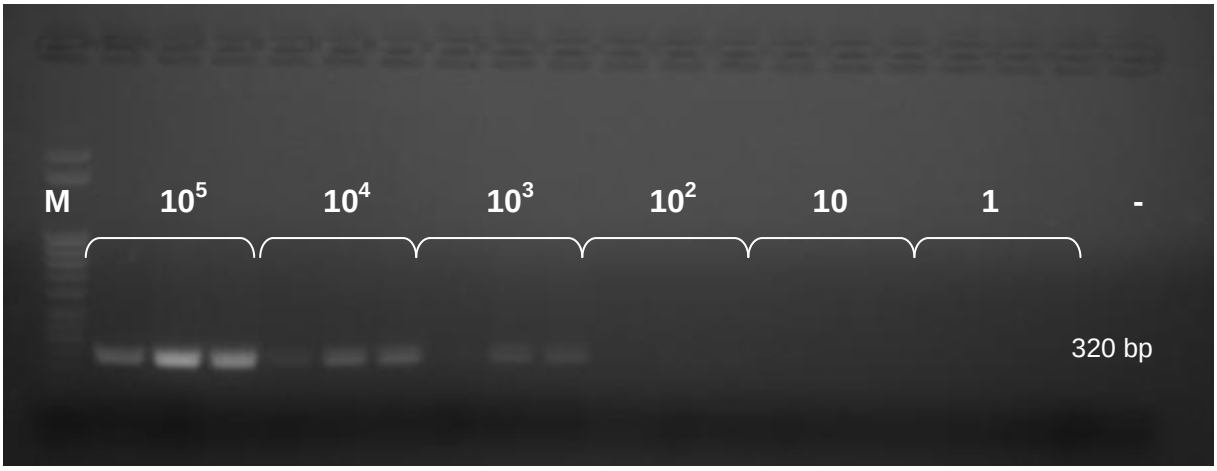


Figure 24: *E. invadens* primer sensitivity testing Inv5.8sf/ Inv5.8sr

4.2.2 Nested PCR:

The specificity of nested PCR using the primer pairs JDP1/ Rhiz3r and Rhiz2/ JDP2 was demonstrated by amplification of *Acanthamoeba* spp. DNA in tissue samples (brain and intestine) from a euthanized *Boa*.

Figure 25 illustrates that the PCR performed at a temperature of 54°C leads barring to the expected ~200bp amplicon, to many unspecific DNA amplicons in different sizes and DNA amount. Furthermore, performing the nested PCR with 20 cycles in the first and second step leads to less increase of DNA amount as shown in Figure 25a. Following this strategy leads to very little DNA amplification of *Acanthamoeba* spp. with a size of ~200bp in difference to performing the PCR with 20 cycles in the first step and 30 cycles in the second step as illustrated in Figure 25b. Unfortunately, also the non specific bands increased with the number of performed cycles. As marked in Figure 25b an amplicon with the size of 150bp was detected right beneath the ~200bp fragments of acanthamobae which turned out to be genotype T5 (*Acanthamoeba lenticulata*)

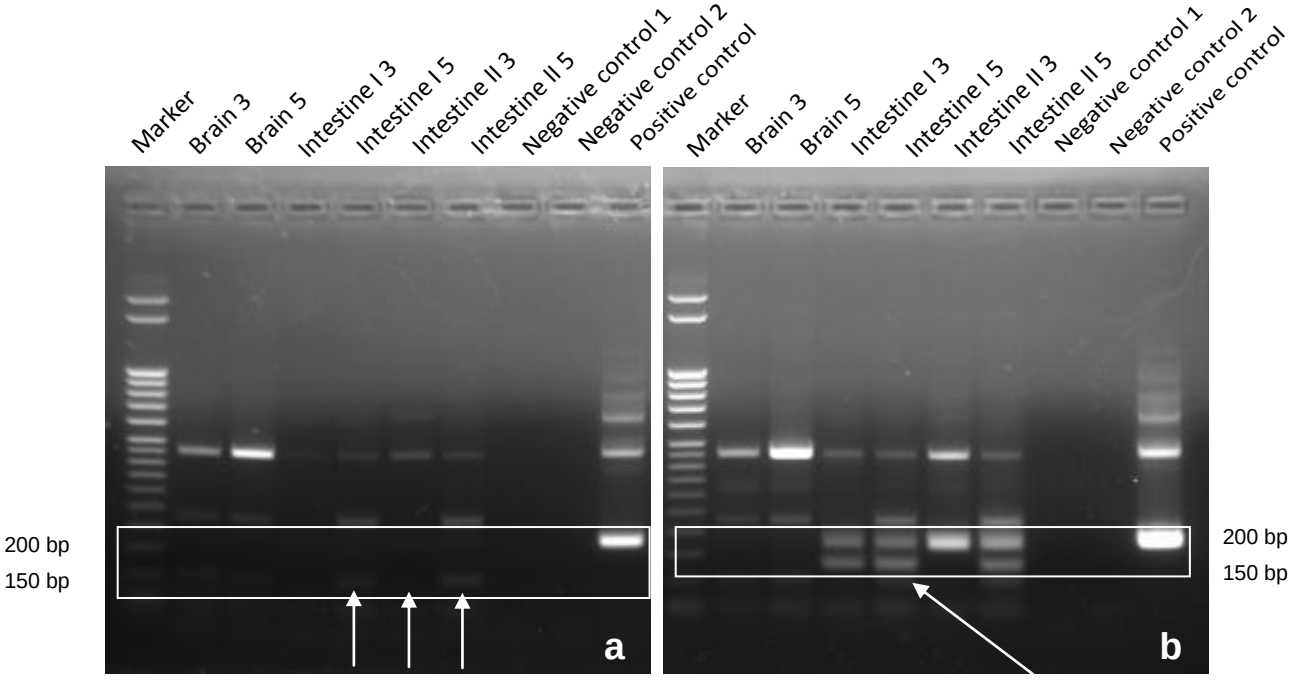


Figure 25: Gelelectrophoresis nested PCRGastB54°C a) 20 cycles; b) 30 cycles

Performing GastB 56°C with 30 cycles in the first and second step of the PCR turned out to be the most capable combination of cycle numbers and temperature of this nested PCR establishment. With this program the unspecific DNA amplification decreased and fragments with 150 bp length are a detectable as illustrated in Figure 26a, b.

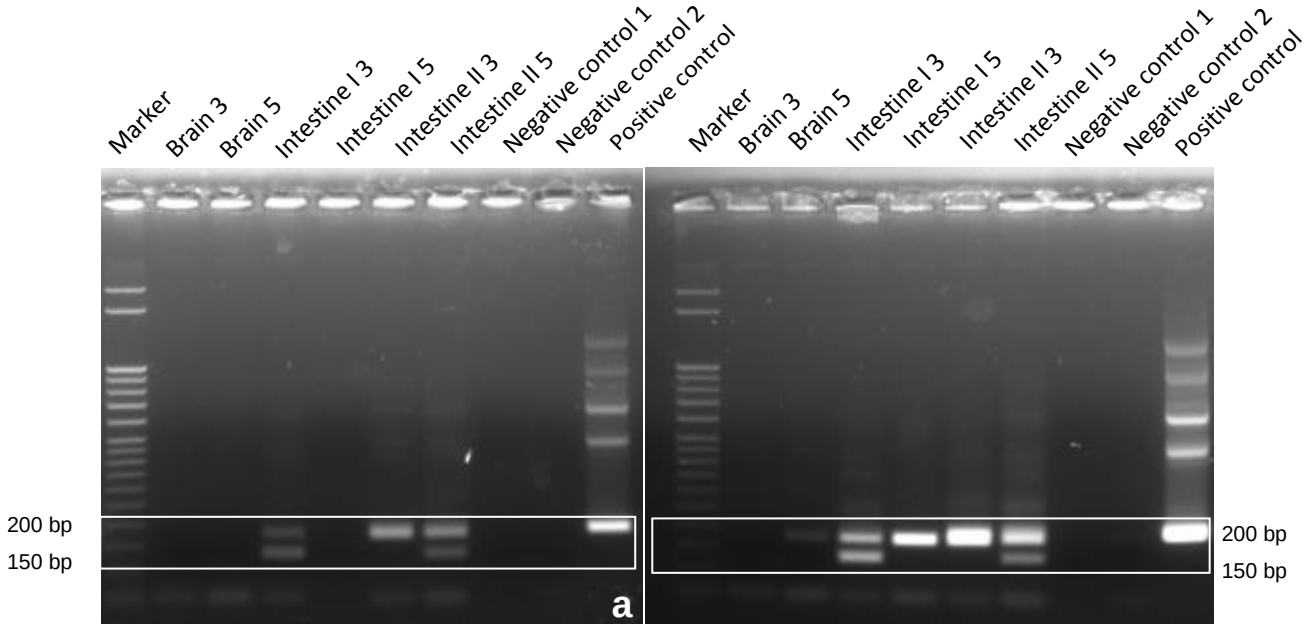


Figure 26: Gelelectrophoresis nested PCR GastB56°C; a) 20 cycles; b) 30 cycles

4.2.3 Snakes

4.2.3.1 Snakes: first sample drawing (6th of November 2007)

Mouth and cloaca swabs from nine snakes were collected during this sample drawing.

Table 12: PCR results snakes: first sample drawing (06.11.07)

Sample		*Acanthamoeba* spp.	*Acanthamoeba* spp. nested PCR	*B. mandrillaris*	*N. fowleri*	*E. invadens*
KP M	mouth	-	-	-	-	-
KP K	cloaca	-	-	-	-	-
FPu M	mouth	-	-	-	-	-
FPu K	cloaca	-	-	-	-	+
FPo M	mouth	-	-	-	-	+
FPo K	cloaca	-	+	-	-	-
Ana M	mouth	-	-	-	-	+
Ana K	cloaca	-	-	-	-	-
La M	mouth	-	-	-	-	+
La K	cloaca	-	+	-	-	+
BZ M	mouth	-	+	-	-	-
BZ K	cloaca	-	+	-	-	+
Bk M	mouth	-	-	-	-	+
Bk K	cloaca	-	-	-	-	-

TP M	**mouth**	-	+	-	-	+
TP K	**cloaca**	-	-	-	-	+
AG M	**mouth**	-	-	-	-	+
AG K	**cloaca**	-	+	-	-	+

Aerobic culture:

Due to the fact, that the *Acanthamoeba* spp. PCR (JDP) is not suitable for the purposes of detecting amoebas from swabs a nested PCR was performed. 5 of 9 snakes turned out to be *Acanthamoeba* spp. positive in the nested PCR (Figure 27 and Figure 28) in contrast to JDP PCR (Schröder et al. 2001), where no positive results were detectable. Five of nine snakes (FPo= *Python sebae* upper; La= *Langaha madagascariensis*; TP= *Python morulus*; BZ= *Boa* accordion disease and AG= *Eunectes notaeus*) showed an infection with acanthamoebas. Additionally to the ~200bp amplicon another signal with 150bp was detectable on the agarose gel. Sequencing of the 150bp amplicons showed that these PCR products are *Acanthamoeba* spp. genotype T5 (*A. lenticulata*). As demonstrated in Figure 29 four snakes (La= *Langaha* sp; BZ= *Boa* "accordion disease"; TP= *Python morulus* and FPo= *Python sebae* "upper") are co infected with different genotypes of acanthamoebas and one snake (AG= *Eunectes notaeus*) only with genotype T5.

Furthermore, the PCR performed to detect *B. mandrillaris* and *N. fowleri* were negative for all collected samples from this sample drawing as shown in Table 12.

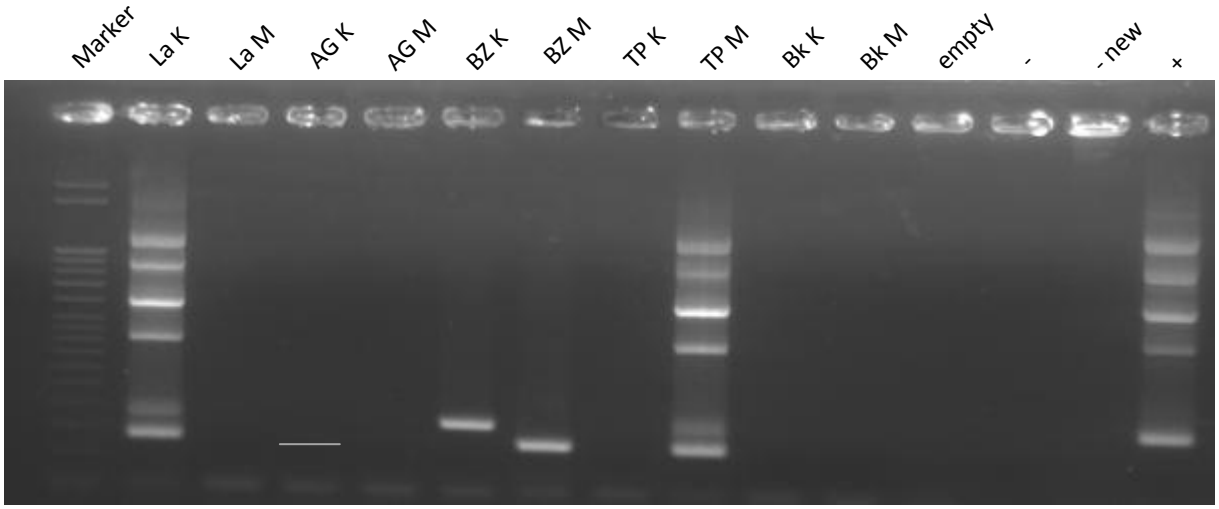


Figure 27: nested PCR results snakes: first sample drawing (16.11.2007)

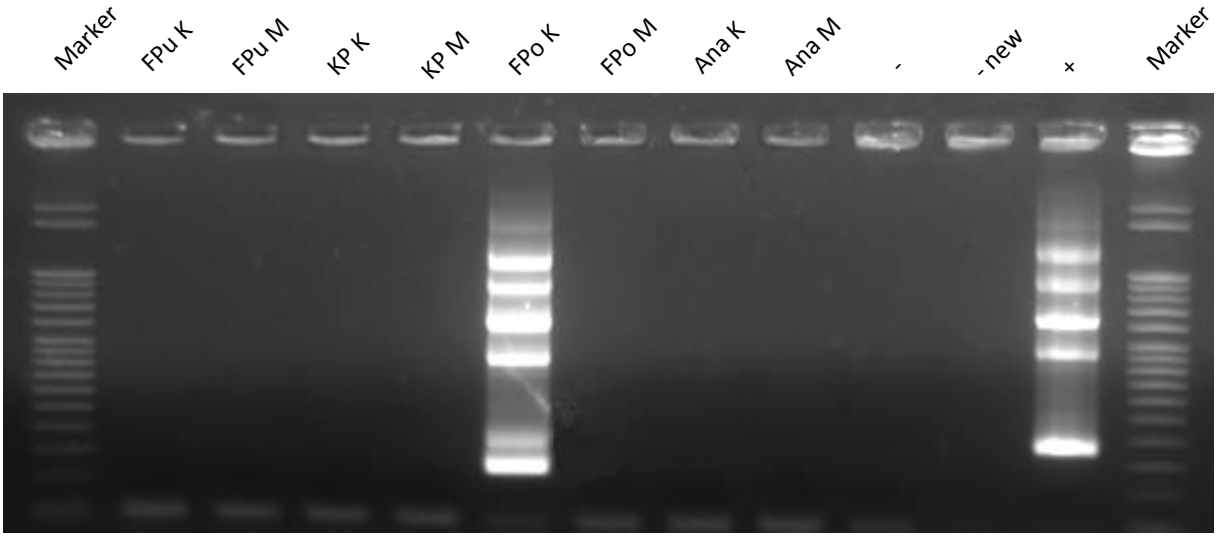


Figure 28: nested PCR results snakes: first sample drawing (16.11.2007)

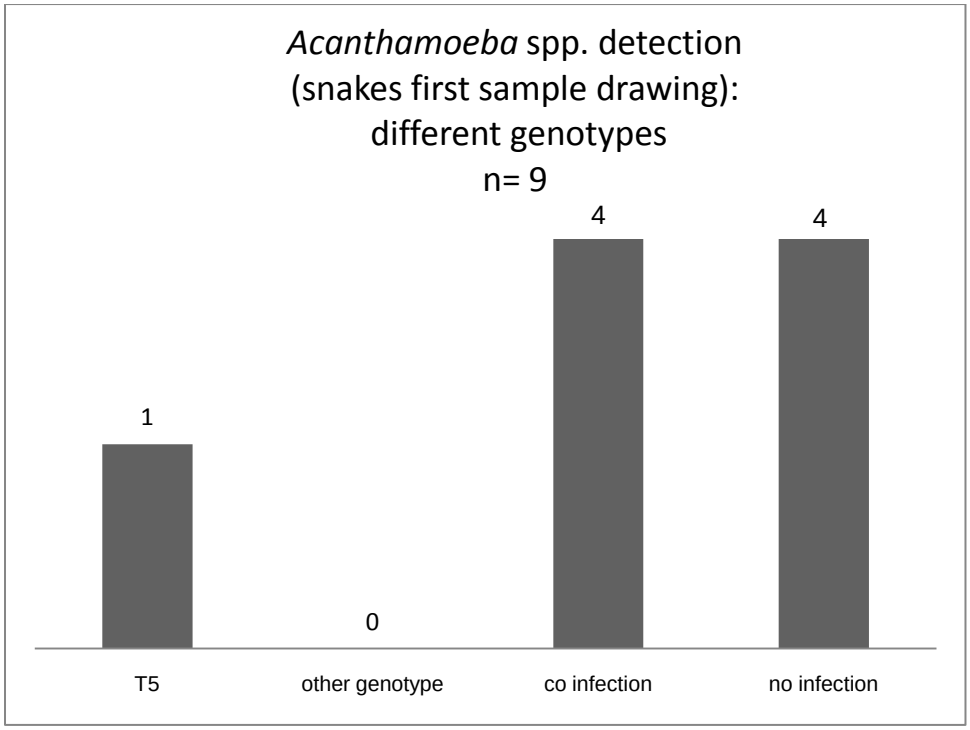


Figure 29: *Acanthamoeba* spp. co- infection in snakes, first sample drawing (06.11.2007)

Anaerobic culture:

Eight of nine snakes (88%) are *E. invadens* positive. With the exception of KP (*Python regius*) all snakes showed a positive signal (Figure 30 and Figure 31) in PCR performance as demonstrated in Table 12.

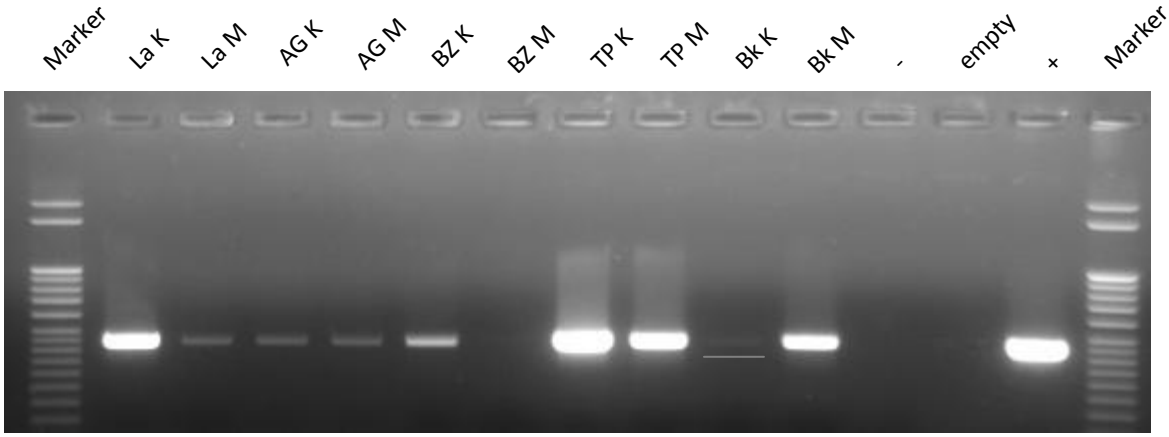


Figure 30 *E. invadens* PCR results snakes: first sample drawing (06.11.07)

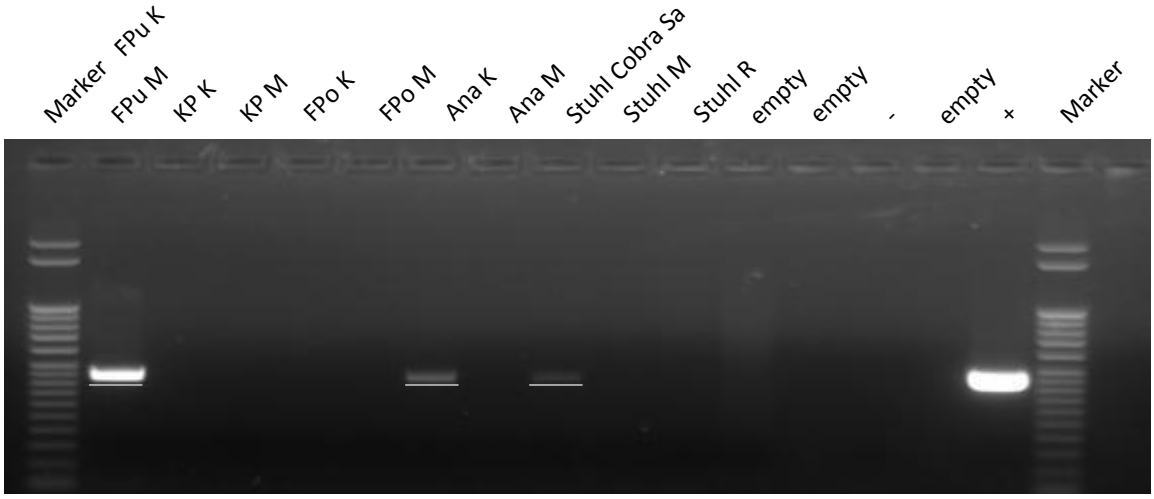


Figure 31 *E. invadens* PCR results snakes: first sample drawing (06.11.07)

Co- infection *E. invadens* and *Acanthamoeba* spp.:

Figure 32 illustrates co infections of the analyzed snakes. Five of nine (56%) are co-infected with *E. invadens* and *Acanthamoeba* spp. Three of nine snakes (33%) are only infected by *E. invadens*. No snake was detected to be infected only by *Acanthamoeba* spp. One snake (11%) shows no positive signal in PCR performance for *E. invadens* as for *Acanthamoeba* spp. respectively (*Python regius* = KP).

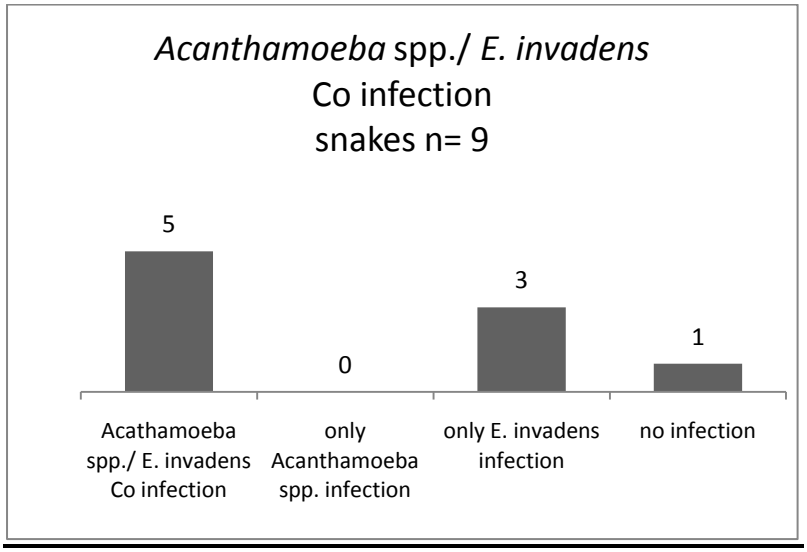


Figure 32: Co- infection *Acanthamoeba* spp./ *E. invadens* in snakes first sample drawing (06.11.07)

4.2.3.2 Snakes: second sample drawing (13th of December 2007)

Mouth and cloaca of eight snakes were swabbed during the second sample drawing.

Table 13: PCR results snakes: second sample drawing (13.12.2007)

sample		*Acanthamoeba* spp.	*Acanthamoeba* spp. nested PCR	*B. mandrillaris*	*N. fowleri*	*E. invadens*
KP M	mouth	-	-	-	-	-
KP K	cloaca	-	-	-	-	-
FP2 M	mouth	-	-	-	-	-
FP2 K	cloaca	-	-	-	-	+
Ana Gr. M	mouth	-	+	-	-	+
Ana Gr. K	cloaca	-	+	-	-	-
FP1 M	mouth	-	+	-	-	+
FP1 K	cloaca	-	-	-	-	+
ASN M	mouth	-	-	-	-	+
ASN K	cloaca	-	+	-	-	+
BZ M	mouth	-	+	-	-	+
BZ K	cloaca	-	-	-	-	+
Bk M	mouth	-	+	-	-	+
Bk K	cloaca	-	+	-	-	+
AG M	mouth	-	+	-	-	+
AG K	cloaca	-	+	-	-	+

Aerobic culture:

Figure 33 demonstrates that 6 of 8 snakes (75%) are positive for *Acanthamoeba* spp. in PCR. Furthermore, all of these 6 *Acanthamoeba* spp. positive snakes were positive for genotype T5 and another genotype as shown in Figure 34.

As demonstrated in Table 13 all snakes were negative for *B. mandrillaris* and *N. fowleri* in PCR.

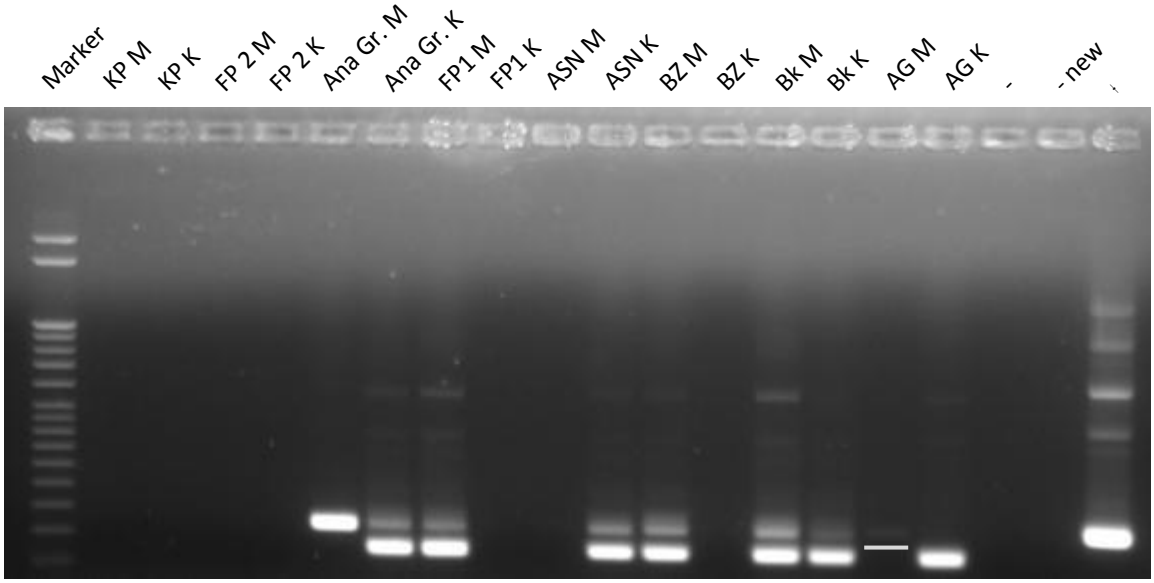


Figure 33: *Acanthamoeba* spp. nested PCR results: snakes second sample drawing (13.12.2007)

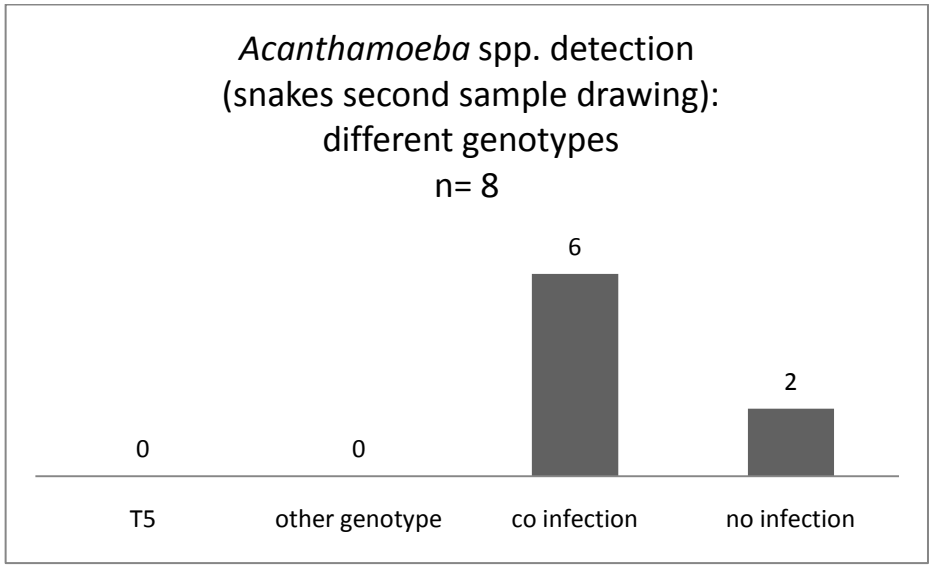


Figure 34: *Acanthamoeba* spp. co infection in snakes, second sample drawing (13.12.2007)

Anaerobic culture:

Seven of eight snakes (87.5%) turned out to be *E. invadens* positive in PCR (Figure 35). Again, the only snake without an *E. invadens* infection is *Python regius* (KP).

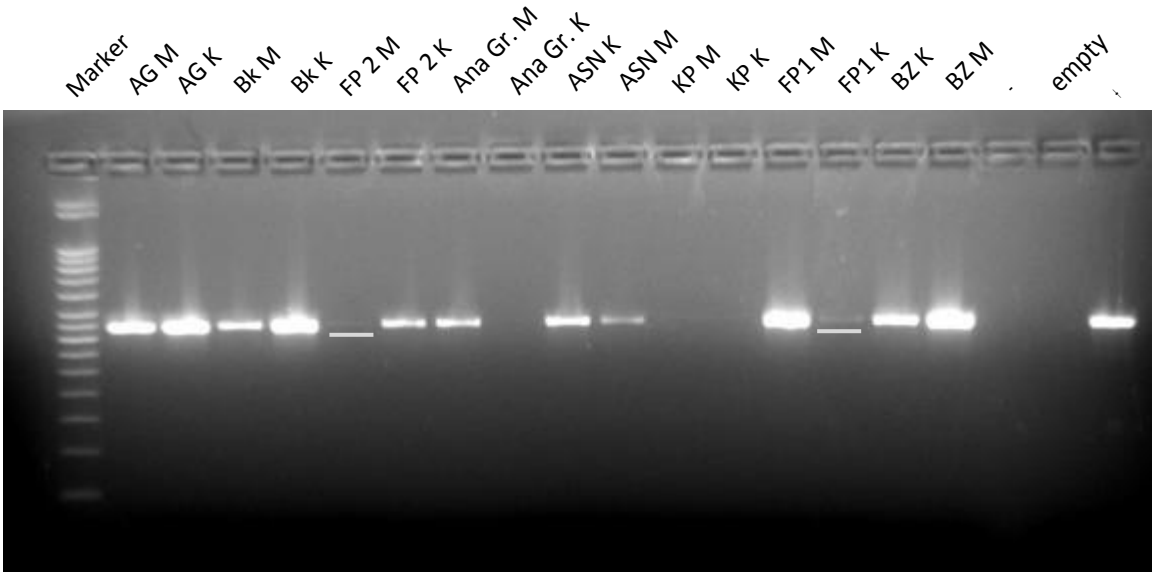


Figure 35: *E .invadens* PCR results snakes: second sample drawing (13.12.07)

Co-infection *E. invadens* and *Acanthamoeba* spp.:

Figure 36 illustrates co infection of *Acanthamoeba* spp. and *E. invadens* in snakes from the second sample drawing. Six of eight snakes (75%) are infected with *Acanthamoeba* spp. and *E. invadens.* One snake (12%) shows only an *Acanthamoeba* spp. infection, but no infection with *E. invadens*. Again, the only snake (13%) that shows no positive signals in PCR performances for *E. invadens* as well as *Acanthamoeba* spp. is *Python regius* (KP).

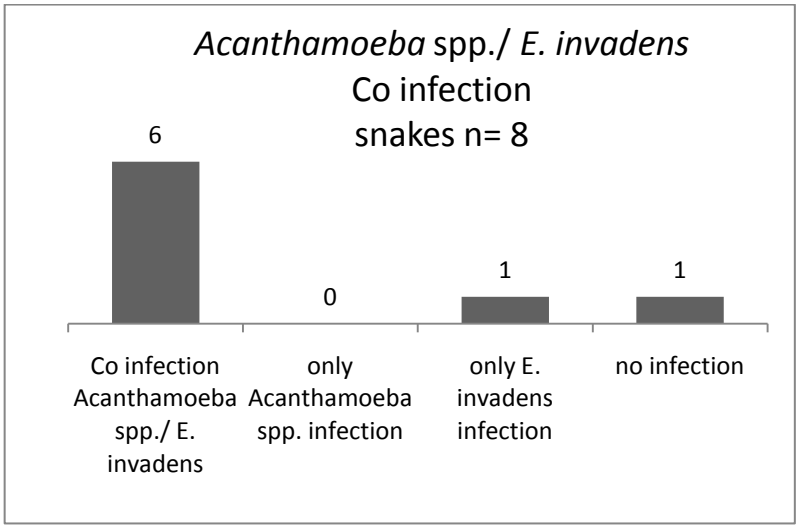


Figure 36: Co- infection *Acanthamoeba* spp./ *E. invadens* in snakes second sample drawing (13.12.2007)

4.2.3.3 Summary: snakes

11 of 17 (64%) of swabbed snakes turned out to be *Acanthamoeba* spp. positive. Furthermore, 15 of 17 snakes (88%) were positive for *E. invadens* in PCR performance.

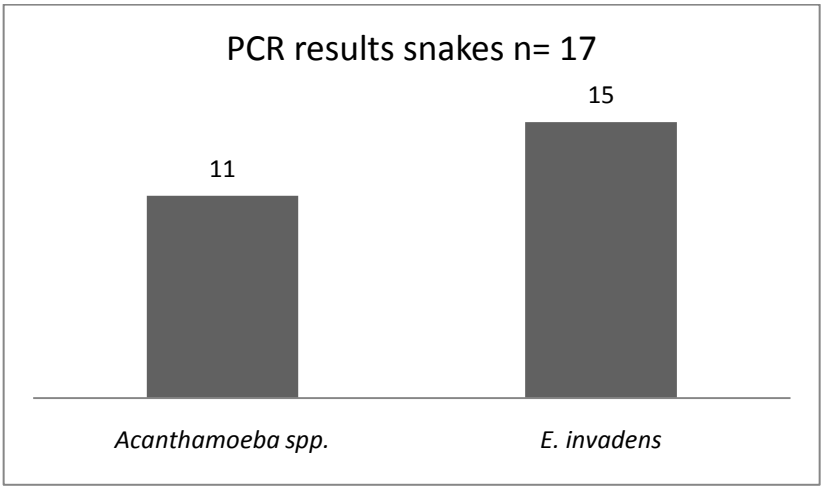


Figure 37: PCR results snakes: summary

PCR results: comparison of the first and second sample drawing from snakes

Python regius (KP) was negative for *Acanthamoeba* spp. and *E. invadens* in both sample drawings which points out that no change of infection status occurred.

Boa accordion disease (BZ) was *Acanthamoeba* spp. and *E. invadens* positive in both sample drawings. However, analyzing of the cloaca swabs resulted in the first sample drawing during PCR performance that this snake is *Acanthamoeba* spp. positive unlike to the second sample drawing. Though, the mouth swab in the first sample drawing was *E. invadens* negative in PCR performance in difference to the second sample drawing.
In the first sample drawing one snake "*Boa* small" (Bk) turned out to be *Acanthamoeba* spp. negative in mouth as well as in cloaca swabs in difference to the second sample drawing, where this snake was positive for *Acanthamoeba* spp. Detected amoebas from swabs of AG (*Eunectes notaeus*) were in both sample drawings *E. invadens* and *Acanthamoeba* spp. But, mouth swab was negative for acanthamoebas in the first collection of samples which changed in the second.

Table 14: Comparison of PCR results from the first and second sample drawing from snakes

Sample		First sample drawing *Acanthamoeba* spp. nested PCR	First sample drawing: *E. invadens*	Second sample drawing: *Acanthamoeba* spp. nested PCR	Second sample drawing: *E. invadens*
KP M	**mouth**	-	-	-	-
KP K	**cloaca**	-	-	-	-
BZ M	**mouth**	+	-	+	+
BZ K	**cloaca**	+	+	-	+
Bk M	**mouth**	-	+	+	+
Bk K	**cloaca**	-	-	+	+
AG M	**mouth**	-	+	+	+
AG K	**cloaca**	+	+	+	+

4.2.3.4 *Boa* "accordion disease": tissue samples

The tissue samples from the intestine of the *Boa* "accordion disease" turned out to be *E. invadens* positive as shown in Figure 38. Furthermore, tissue samples form the brain and

the intestine were positive for *Acanthamoeba* spp. as demonstrated in Figure 26. All samples turned out to be *B. mandrillaris* and *N. fowleri* negative.

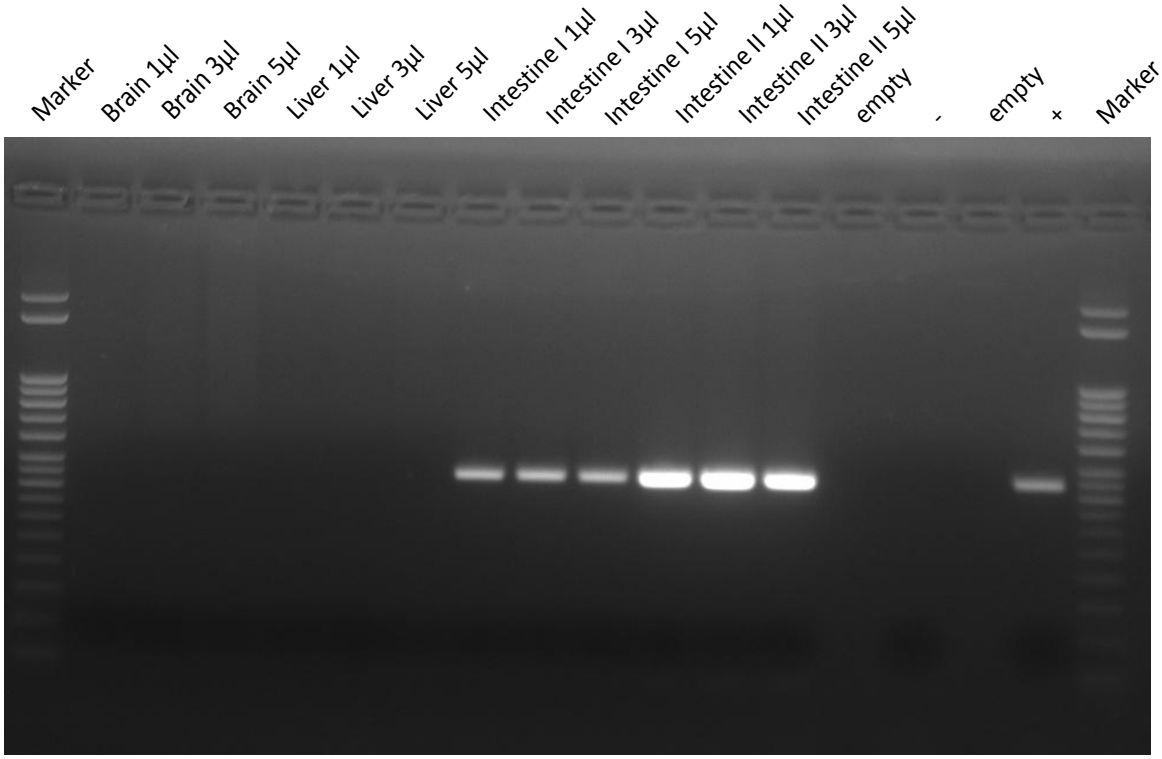


Figure 38: *E. invadens* PCR results *Boa* "accordion disease" (tissue samples)

4.2.4 Chelonians

4.2.4.1 Chelonians: first sample drawing (6th of February 2008)

Twenty mouth and cloaca swabs and one faeces sample from 10 chelonians were analyzed.

Table 15: PCR results chelonians first sample drawing (06.02.2008)

Sample		*Acanthamoeba* spp.	*Acanthamoeba* spp. nested PCR	*B. mandrillaris*	*N. fowleri*	*E. invadens*
1K	cloaca	-	+	-	-	+
1M	mouth	-	+	-	-	+
2K	cloaca	-	+	-	-	+
2M	mouth	-	+	-	-	+
2 Kot	faeces	-	+	-	-	+
3K	cloaca	-	+	-	-	+

3M	**mouth**	-	+	-	-	+
4K	**cloaca**	-	+	-	-	+
4M	**mouth**	-	+	-	-	+
5K	**cloaca**	-	-	-	-	-
5M	**mouth**	-	+	-	-	+
6K	**cloaca**	-	+	-	-	+
6M	**mouth**	-	+	-	-	+
7K	**cloaca**	-	+	-	-	+
7M	**mouth**	-	-	-	-	+
8K	**cloaca**	-	+	-	-	+
8M	**mouth**	-	+	-	-	+
9K	**cloaca**	-	+	-	-	+
9M	**mouth**	-	-	-	-	+
10K	**cloaca**	-	-	-	-	+
10M	**mouth**	-	-	-	-	+

Aerobic culture:

In the first sample drawing from chelonians, mouth and cloaca swabs from 10 individual animals were collected and one faeces sample additionally. Nine of ten (90%) chelonians (1= *Trachemys scripta elegans*; 2= *Astrochyles radiata*; 3= *Heosemys*; 4= *Testudo kleinmanni*; 5= *Heosemys, juvenile*; 6= *Emys orbicularis*; 7= *Heosemys,* dam; 8= *Phrynops hilarii*; 9= *Dipsochelys dussumieri*) were positive for *Acanthamoeba* spp. in nested PCR (Figure 39 and Figure 40).

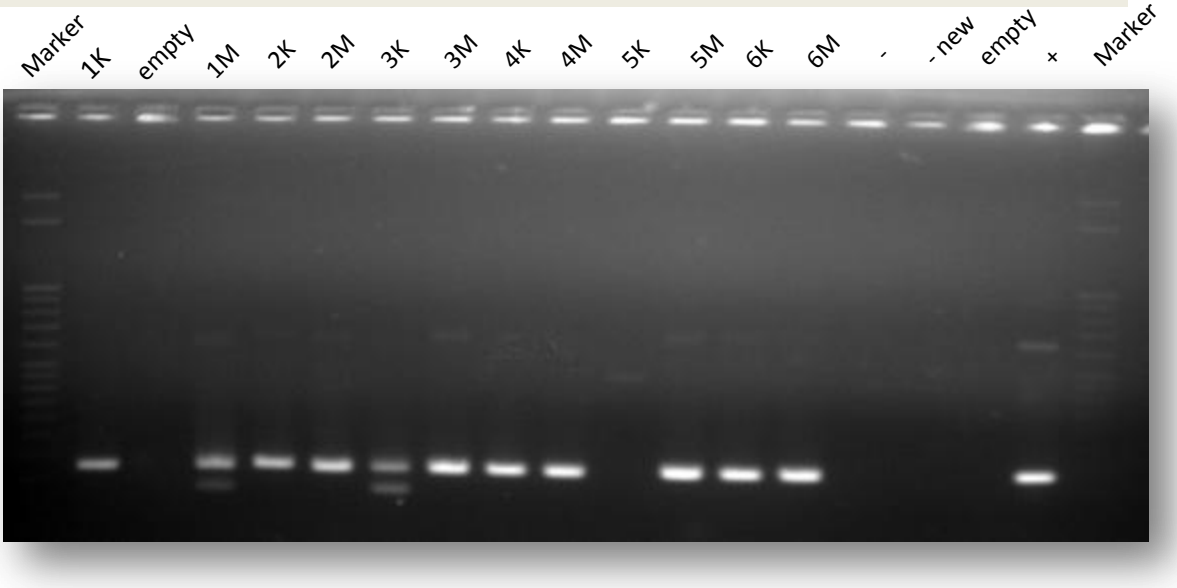


Figure 39: nested PCR results chelonians: first sample drawing (06.02.2008

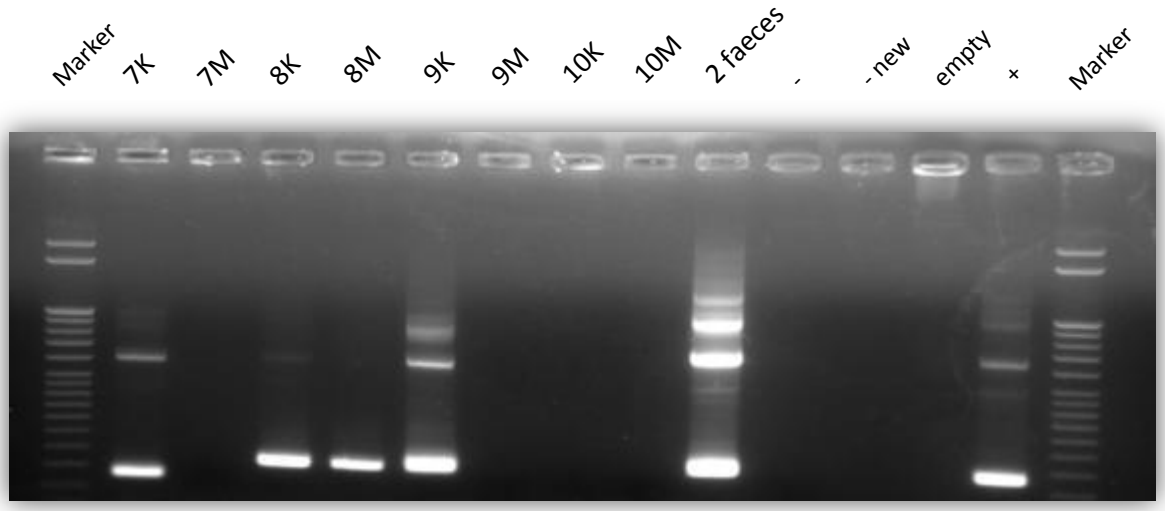


Figure 40: nested PCR results chelonians: first sample drawing (06.02.2008)

As demonstrated on the agarose gel, different *Acanthamoeba* spp. genotypes are detected in nested PCR (

Figure 39 and Figure 40). Two of nine (22%) chelonians, *Trachemys scripta elegans* (1) and *Heosemys* (3) are co-infected with the genotype T5 and other genotypes of *Acanthamoeb*a spp. One chelonian, *Heosemys,* dam (7) was positive for genotype T5. However, the six remaining chelonians *Astrochyles radiata* (2), *Testudo kleinmanni* (4), *Heosemys*, juvenile (5), *Emys orbicularis* (6), *Phrynops hilarii* (8) and *Geochelone elephantopus* A (9) where *Acanthamoeba* spp. was detected are positive for other genotypes than T5.

All 20 mouth and cloaca swabs were negative for *B. mandrillaris* and *N. fowleri*.

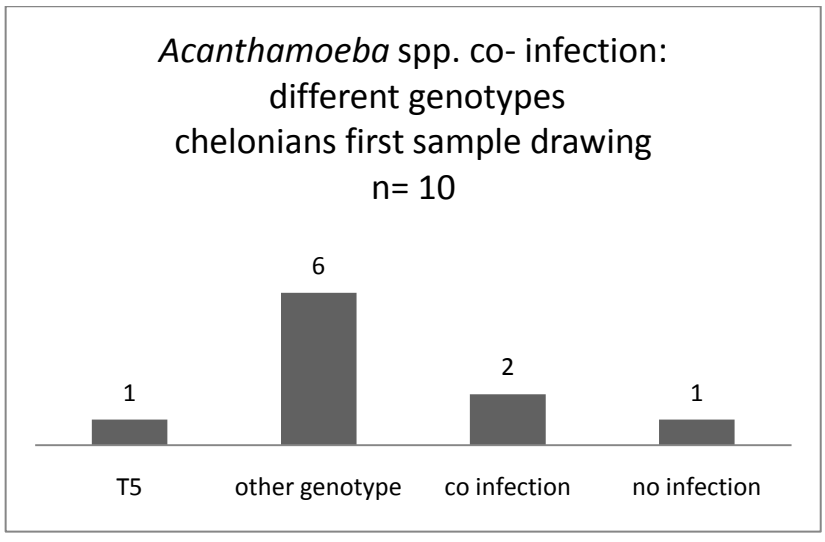


Figure 41: *Acanthamoeba* spp. co- infection chelonians first sample drawing (06.02.2008)

Anaerobic culture:

Mouth and cloaca swabs from ten chelonians were analyzed in this sample drawing to detect *E. invadens*. All chelonians (100%) turned out to be *E. invadens* positive in PCR performance, although the signals on agarose gel were weak as shown in Figure 42 and Figure 43. Faeces sample from *Astrochyles radiata* (sample 2 faeces) has been found to produce the strongest signal in PCR performance for these anaerobic amoebas (Figure 42 and Figure 43).

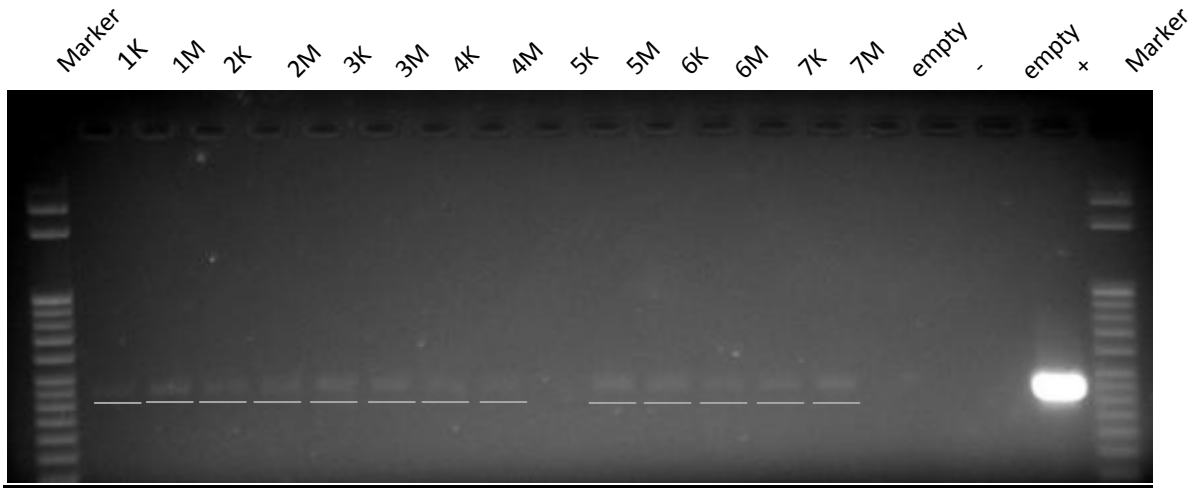


Figure 42: *E. invadens* PCR results chelonians: first sample drawing (06.02.2008)

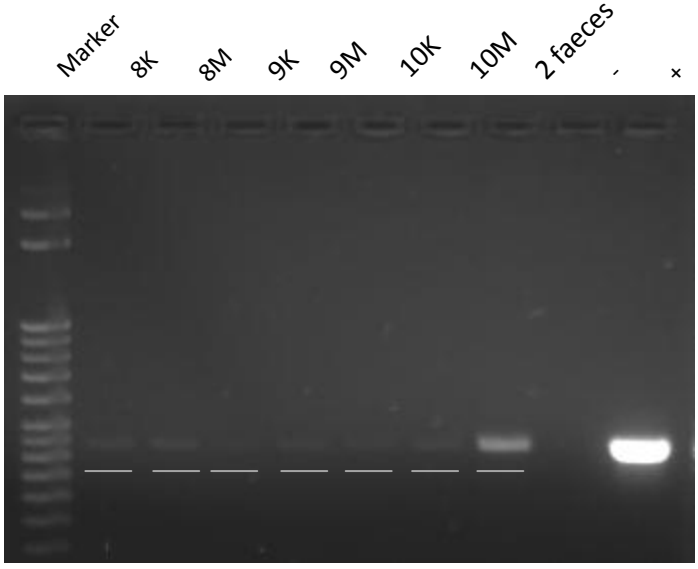


Figure 43: *E. invadens* PCR results chelonians: first sample drawing (06.02.2008)

Co-infection *E. invadens* and *Acanthamoeba* spp.:

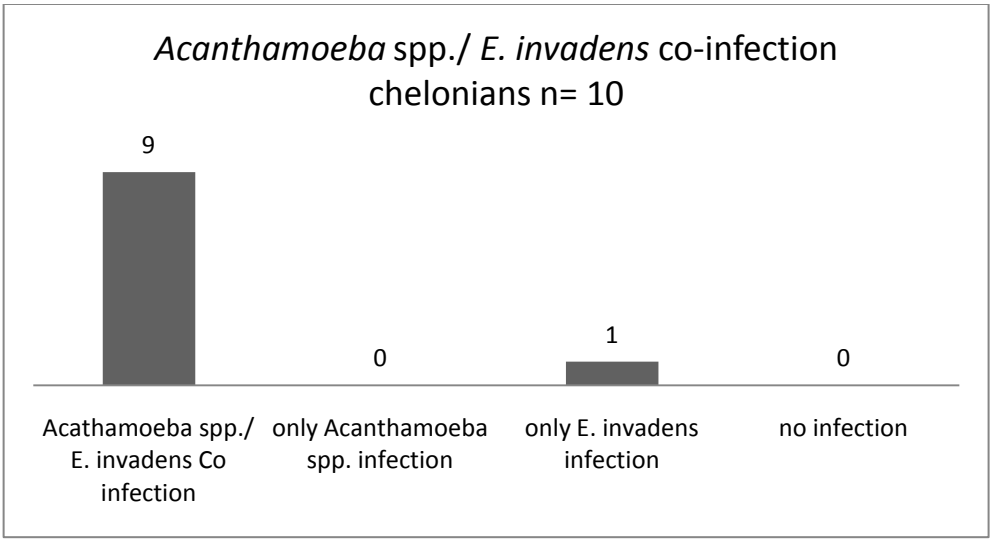


Figure 44: *Acanthamoeba* spp. /*E. invadens* co-infection chelonians first sample drawing (06.02.2008)

Figure 44 illustrates co-infections of *Acanthamoeba* spp. and *E. invadens* in samples collected from chelonians in the first sample drawing. Nine of ten chelonians (90%) showed positive signals in *Acanthamoeba* spp. nested PCR and *E. invadens* PCR performance. One chelonian (10%) shows only an infection with *E. invadens* but no infection with *Acanthamoeba* spp.

4.2.4.2 Chelonians: second sample drawing (7th of May 2008)

Mouth and cloaca of 10 chelonians were swabbed in the second sample drawing.

Table 16: PCR results chelonians: second sample drawing (07.05.2008)

Sample		*Acanthamoeba* spp.	*Acanthamoeba* spp. nested PCR	*B. mandrillaris*	*N. fowleri*	*E. invadens*
1K	cloaca	-	-	-	-	-
1M	mouth	-	+	-	-	-
2K	cloaca	-	-	-	-	-
2M	mouth	-	-	-	-	+
3K	cloaca	-	+	-	-	+
3M	mouth	-	+	-	-	+

4K	**cloaca**	-	+	-	-	+
4M	**mouth**	-	-	-	-	+
5K	**cloaca**	-	+	-	-	+
5M	**mouth**	-	+	-	-	-
6K	**cloaca**	-	+	-	-	-
6M	**mouth**	-	+	-	-	-
7K	**cloaca**	-	-	-	-	-
7M	**mouth**	-	+	-	-	-
8K	**cloaca**	-	-	-	-	-
8M	**mouth**	-	-	-	-	-
9K	**cloaca**	-	-	-	-	-
9M	**mouth**	-	-	-	-	-
10K	**cloaca**	-	-	-	-	-
10M	**mouth**	-	-	-	-	-

Aerobic culture:

Six of ten (60%) chelonians were positive in nested PCR performance for the detection of *Acanthamoeba* spp. The positive animals are *Trachemys scripta elegans* (1); *Heosemys* (3); *Testudo kleinmanni* (4); *Heosemys,* juvenile (5); *Emys orbicularis* (6) and *Heosemys*, dam (7). Again, different genotypes of *Acanthamoeba* spp. were detected on the agarose gel as demonstrated in Figure 45 and Figure 46.

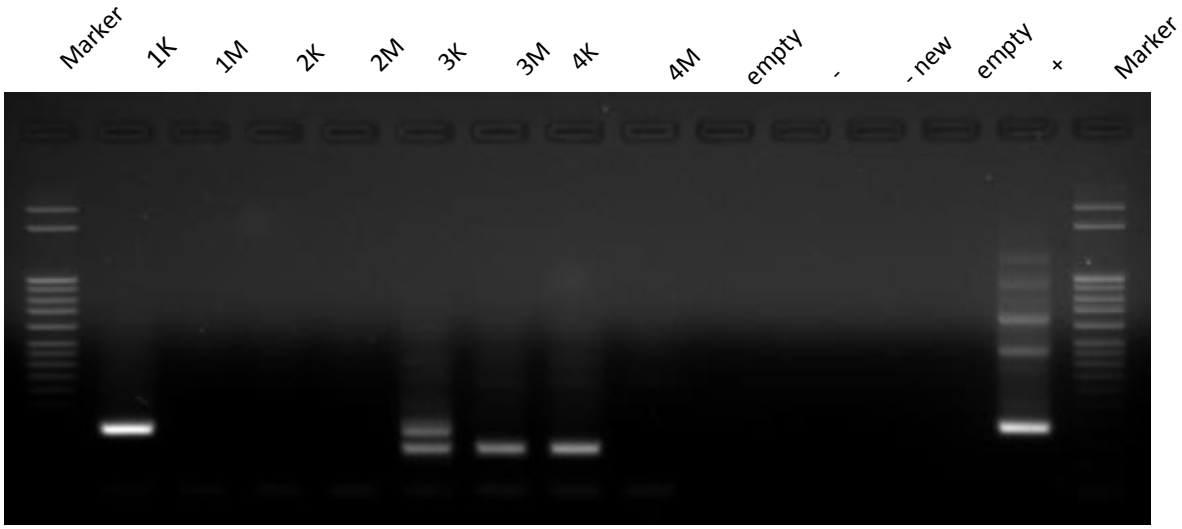


Figure 45: nested PCR results chelonians second sample drawing (07.05.2008)

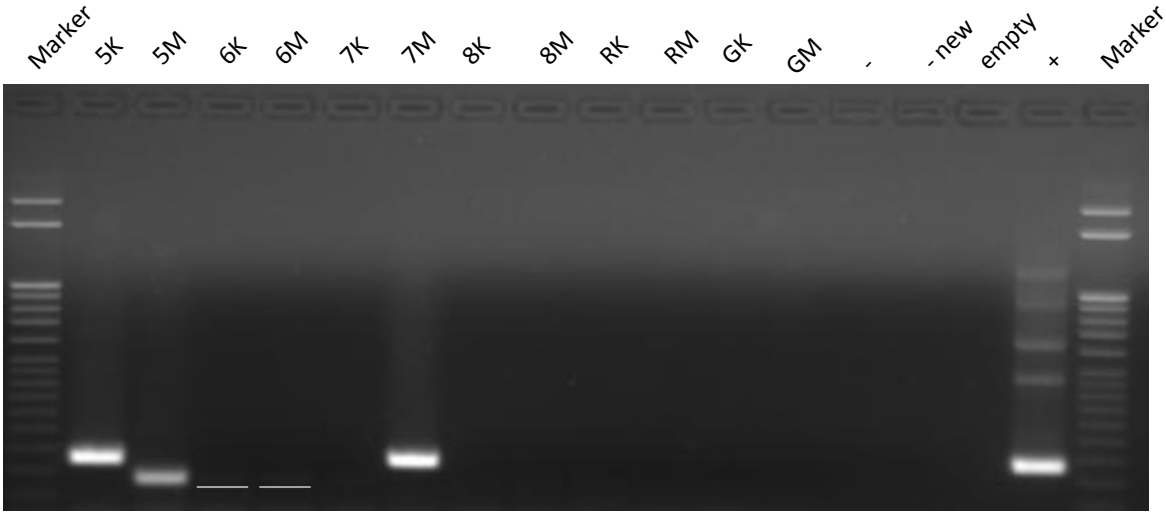


Figure 46: nested PCR results chelonians: second sample drawing (07.05.2008)

As demonstrated on the agarose gel (Figure 45 and Figure 46), positive signals for different *Acanthamoeba* spp. Genotypes were detectable under UV exposure.

As shown in Figure 47 two chelonians *Testudo kleinmanni* (4) and *Emys orbicularis* (6) turned out to be positive for genotype T5. Moreover, two chelonians *Trachemys scripta elegans* (1) and *Heosemys*, dam (7) were positive for other genotypes. Co-infection was detectable in two chelonians *Heosemys* (3) and *Heosemys,* juvenile (5).

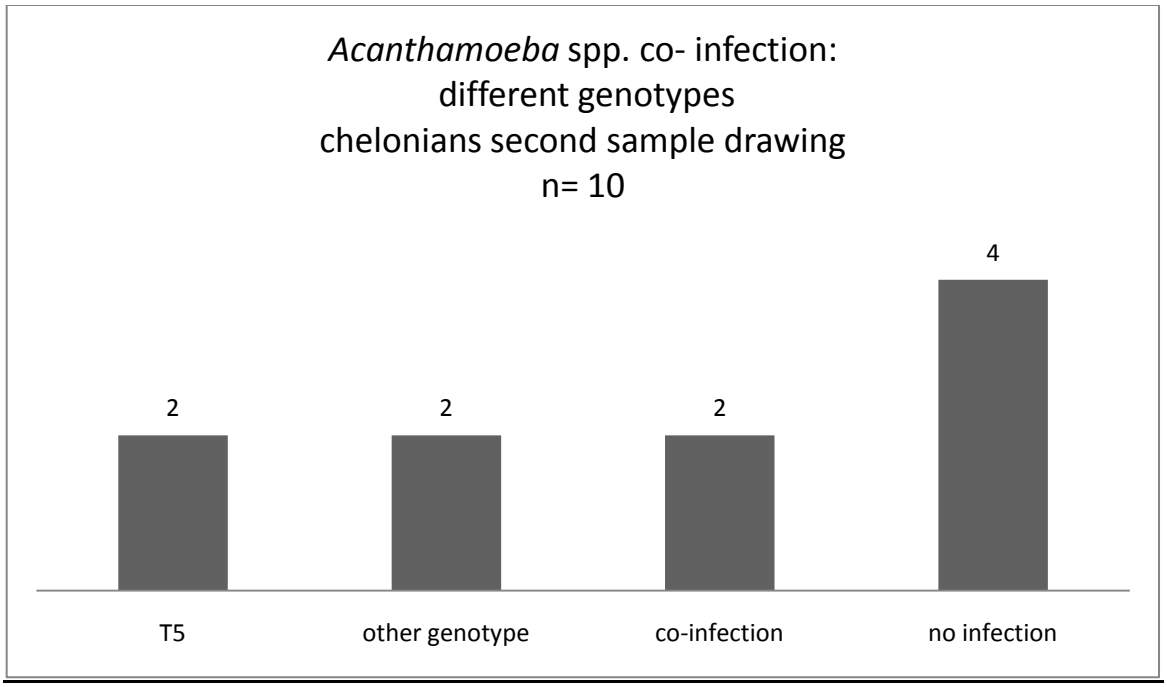


Figure 47: *Acanthamoeba* spp. co-infection chelonians: second sample drawing (07.05.2008)

Anaerobic culture:

The infection with *E. invadens* in chelonians from the second sample drawing was demonstrated by using the primer pair Inv18sf/ Inv18sr to amplify DNA during PCR performance. Four of ten chelonians (40%) turned out to be *E. invadens* positive: *Astrochyles radiata* (2); *Heosemys* (3); *Testudo kleinmanni* (4) and *Heosemys* juvenile (5) as shown in Figure 48 and Figure 49.

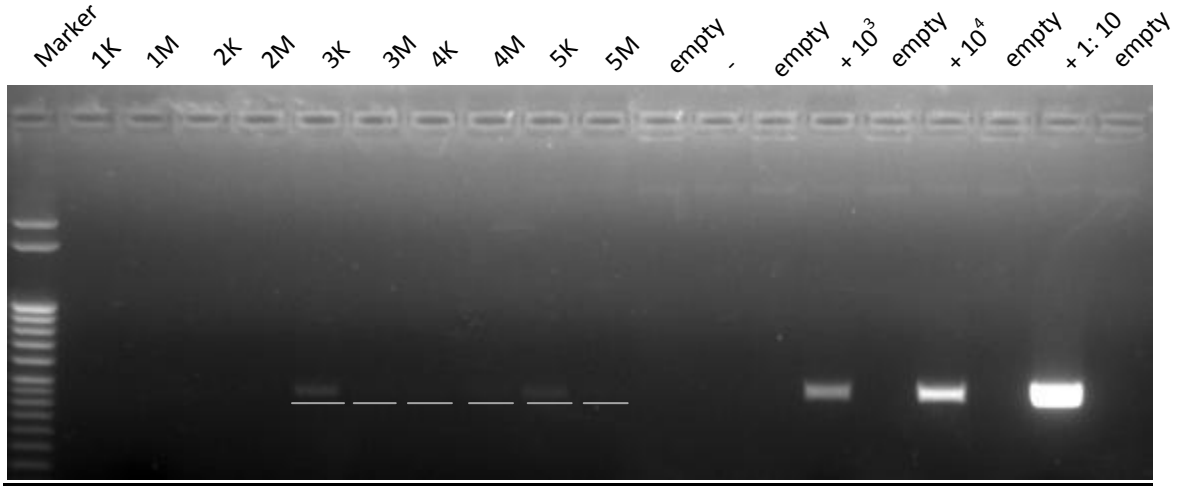


Figure 48: *E. invadens* PCR results chelonians: second sample drawing (07.05.2008)

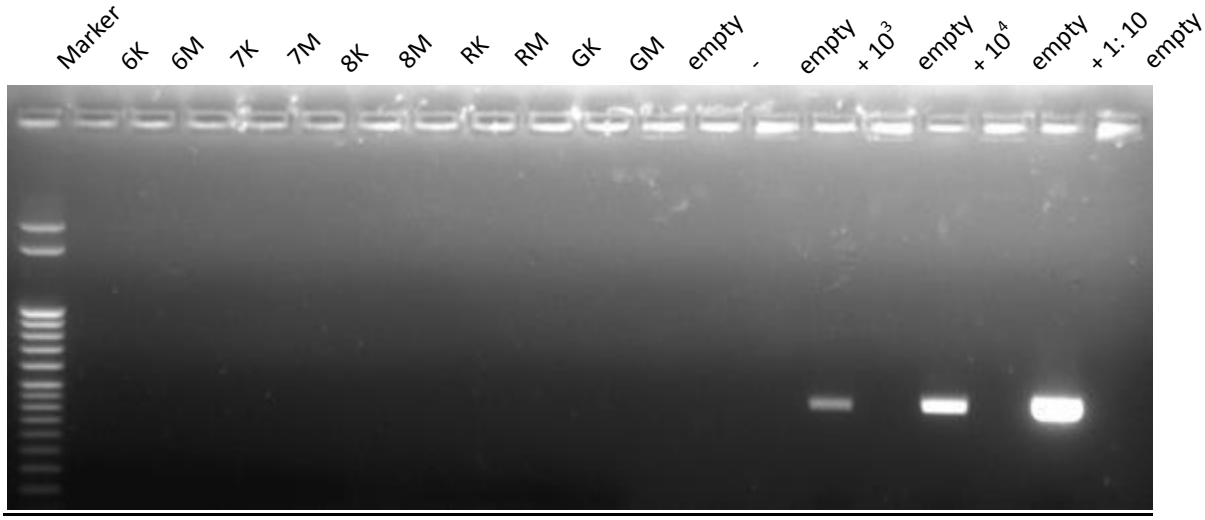


Figure 49: *E. invadens* PCR results chelonians: second sample drawing (07.05.2008)

Co-infection *E. invadens* and *Acanthamoeba* spp.:

As demonstrated in Figure 50 three chelonians (30%) from the second sample drawing showed positive results in *Acanthamoeba* spp. nested PCR and *E. invadens* PCR performance. Furthermore, three chelonians (30%) were only positive for *Acanthamoeba* spp. and only one (10%) for *E. invadens*. The figure below also shows that three chelonians (30%) were negative for acanthamoebas and entamoebas.

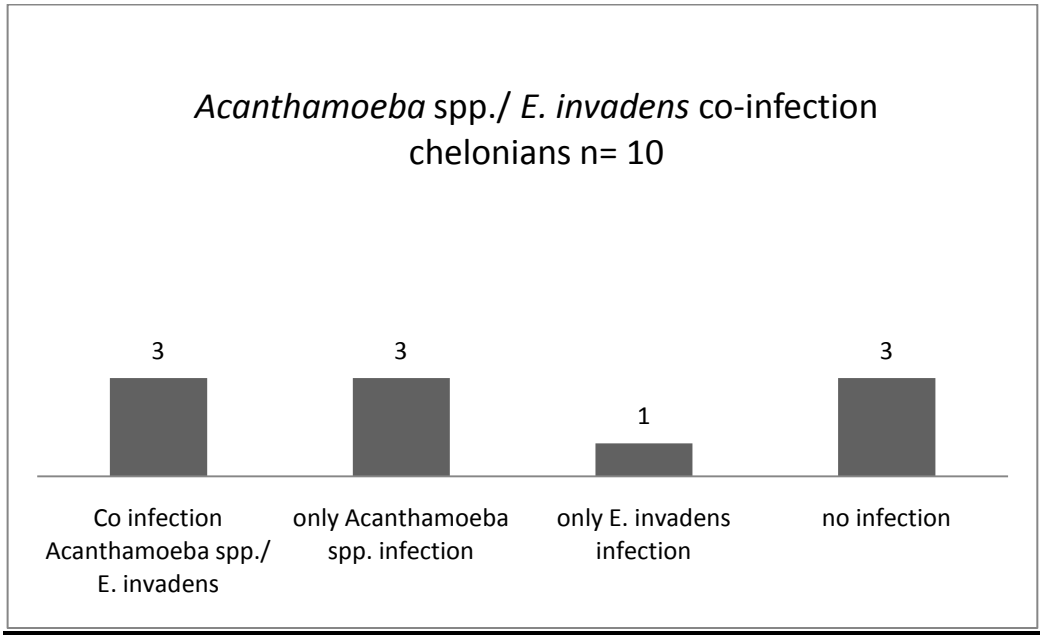


Figure 50: *Acanthamoeba* spp. /*E. invadens* co-infection chelonians: second sample drawing (07.05.2008)

4.2.4.3 Summary chelonians:

PCR results: comparison of the first and second sample drawing from chelonians

A direct comparison of PCR results is not possible due to the fact, that it is unclear if exactly the same individual has been swabbed in both sample drawings.

In summary 15 of 20 (75%) chelonians showed a positive signal in nested PCR performance for the detection of *Acanthamoeba* spp. Furthermore, 14 of 20 (70%) chelonians were positive in *E. invadens* PCR performance as demonstrated in Figure 51.

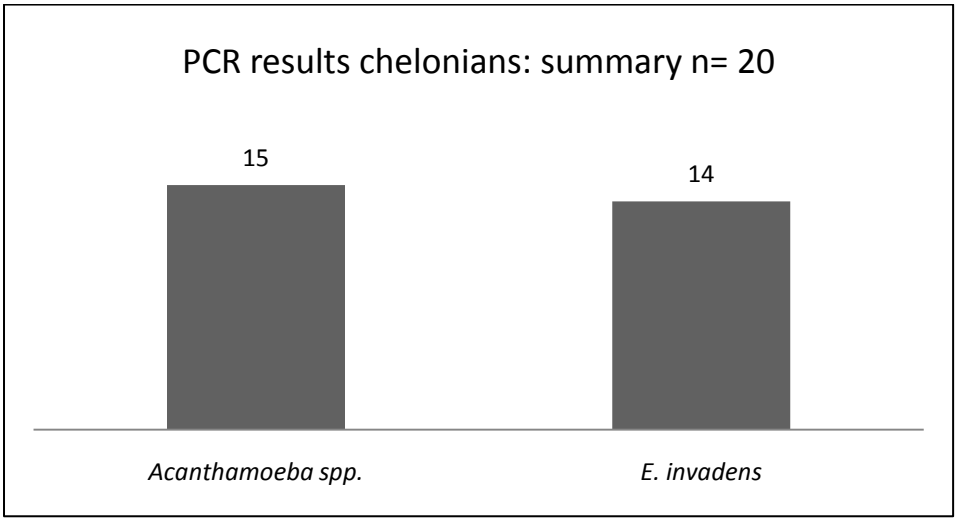


Figure 51: summary PCR results chelonians

4.2.5 Lizards

4.2.5.1 Lizards: first sample drawing (8th of May 2008)

Mouth and cloaca of nine lizards were swabbed in the first sample drawing.

Table 17: PCR results lizards first sample drawing (08.05.2008)

sample		*Acanthamoeba* spp.	*Acanthamoeba* spp. nested PCR	*B. mandrillaris*	*N. fowleri*	*E. invadens*
IM	mouth	-	-	-	-	-
IK	cloaca	-	-	-	-	-
IIM	mouth	-	+	-	-	-
IIK	cloaca	-	+	-	-	-
IIIM	mouth	-	-	-	-	-
IIIK	cloaca	-	-	-	-	-
IVM	mouth	-	-	-	-	-
IVK	cloaca	-	-	-	-	-
VM	mouth	-	-	-	-	-
VK	cloaca	-	-	-	-	-
VIM	mouth	-	+	-	-	-
VIK	cloaca	-	-	-	-	-
VIIM	mouth	-	-	-	-	-
VIIK	cloaca	-	-	-	-	-
VIIIM	mouth	-	-	-	-	-
VIIIK	cloaca	-	-	-	-	-
IXM K11	mouth	-	-	-	-	-
IXK K11	cloaca	-	-	-	-	-

Aerobic culture:

In this sample drawing mouth and cloaca swabs from nine lizards were collected and investigated for *Acanthamoeba* spp., *B. mandrillaris*, *N. fowleri* and *E. invadens*.

Again, the JDP PCR for detecting *Acanthamoeba* spp. was not suitable for this kind of samples so a nested PCR was performed. The nested PCR demonstrated that two of nine (22%) lizards (*Corucia zebrata* = II and *Crotaphytus collaris* = VI) are *Acanthamoeba* spp. positive without co- infections of different genotypes as clarified in Figure 52 and Figure 53 Furthermore, all lizard samples were negative for *B. mandrillaris* and *N. fowleri* in PCR performance as shown in Table 17.

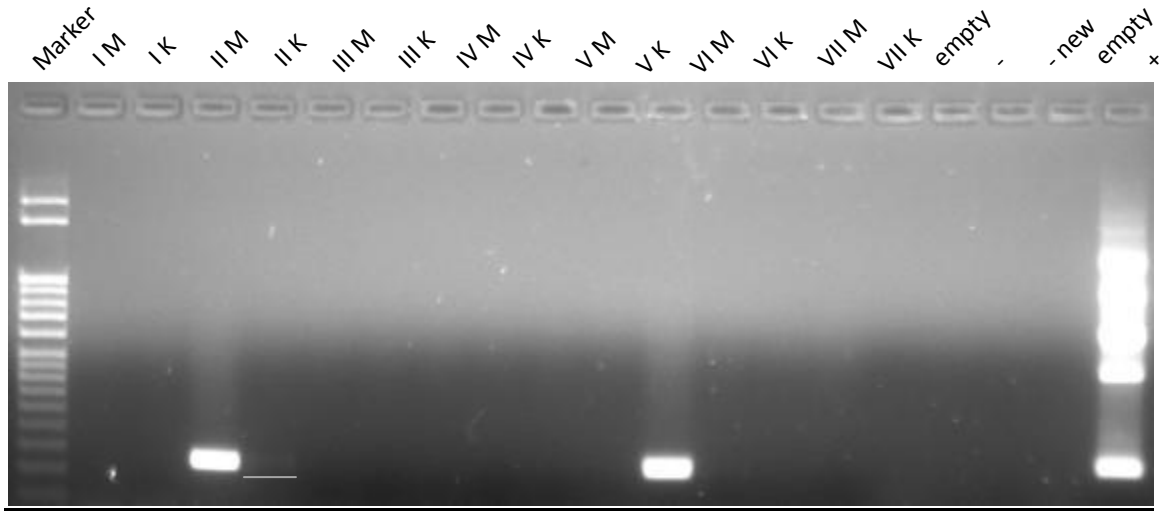


Figure 52: nested PCR results lizards: first sample drawing (samples 08.05.08)

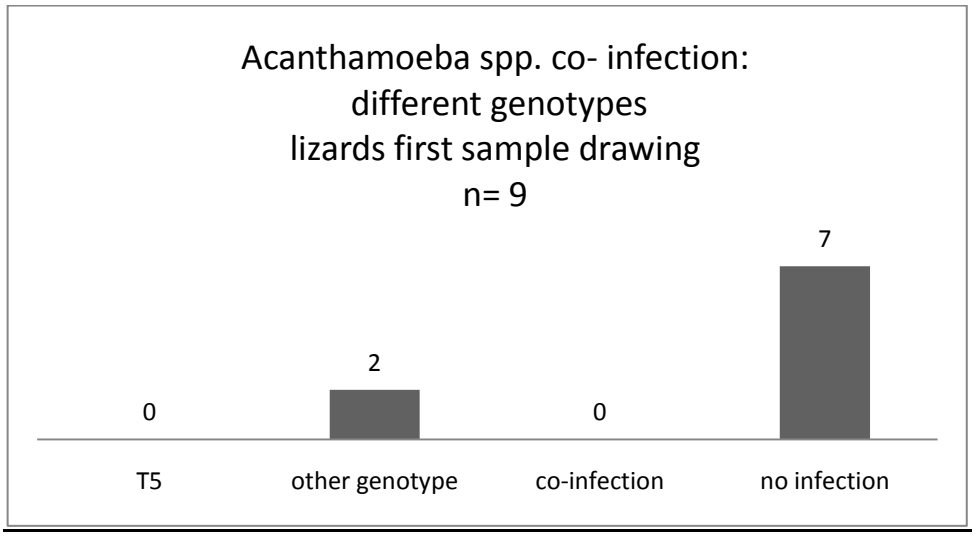


Figure 53: *Acanthamoeba* spp. co-infection lizards: first sample drawing (08.05.2008)

Anaerobic culture:

All lizard samples from the first sample drawing turned out to be *E. invadens* negative in PCR performance.

4.2.5.2 Lizards: second sample drawing (19th of August 2008)

Table 18: lizards PCR results second sample drawing (19.08.2008)

Sample		*Acanthamoeba* spp.	*Acanthamoeba* spp. Nested PCR	*B. mandrillaris*	*N. fowleri*	*E. invadens*
IM	mouth	-	+	-	-	-
IK	cloaca	-	+	-	-	-
IIM	mouth	-	+	-	-	-
IIK	cloaca	-	+	-	-	-
IVM	mouth	-	+	-	-	-
IVK	cloaca	-	-	-	-	-
VM	mouth	-	-	-	-	-
VK	cloaca	-	+	-	-	-
VIM	mouth	-	-	-	-	-
VIK	cloaca	-	+	-	-	-
VIIM	mouth	-	-	-	-	-
VIIK	cloaca	-	+	-	-	-
VIIIM	mouth	-	+	-	-	-
VIIIK	cloaca	-	+	-	-	-
IXM K11	mouth	-	-	-	-	-
IXK K11	cloaca	-	-	-	-	-

Aerobic culture:

In this sample drawing mouth and cloaca swabs of eight lizards were collected and analyzed. PCR performance of these 16 swabs showed that no lizard is infected by *B. mandrillaris* or *N. fowleri* as demonstrated in Table 18. However, *Acanthamoeba* spp. was detected by performing a nested PCR due to the fact that the JDP PCR for the detection of *Acanthamoeba* spp. is not suitable for swabs. Seven of eight (87.5%) lizards (I = *Chamaeleon calyptratus*; II= *Corucia zebrata*; IV= *Cyclura cornuta*; V= *Varanus exanthemicus*; VI= *Crotaphytus collaris*; VII= *Tiliqua gerardii;* VIII= *Diposaurus dorsalis*)

were positive for *Acanthamoeba* spp. other genotypes as demonstrated in Figure 54 and Figure 55. No PCR amplicons with a size of ~150bp were detected on the agarose gel, so there were no co infections of genotype T5 with other genotypes of *Acanthamoeb*a spp. found in this samples drawing.

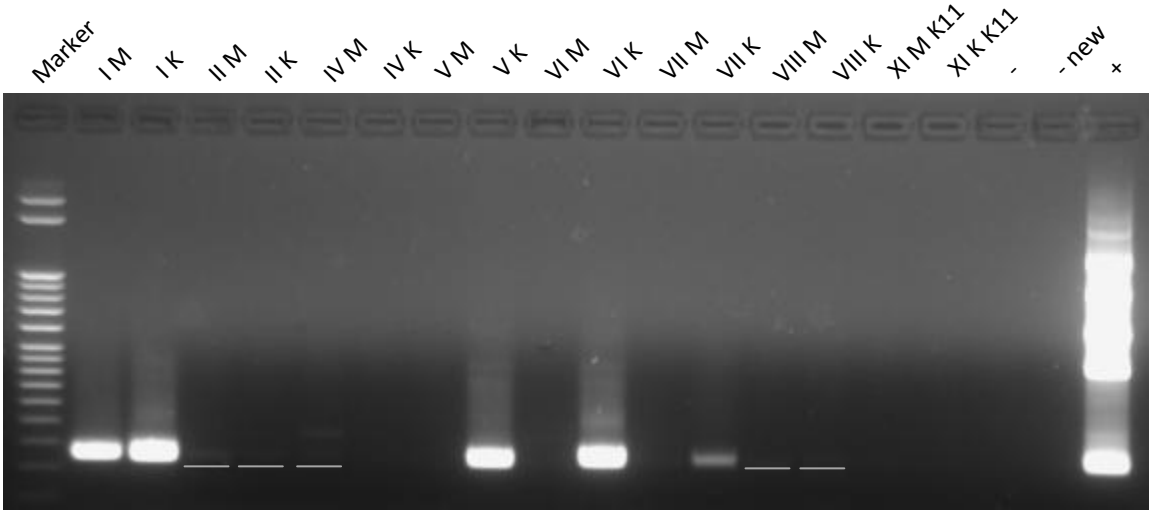


Figure 54: nested PCR results lizards: second sample drawing (19.08.2008)

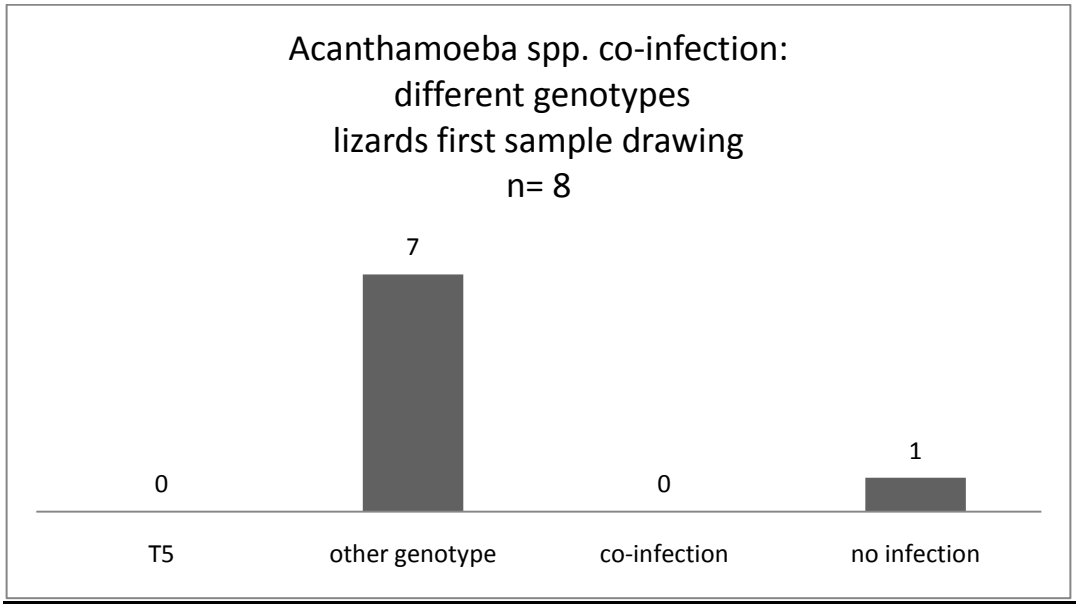


Figure 55: *Acanthamoeba* spp. co-infection lizards: second sample drawing (19.08.2008)

Anaerobic culture:

All analyzed samples from this sample drawing were negative for *E. invadens* as shown in Table 18.

1.1.1.1 Summary: lizards

In summary nine of 17 (53%) lizards showed positive signals in nested PCR for detection of *Acanthamoeba* spp. The investigation for *B. mandrillaris*, *N. fowleri* and *E. invadens* was negative for all collected samples.

PCR results: comparison of the first and second sample drawing from lizards

With the excuse of one lizard, *Chamaeleon calyptratus* (I) it is unclear if exactly the same individual has been swabbed in both sample drawings. Therefore, a direct comparison of PCR results is not possible for the other lizards in this study.

As demonstrated in Table 19 the lizards' swabs from the first sample drawing were negative for *Acanthamoeba* spp. in PCR performance in difference to the analyzed swabs from the second sample drawing. These swabs turned out to be *Acanthamoeba* spp. positive.

Table 19: Comparison of PCR results from the first and second sample drawing from lizards

Sample		First sample drawing *Acanthamoeba* spp. nested PCR	Second sample drawing: *Acanthamoeba* spp. nested PCR
I M	mouth	-	+
I K	cloaca	-	+

4.2.6 Nematodes:

Figure 56 illustrates that the PCR performance of isolated nematodes from soil samples from a terrarium of a dead cobra, with 3 different primer pairs showed that that the first primer pair Rhiz1r/ SSU1 and the third primer pair Rhiz1/ Rhiz2r amplified the isolated DNA. The second primer pair Wurm18sf/ Wurm5,8rev produced no amplicon. Sequencing and blasting of the amplified DNA showed that the isolated nematode is *Pelodera strongyloides dermatica*, *Pelodera punctata* or an uncultured nematoda. By means of an alignment the nematodes species was identified as *Pelodera strongyloides dermatica*, a parasitic saprophage nematode that can cause a CLM (Cutaneous larva migrans) and folliculitis in humans and animals.

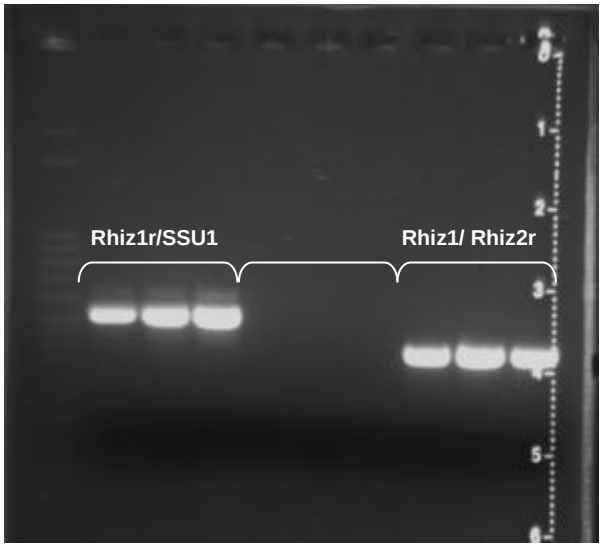


Figure 56: nematodes PCR performed with 3 primer pairs

5 Discussion

In this study, we evaluated the infection rates of asymptomatic reptiles from a zoo with the free-living amoebae *Acanthamoeba* spp., *B. mandrillaris*, *N. fowleri* and with *Entamoeba invadens*. Thereby culture techniques and molecular biological methods were used in parallel.

Altogether, the genus *Acanthamoeba* proved to be the genus with the highest prevalence followed by *E. invadens* investigating altogether 54 reptiles in this study. The investigation of the sampled animals showed no positive results for *Balamuthia mandrillaris* and *Naegleria fowleri*. The predominance of *Acanthamoeba* spp. might be due to the ubiquity of FLA and the resistance of the cysts to harsh environmental conditions.

Also several other authors reported the occurrence of FLA in wild animals as well as in animals kept in zoological collections, these were usually diseased animals. To our knowledge, only a few comparative data on the occurrence of FLAs and *E. invadens* in asymptomatic in exist to date.

5.1 Overall infection rates in the zoo

All animals were investigated in two independent sampling rounds and each time by mouth and cloacal swabs. Interestingly, there is no big discrepancy between mouth and cloacal swabs in case of positive samples. In detail, in case of *Acanthamoeba* spp. 3.7% (4 of 108) mouth swabs and 7.4% (8 of 108) cloacal swabs were positive in culture. Due to the fact, that PCR is much more sensitive than culture, we were able to detect 23.1% (25 of 108) mouth swabs and 25% (27 of 108) cloacal swabs as positive for *Acanthamoeba* spp. in PCR. *E. invadens* PCR led to similar results, 23.1% (25 of 108) mouth swabs and 20.3 % (22 of 108) cloacal swabs were positive for *E. invadens*.
The suggestion that FLAs might be commensals in some animals, which only pass the gut without causing disease, was reported by several authors (Frank and Bosch, 1972; Bosch and Deichsel, 1972 and Hassl et al., 2000). Madrigal Sesma et al. (1988) suggested that a true colonization might be indicated by the findings of amoeba-cysts in gut contents.

Collecting samples by swabbing might have triggered stress in the investigated animals, so many reptiles particularly chelonians refused to open their mouth during sample

drawing. Some animals were attracted and provoked to open their mouth with food. In some cases, small food particles were collected with the samples, which eventually led to contamination of the agar plates and of the samples collected for DNA isolation. Moreover, possibly in some cases the outer skin of the cloaca was swabbed in addition to the mucosa. Furthermore, some animals especially chelonians defecated during sample drawing, which led to the collection of a significant amount of excrement and of coexisting species within the faeces.

During this study, more FLAs than entamoebas were detected, in culture as well as in PCR. Investigation of swab samples by PCR showed that 64.8% were positive for *Acanthamoeba* spp. and 53.7% for *E. invadens*.
In detail, of the snakes 64% showed positive results for *Acanthamoeba* spp. and 83% for *E. invadens*. The snakes were the only group where the infection rates with *E. invadens* were higher than with acanthamoebas. Of the chelonians 75% were positive for *Acanthamoeba* spp. and 70% for *E. invadens*. The lizards showed only positive results for acanthamoebas (53%).

Of the FLA only *Acanthamoeba* spp. were detected equally in all samples. No *Balamuthia mandrillaris* and *Naegleria fowleri* were detected during this study.

Beside acanthamoebas, also *Echinamoeba* spp. and Valkampfiidae were detected. The detection rates of *Echinamoeba* spp. and Valkampfiidae in reptiles during this study were comparable to findings of Hassl and Benyr (2003).

Several studies showed that free-living amoebas in reptiles are rather frequent (Frank and Bosch, 1972, Bosch and Deichsel, 1972, Madrigal Sesma et al., 1988, Walochnik et al., 1999). Frank and Bosch (1972) demonstrated the dominance of the genus *Acanthamoeba* and a high prevalence of *Entamoeba* spp. in faecal samples or swabs from reptiles. This was in a project on amoebas from the "*Hartmannella-Acanthamoeba-Naegleria*" complex in cold-blooded animals.

The animals investigated in our study showed no clinical signs and seemed to be in a good state of health, although FLAs and and *E. invadens* were detected. Several authors suggested that FLAs might be commensals in some animals, which only pass the gut without causing diseases (Frank and Bosch, 1972; Bosch and Deichsel, 1972 and Hassl et al., 2000).

Ebani and Fratini (2005) suggested, that infections with *Chlamydophila* spp. is more frequent than reported due to missing clinical signs.

5.2 Infection rates per host

Apparently, an infection with FLA is not depending on the respective taxon of the reptilian host. As suggested by Hassl and Benyr (2003), the physiological and anatomical differences of reptiles do not affect the colonization of the intestines by FLAs. The authors investigated faeces samples of 80 individuals (20% amphibians, 25% snakes, 47% lizards and 8% chelonians) for the occurrence of FLA, *Cryptosporidia* and *Salmonella* with the result, that 16 strains of FLA could be isolated from snakes, chelonians and lizards. This is in concordance with the results obtained in the current study.

Although in the current study, no *B. mandrillaris* and *N. fowleri* could be detected *Acanthamoeba* spp. were detected in many animals from the three investigated reptile groups, namely snakes, chelonians and lizards.

5.2.1 Snakes

In the present study, 88% (eight of nine) snakes from the first and 87% (seven of eight) snakes from the second sample *drawing* showed positive signals for *E. invadens* in PCR. Entamoebas can be highly pathogenic to reptiles. Kojimoto et al. (2001) reported on severe colitis in reptiles, especially in carnivorous snakes. In that study, four ball pythons (*Python regius*) showing clinical signs like anorexia, died during September and November 1999 in a zoo in Japan probably due to *Entamoeba* sp. The authors investigated tissue samples from these four snakes during necropsy and observed swollen parts in the large intestine, necrotic tissues and lesions in the livers. Characterization of observed amoebas during that study, based on microscopy and not on PCR.

Interestingly, the investigated and *Entamoeba* positive analyzed snakes in my study, which were all herbivorous, showed no clinical signs and demonstrated a good state of health. With the exception of one snake (*Boa* "accordion disease"), which was euthanized in the course of this project all animals were healthy and asymptomatic.

The *Boa* "accordion disease" showed apathetic staring, lethargy and an oversized skin, wrinkled like an accordion. The isolation of *Acanthamoeba* spp. from intestine tissue samples on NN agar plates was successful. Furthermore, the detection of *E. invadens* in intestine tissue samples by PCR was possible. The apathetic starring and lethargy of the

animal provide potential evidence for the colonization of the brain by amoebae, however, neither PCR nor plate culture detected FLAs or *Entamoeba* in brain tissue samples. Unfortunately, I have no information on lesions or histological changes in the brain or other organs.

For comparison, a study on the detection of *E. invadens* in 182 examined captive snakes belonging to 23 different genera (e.g. *Boa*, *Python* and *Vipera*) by ISH (in situ hybridization) generated positive signals in nine snakes (Richter et al., 2008a). Interestingly, only one of these nine snakes (three *Boa*, one *Crotalus*, two *Eunectes* and three *Python*) had a history of bloody vomitus and diarrhoea but the other eight snakes were apparently asymptomatic despite positive results for *E. invadens*. However, at examination severe diphtheroid colitis in all nine snakes and necrotizing hepatitis in five snakes was observed. Furthermore, Richter et al., (2008b) detected an amphibian *Entamoeba* species in a *Boa constrictor* that was found dead in a public zoo. Infection of the *Boa* that suffered from a diphtheroid colitis with E. *ranarum* was detected from tissue samples by ISH. The detection of entamoebas in reptiles also was the topic of a study published by Frank and Bosch (1972). In that study, 20 reptiles were analyzed for the occurrence of entamoebas with the findings, that one of two investigated snakes was positive for entamoebas in culture.

The first investigation of the snakes in November 2007 revealed 55% (five of nine) positive for *Acanthamoeba* spp. during the first sample drawing and 75% (six of eight) during the second sample drawing, all in PCR. Generally, PCR was more sensitive that culture. In addition to the greater sensitivity of PCR, this might also be due to the fact, that not only living amoebas can be detected, but also parts of amoebas and dead amoebas.

Python regius is the only snake that showed no positive signals in PCR, neither for *E. invadens* nor for free-living amoebas. Interestingly, this also was the last snake introduced to the zoological collection. Moreover, this snake had never been kept together with other snakes in one terrarium so a possible transfer of amoebas from one snake to the other was unlikely.

In culture, 22% (two of nine) sakes turned out to be positive for *Acanthamoeba* spp. in the first and 12% (one of eight) in the second sample drawing. Frank and Bosch (1972) published a study, suitable for comparison to our results, as mentioned above. The

authors investigated faeces and swab samples from 20 reptiles among two snakes for the occurrence of *Acanthamoeba* spp. and found both snakes positive for *Acanthamoeba* spp.

Interestingly, also other FLAs than those searched for were detected during this study. In culture, 33% (three of nine) samples were positive for *Echinamoeba* and 11% (one of nine) for *Vanella* during the first sample drawing. In the second sample drawing, the cultivation of collected samples showed that 25% (two of eight) of the samples were positive for *Echinamoeba*.

5.2.2 Chelonians

The first chelonian sample drawing was in February 2008. Interestingly, all chelonians (10 of 10) in this first sample drawing were *E. invadens*-positive in PCR. That stands in contrast to the second sample drawing in May 2008, where only 40% (four of 10) were positive. However, as in the snakes, it is not clear, if exactly the same individuals were swabbed in both sample drawings, because several chelonians were housed together in one enclosure moreover the composition of the livestock within one terrarium changed during the sample period. Another possibility for the discrepancy between the first and the second sample drawing might be that the *E. invadens* positive chelonians did not have an amoebosis but only a temporal colonization.

Altogether, 70% of the chelonians investigated during this study were positive for *E. invadens* without showing any clinical signs. A possible explanation could be that *E. invadens* can exist without causing disease in herbivorous turtles. The reason for that is, that *E. invadens* is enabled to finish the life cycle by the uptake of plant nourishment, ingested by turtles. Without the specific nourishment available in herbivorous chelonians, the entamoebas have to invade the chelonians' intestinal mucosa with the outcome of an amoebosis in carnivorous reptiles (Meerovitch, 1960 in Kojimoto et al., 2001). During this study, from 20 investigated chelonians, eight chelonians (*Astrochyles radiata, Testudo kleinmanni*, *Dipsochelys dussumieri*) were strictly herbivorous and 12 animals (*Trachemys scripta elegans, Heosemys, Emys orbicularis, Phrynops hilarii*) omnivorous. Interestingly, neither herbivorous nor omnivorous chelonians showed clinical signs of amoebosis even if the animals were positive for *E. invadens*.

In the study by Frank and Bosch (1972), three of nine chelonians (33%) turned out to be positive for entamoebas, but it has to be mentioned, that these results based exclusively on microscopic investigation.
A study on histopathological investigation of tissue samples of 40 dead wood turtle hatchlings was published by MacNeill et al. (2002). The authors observed amoebas consistent with *Entamoeba* sp. in tissue samples by microscopy, but there were no molecular biological investigations performed to confirm these results. Moreover, a co-infection with *Aeromonas sobria*, a gram-negative bacterium that causes gastroenteritis was detected in involved turtles but might not have been the causative agent of the disease in these cases.

Strikingly, in the current study 90% (nine of 10) of the chelonians were positive for *Acanthamoeba* spp. in the first and 60% (six of 10) in the second sample drawing by PCR. This high positivity for *Acanthamoeba* spp. is corroborated by Frank and Bosch (1972), who investigated swab and faeces samples of reptiles as mentioned above. The authors detected *Acanthamoeba* spp. in 100% (nine of nine) of the samples collected from chelonians even though the detection of acanthamoebas based exclusively on microscopy and not on PCR.

Additionally to the detected entamoebas and acanthamoebas, 40% (four of 10) chelonians showed positive results for *Echinamoeba* in culture and 30% (three of 10) for Valkampfiidae. A possible reason for this high infection rate with FLAs could be the appearance of free-living amoebas in animals as commensals (Frank and Bosch, 1972 and Hassl et al. 2000).

5.2.3 Lizards

Interestingly, it was not possible to detect *E. invadens* in any of the swab samples collected from lizards during this study neither by culture nor by PCR. As mentioned above a similar study was published by Frank and Bosch (1972). In that study, swab and faeces samples of 11 lizards were investigated for FLAs and entamoebas, with the result that three of 11 lizards (27%) were positive for entamoebas in microscopy. To our knowledge, there are no reports on screenings for FLAs and entamoebas in apparently healthy reptiles beyond that study.

Investigating the lizards from the zoo by PCR resulted in the detection of *Acanthamoeba* spp. in 22% (two of nine) of the lizards from the first and in 87% (seven of eight) from the second sample drawing. Additionally, in the second sample drawing 12% (one of eight) lizards were also *Acanthamoeba* spp.-positive in culture. Again, PCR was much more sensitive than culture.
The occurrence of FLAs in lizards was also published by several authors. For example, Walochnik et al., (1999) published the detection of *Acanthamoeba* spp. in a necrotic lesion on the tail of a *Basiliscus plumifrons*. The molecular biological characterization of the *Acanthamoeba* revealed in *A. castellanii, which* is genotype T4. Madrigal Sesma (1988) investigated gut samples from 110 lizards for *Acanthamoeba* spp. and other FLAs. The authors demonstrated that 58% (64 of 110) samples were positive.

As expected, also other FLAs besides *Acanthamoeba* spp. were detected in lizards' samples. 22% (two of nine) lizards from the first sample drawing were positive for *Echinamoeba*, 11% (one of nine) for Valkampfiidae and *Hartmannella*.
As for the snakes and the chelonians also for the lizards it cannot be granted that the same individuals had been swabbed in both sample drawings. On average three animals were in one terrarium, so also in case of the lizards a direct comparison of the results is not possible. All lizards, from both sample drawings were found to be *B. mandrillaris* and *N. fowleri* negative.
Walochnik et al. (1999) had compareable findings. In that study, the authors were also able to detect other FLAs beside *Acanthamoeba* spp. The investigation of tissue samples from the basilisk resulted in the detection of *Naegleria gruberi*, *Hartmannella vermiformis* and *Echinamoeba* sp. Another study, published by Madrigal Sesma et al. (1988), showed the occurrence of FLA like *Hartmannella*, *Naegleria* and *Valkampfiidae* in lizards by incubating gut contents on agar plates. *Paravahlkampfia* sp. was isolated from the intestine of lizards in a study by Schuster et al. (2003). The reptiles belonged to a zoological collection in the USA, were moribund but showed no lesions in the intestinal tissue. Because of this, the authors suggested that this amoeba might be part of the animals' normal flora.

It is a fact that reptiles are carriers of different pathogens, not only amoebae. Lizards are known as asymptomatic carriers of different *Salmonella* species infection rates of about 77% have been reported (Iveson et al., 1969 in Mermin et al., 1997).

5.3 Infection rates per habitat

In this study, the animals were housed in enclosures with arid conditions filled with sand and soil as well as in plastic tanks filled with water, depending on the animals' physiological and anatomical characteristics. Some reptiles use to burrow in the soil where they might be exposed to cysts or trophozoites of FLA. The *Acanthamoeba*-specific PCR did not reveal differences in colonization between animals kept in arid terrariums and terrariums with a moist climate. Hassl and Benyr (2003) postulated, that FLAs colonize animals kept in terrariums with arid climate less frequently than animals from other types of terrariums. This might certainly be true for *Naegleria* having less resistant cyst than *Acanthamoeba*.

Animals in zoological collections are usually exposed to stress factors from different potential sources. The artificial habitat and with it several stimuli are foreign to these animals and might lead to stress and affect the immune system or other functions of these animals (French et al., 2006). An important aspect of keeping animals in private households as well as in zoological collections is to reduce stress, which is the main reason for susceptibility to infections (Ebani and Fratini, 2005).
Captive animals are confronted daily with noise produced by visitors, keepers or technical equipment like cameras and monitors. Furthermore, the light conditions are artificial and suited to human convenience and not to the animals' needs. Moreover, the temperature animals are exposed to is often not adequate and there are even more stimuli that affect stress in captive animals like food, composition of animal groups, contact to humans, small enclosures and odors (Morgan et al., 2007). French et al. (2006) reported that stressed lizards had higher levels of corticosterone than control animals, which partially affects the immune system, resulting in immunosuppression of the animals. The effect of social stress as immunosuppressor was suggested by Rideout et al. (1997). The author reported on five cases of GAE due to *B. mandrillaris* in old world primates at the zoo in San Diego. In that study, one affected gorilla was under social stress because of the death of an older male silverback.

Another source of infection or transmission of FLA and entamoebas can be central water supply for the terrariums. However, the enclosures of the reptiles investigated during this study were not provided with water distributed by a central water system. Therefore, transfer pathogens from one animal to another via the water supply is not possible.
In the MacNeill paper on the 40 dead hatchling turtles, the animals showed symptoms like lethargy and inactivity before death occurred. 10 to 20 turtles were kept together in

one outdoor tank above ground and the water was changed daily. The author assumed that, the installation of a new water supply system was the reason for the outbreak.

5.4 Infection rates per amoeba species

The detection of more FLA than entamoebas in reptiles in this study might be explained by the fact that entamoebas are obligatory parasitic in contrast to FLA, which are facultatively parasitic. FLAs are ubiquitous and can be detected in many environmental samples and different materials like soil, dust and fresh water (Marciano-Cabral and Cabral, 2003).

5.4.1 *Acanthamoeba* spp.

During this study, 64.8% (35 of 54) reptiles were *Acanthamoeba* spp. positive in PCR and 20.3% (11 of 54) in culture.

The occurrence of *Acanthamoeba* spp. in reptiles was also the topic of a study in the early 1970s from Frank and Bosch (1972) as mentioned above. The authors demonstrated the occurrence of "*Hartmannella-Acanthamoeba"* and *Naegleria* in faeces samples, cloaca swabs or tissue samples collected from snakes, chelonians and lizards. 20 animals (two snakes, nine chelonians, three lizards and five animals without genus description) were investigated by swabbing the cloaca or collecting faeces samples with the result that all of them were positive for "*Hartmannella-Acanthamoeba"* and 50% positive for *E. invadens*. It was not stated whether the animals showed any pathological changes or if they appeared to be healthy.

When comparing the six sample drawings, the genus composition of free-living amoebas was always different. However, throughout all sample rounds all samples were negative for *B. mandrillaris* and *N. fowleri*.

The occurrence of acanthamoebas in contrast to *B. mandrillaris* and *N. fowleri* in our study might be because of the higher resistance in case of adverse environmental conditions. Acanthamoebas have a thicker cyst wall than *Balamuthia* and *Naegleria*.

5.4.2 *B. mandrillaris*

During this study, all samples were negative for *B. mandrillaris.* To our knowledge, also in the literature, there are no reports on the occurrence of *Balamuthia* in cold-blooded animals.

During this study, it was not possible to isolate or detect *B. mandrillaris* in samples collected from reptiles. It is to assume that the genus *Balamuthia* is not part of the normal intestinal flora of reptiles because no amoebas of this genus were isolated or detected from sampled animals during this study, which were obviously healthy. *Balamuthia* has until now never been isolated from reptiles and to our knowledge, there is no study on the occurrence of *B. mandrillaris* in cold-blooded animals altogether. Nevertheless, several authors reported on the occurrence of *Balamuthia* in animals. Apart from the first isolation of *B. mandrillaris* from the brain of a pregnant mandrill (Visvesvara et al., 1993) a few reports on the occurrence of *Balamuthia* in primates from zoological collections have been published. Rideout et al. (1997) revealed that all known cases of GAE in primates in the past 30 years at the zoo of San Diego were due to *B. mandrillaris*. In that study, additionally to paraffin embedded tissue samples of affected old world primates, environmental samples from water and moist soil were investigated for the occurrence of *Balamuthia*, which concerns samples. It was not possible to isolate *B. mandrillaris* from one of the collected environmental samples, so the infection source is still unclear. Also, the route of infection remains unknown, but the authors suggested that the infection was hematogenous from skin lesions.
Actually, only in three cases, the isolation of *Balamuthia* from environmental samples was successful. Two times, the amoebas were isolated from a flowerpot from the household of children affected by *B. mandrillaris* GAE (Schuster et al., 2003 and Dunnebacke et al., 2004). A recently published study on the occurrence of FLA in public buildings in Tehran (Iran) characterized an amoeba isolated from a dust sample as *Balamuthia mandrillaris* by PCR (Niyyati et al., 2009).

Isolation of *Balamuthia mandrillaris* is altogether not as simple as in the case of *Acanthamoeba* spp. or *N. fowleri*. *Balamuthia* cannot be isolated on NN agar plates because these FLA do not feed on bacteria. *B. mandrillaris* requires mammalian cells like monkey kidney cells (Vero cells), rat glioma cells or human brain microvascular endothelial cells as food source as published by Schuster (2002). Matin et al. (2006) demonstrated that the uptake of *E. coli* by *B. mandrillaris* does not lead to growth but to the remaining in the trophic stage. However, Schuster et al. (2002) reported that Balamuthia can feed on other amoebas presenting on NN agar plates.

In our study, the swab samples were stored for several weeks and checked occasionally for the appearance of *B. mandrillaris* because Schuster et al. (2002) reported on the

occurrence of this genus on agar plates after incubating the samples for a couple of weeks. Thus in the current study, growth of the amoebas might have been possible on agar plates where other FLAs were isolated. As reported by Niyyati et al. (2009), the isolation of *B. mandrillaris* on NN agar plates was successful in a study on the occurrence of FLA in public buildings in Iran. In that study, *Acanthamoeba* spp., vahlkampfids and *Thecamoeba* were observed a few days after streaking out the swab samples on agar plates. After a couple of days, also *Balamuthia mandrillaris* was detected. By using other isolated amoebae as a food source, *Balamuthia* was able to grow on NN agar plates during that study. In our study, all samples remained negative for *Balamuthia mandrillaris* even after a several weeks of culture.
Because of this, a specific PCR for the detection of *B. mandrillaris* was established.

Worldwide, isolation of *B. mandrillaris* from environmental samples was successful in only two cases, both times from soil collected from plant pots of households from GAE patients in the USA (Schuster et al., 2002 and Dunnebacke et al., 2004). Water as source of infection with *B. mandrillaris* was suggested by Foreman et al. (2004). In that case, a Great Dane dog in California showed first clinical signs of GAE 10 days after swimming in a stagnant pond, where the dog used to swim periodically. A stagnant freshwater pond or dam have been the source of infection of another affected dog, in Australia. The golden retriever had possibly been infected with *Balamuthia* via the nasal cavity and olfactory nerve pathway while swimming in the pond. A skin lesion as infection site was not evident as reported by Finnin et al. (2007). Rideout et al. (1997) investigated environmental samples (water and moist soil) from the Zoo in San Diego for the occurrence of *B. mandrillaris* without success. In that study, environmental samples were collected with the aim to identify the source of infection in five cases of GAE due to *Balamuthia* in old world primates as mentioned above.

5.4.3 *N. fowleri*

Also N. fowleri was not detected in the current study. There are only a few reports on *Naegleria spp.* in reptiles. Walochnik et al. (1999) demonstrated the occurrence of *Naegleria gruberi* in a *Basiliscus plumifrons* (Sauria: Iguanidae). The broad temperature spectrum of *N. gruberi* with an optimum growth temperature of 28°C to 30°C and the ability to grow at lower temperatures might be the reason for the occurrence of this species in cold-blooded animals. Hassl and Benyr (2003) reported the detection of *Naegleria* spp. in faecal samples of three lizards and two snakes. Furthermore, the

authors reported on the occurrence of the thermo-tolerant *N. australiensis* in a lizard (*Iguana iguana*).
In the publication of Frank and Bosch (1972), it is not clear whether *Naegleria* was actually detected in reptiles or not. It is possible that the authors did not differentiate exactly between FLAs from different genera, since the term "*Hartmannella-Acanthamoeba-Naegleria*" complex was used throughout that study. Obviously, Frank and Bosch summarized the detected limax amoebae by using the terms "*Hartmannella-Acanthamoeba*" complex and "*Hartmannella-Acanthamoeba-Naegleria*" complex.

In case of *N. fowleri* the sensitivity to environmental conditions like desiccation because of the thin cyst-wall can be a reason for absence of these amoebas. To some extent, the reptiles' enclosures were equipped with sand, stones, wood and a small basin as a water supply. Terrestrial chelonians were housed in crates filled with sand as substrate and heating lamps above the enclosures. The only water sources in these crates were dishes with a little water. However, no *N. fowleri* and no other Naegleria species were detected. in animals housed in water basins or in terrariums filled with soil. Another important aspect is that *Naegleria fowleri* is a thermophilic FLA and therefore may generally not occur in cold-blooded animals. However, also no other, non-thermophilic *Naegleria* species were detected.

5.4.4 *E. invadens*

Due to the fact that no lizard turned out to be *E. invadens* positive, the infection rates for entamoebas in this study rely on data from snakes and chelonians. Altogether, 53.7% (29 of 54) animals were positive for *E. invadens* at least it in PCR. However, was not possible to isolate entamoebas from any of the samples

During this study, living cold-blooded animals were screened for the occurrence of *E. invadens,* in difference to most other reports on captive reptiles where only dead animals were investigated. Moreover, in the current study, swab samples were investigated while in most other studies tissue samples were investigated.
To our knowledge, the only report on *E. invadens* in swab and faeces samples collected from living reptiles is the one by Frank and Bosch (1972). The authors investigated 20 animals (two snakes, nine chelonians, three lizards and five animals without genus description) for the occurrence of *Entamoeba* sp. in cloaca swabs and faeces samples. *Entamoeba* spp. was detected in ten of 20 (50%) swabs or faeces samples with one

case of co-infection with two different *Entamoeba* types, namely E1 and E2 as described by Bosch and Deichsel (1972).

Kojimoto et al. (2001) reported on four ball pythons kept in the same display in a zoological park in Japan. These snakes showed signs of anorexia and three snakes died within three to 30 days after showing the first anorectic signs. The last snake was treated with metronidazole for 26 days but also this reptile died nevertheless. The necropsy of lung, heart, liver, stomach, intestine and kidney revealed lesions in the liver and intestine. However, it remains unclear whether these four snakes died due to an *E. invadens* infection, because the detected amoebas were identified morphologically only and thus also another *Entamoeba* sp. might have been the causative agent.
For example, a case of an infection with an amphibian *Entamoeba* species, probably *E. ranarum* in a snake was reported by Richter et al. (2008b). A *Boa constrictor* with no anorectic signs was found dead in its enclosure in a zoological collection. Necropsy showed multifocal diphtheroid colitis, pneumonia and protozoa in tissue samples from the intestine. To identify the observed protozoa an ISH was performed with the result that the sample showed positive signals with the general *Entamoeba* probe but not with the specific probe for *E. invadens*. A gene sequence analysis then demonstrated highest sequence similarity to *E. ranarum*.

5.4.5 Co-infections

The co-infection rate with *Acanthamoeba* spp. and *E. invadens* in the investigated was 64% (11 of 17) in snakes and 60% (12 of 20) in chelonians. As no *E. invadens* was detected in swabs collected from lizards, there also was no co-infection of *Acanthamoeba* spp. and *E. invadens* in lizards. Results from Frank and Bosch (1972) also found, that co-infections with acanthamoebas and entamoebas are frequent in reptiles. In their study co-infections with *Acanthamoeba* spp. and *Entamoeba* spp. occurred in 10 of 20 (50%) swabbed animals.
Moreover, in the same study Frank and Bosch (1972) also investigated tissue samples of 28 dissected reptiles (nine snakes, eight chelonians and 11 lizards) and found *Acanthamoeba* spp. in all tissue samples and in 17 (60%) samples *Entamoeba* spp. were detected additionally, thus revealing co-infections. The authors suggested, that amoebosis in the cases without co-infection was caused solely by *Acanthamoeba* spp.
Due to the fact, that the animals in our study were investigated by swabbing and not by section, there are no histological data. One snake (*Boa* "accordion disease") that was

euthanized during this study was dissected after death but to our knowledge, the organs seemed to be normal without pathological changes, although *E. invadens* was detected in intestine samples by PCR. Additionally, *Acanthamoeba* spp. was detected in tissue samples from the brain and intestine by PCR. The investigation of the tissue samples for FLAs resulted in the isolation of *Acanthamoeba* spp. from the intestine.

5.5 Culture vs. molecular biology

As shown in this study, isolation of amoebas from swabs by culture can be negative in spite of positive PCR results and this was true for FLAs, but even more for entamoebas. However, PCR is a much more sensitive technique than culture on NN agar plates and that the collected amoebas might not all grow and replicate on agar plates. Moreover, in difference to cultivation on NN agar plates where only living amoebas can be found, PCR also detects dead amoebas and parts of dead amoebas.

In this study, *Entamoeba invadens* and *Acanthamoeba* spp were detected in from mouth and cloacal swabs by PCR. In case of *Acanthamoeba* spp. there was a discrepancy of similarly between culture and PCR of about 40%-50%. Using culture to analyze swab samples of snakes gave positive results in 17%. In comparison, the PCR showed positive results in 64% of the samples. The chelonians, here a discrepancy of 45% between culture and PCR was observed. 30% of the cultures and 75% of the PCRs were positive of acanthamoebas. Finally, the lizards' samples were positive in 11% of the cultures in difference to 52% in PCR which leads to a discrepancy of 41% between these two detection techniques.

Our results are corroborated by a study of Bradford et al. (2008) who described a high discrepancy between the numbers of detected amoebas, in that case entamoebas in culture and PCR.

Even if PCR is more sensitive than detection of amoebae from culture, it is recommendable to perform both methods in parallel. Because a positive aspect of cultivating amoebas on NN agar plates is that isolates can then be stored at room temperature over months, and subjected to further studies like genotyping and temperature tolerance tests.

5.5.1 *Acanthamoeba* spp.

Generally, the isolation of *Acanthamoeba* spp. on NN agar plates from swabs was unproblematic as soon as fungi and ciliata, that were co-isolated during swabbing, were

removed by subcultivation of the amoebas. However, culture was significantly less sensitive than PCR.

64.8% of the animals turned out to be *Acanthamoeba* spp. positive in PCR and 20.3% of the reptiles were positive for *Acanthamoeba* spp. in culture. This discrepancy can be explained by the higher sensitive of PCR. In contrast to a study by Walochnik et al. (1999) where a three different genera of amoebae were detected after placing a tissue block of the necrotic tail of a *Basiliscus plumifrons* on NN-Agar plates, the number of isolated amoebas in our study was very low because only swab samples from evidently healthy animals were collected.

Within this study, different *Acanthamoeba* PCRs were performed and altogether the nested PCR established during this study, turned out to be best suited for swab samples. The JDP PCR is the standard PCR for the detection of *Acanthamoeba* spp. from clinical samples. For that reason, this was the first PCR employed to detect acanthamoebas in reptile samples. The primer pair JDP1 and JDP2 is located in the 18S rRNA gene. Beside its high conservation at its 5' and 3' end and the appearance in a high number of copies, this gene shows 12 variable regions and enables the differentiation between the 15 genotypes of *Acanthamoeba* spp. by DNA sequencing of the amplicons.

Schroeder et al. (2001) established the JDP PCR for the detection of *Acanthamoeba* spp. in clinical samples The authors used *Acanthamoeba* spp. from axenic culture or corneal scraps and sewage sludge. The presence of trophozoites in the sample was crucial because DNA isolation from mature cysts results in a lower yield than isolation from trophozoites. The amplification of *Acanthamoeba* spp. DNA from mature cysts using the primer pair JDP1 and JDP2 failed during their study.

In our study, the JDP PCR worked nicely with *Acanthamoeba* spp. trophozoites harvested from agar plates. However, no positive signals were detectable on agarose gel from swab samples not even in the culture-positive samples, although Schroeder et al. (2001) amplified *Acanthamoeba* spp. DNA from a single trophozoite or 1 pg DNA. The samples in our study showed DNA amounts between 1 and 6 ng/µl. However, of course, the total DNA yield also includes DNA from bacteria, fungi and other microorganisms, which are common in swab samples.

Seal (2003) discovered that the primer sequence of JDP2 in the publication of Schroeder et al. (2001) was wrong by one base. However, the missing internal G in the JDP2 primer sequence does not lead to lack efficiency of this PCR assay because either version of this primer works. So the incorrect primer can be eliminated as reason for the disability to amplify our samples.

Actually, Schroeder et al. (2001) published two different versions of the JDP PCR. The standard JDP PCR as used in our laboratory should be performed when a high sensitivity is required. To further increase the specificity of the JPD PCR the authors suggested, modifying the cycler program by changing the cycle number from 45 cycles to a combination of 20 cycles followed by 25 cycles under the same temperature terms and time. Nevertheless, when this was tried in the current study the modification did not lead to higher specificity of the PCR with *Acanthamoeba* spp. trophozoites harvested from agar plates. In the contrary, the gel showed no positive signals except the positive control, although the same samples produced amplicons with the standard JDP PCR.

Depending on amoeba density in the sample, several PCR assays are not sensitive enough to detect microorganisms or amoebas from swabs. For this collecting method, a nested PCR is much more effective than standard PCR assays (Calsamiglia et al., 1999, Vilcek et al., 1994). A nested PCR has a highly increased sensitivity by using two primer sets. The product of the first nested PCR step acts as matrix for further PCR reaction using the second primer pair. However, because of the high sensitivity of a nested PCR it is necessary to prevent contamination with DNA from other species.
In our study, the nested PCR was tested with different numbers of cycles. Performing both PCR steps with 20 cycles led to weaker and less signals on agarose gel under UV exposure than with 20 cycles in the first and 30 cycles in the second step. The best results were achieved with 30 cycles in both steps with a temperature of 56°C, which led to higher specificity of primer binding and no unspecific signals. Performing nested PCR gave *Acanthamoeba* spp. positive signals in all culture-positive samples, moreover altogether in 64.8% of the samples collected by swabbing mouth and cloaca.

Altogether, 7.4% (four of 54) reptiles showed positive results for genotype T5. Co-infection was detected in 25.9% (14 of 54) reptiles and 31.4% (17 of 54) other than. genotypes T5.

Acanthamoeba spp. genotyping

The performance of this PCR with the swab samples led in many cases to two bands instead of one band on agarose gel. The amplicon with a size of 200 bp matched the positive control, which was genotype T4. In some cases however, there was an additional signal with the size of 150 bp. Sequencing of the 150 bp amplicon revealed that the detected smaller bands also belonged to *Acanthamoeba*, but to genotype T5, having a shorter amplicon. Therefore, bands with an approximate size of 150 kb on the agarose gel instantly indicated a sample positive for *Acanthamoeba* genotype T5.

Unfortunately, sequencing of the amplicons was more difficult than expected.

The establishment of a nested PCR for *Acanthamoeba* spp. from swabs led to better sequencing results. Due to the fact that the nested PCR enabled a differentiation between T5 and other genotypes already on the gel instead of one band after JDP PCR, it became possible to purify two amplicons separated from each other.
Random sequencing of *Acanthamoeba* spp. positive PCR samples in order to evaluate the respective genotypes during this study resulted in the identification of the genotypes T4 and T5.

The chelonians were the group most affected by *Acanthamoeba* spp. Three chelonians (15%) were positive for genotype T5. Moreover, eight individuals (40%) were positive for another genotype, most likely T4, and four chelonians (20%) were co-infected with genotype T5 and T4. Sequencing of randomly chosen amplicons showed that one chelonian from the first sample drawing, *Phrynops hilarii*, was positive for genotype T4 in both mouth and cloaca swabs (=8M and 8K).
Hassl and Benyr, (2003) found *Acanthamoeba polyphaga* and *Acanthamoeba lenticulata* in the terrarium of a chelonian (*Chelus fimbriatus*). They did not perform genotyping however, these two species are usually genotype T4 and T5, respectively (Stothard et al., 1998).

One of 17 snakes (5.8%) was positive for *Acanthamoeba* spp. genotype T5 during this study. Moreover, co-infections with genotype T5 and other genotypes were detected in 10 of 17 (58.8%) snakes. However, there was no infection with solely genotype T4.
Random sequencing of *Acanthamoeba* spp. amplicons from snake samples showed that two snake samples possibly from the same snake, namely *P. sebae* were positive for

genotype T5. Moreover, two samples (AnaGrM= *Eunectes murinus*, mouth swab and BZM= *Boa* "accordion disease", mouth swab) turned out to be genotype T4.

In case of the lizards, only genotypes other than T5 were found. Interestingly, no co-infections with more than one genotypes occurred in this group. Seven of 17 (41.1%) lizards showed positive results in PCR for other genotypes than T5.
Randomly sequencing of positive *Acanthamoeba* spp. samples collected from lizards (VIM= *Crotaphytus collaris*, mouth swab; IK= Chamaeleon calyptratus, cloaca swab and IIM= *Corucia zebrata*, mouth swab) resulted in the identification of genotype T4.
Walochnik et al. (1999) found *Acanthamoeba castellanii* (genotype T4) in the necrotic tail of *Basiliscus plumifrons* and characterized as genotype as mentioned above.

Altogether, in this study it became apparent that the investigated reptiles were infected with *Acanthamoeba* spp. genotype T4 and T5. This had actually been expected because genotype T4 is the most frequent genotype found in environmental samples followed by genotype T5 (Spanakos et al., 2006). In contrast to genotype T4, there are only a few reports on genotype T5 from clinical samples. Spanakos et al. (2006) reported on a case of AK due to genotype T5 in Greece. In that case, *Acanthamoeba* spp. isolated from clinical samples were investigated for their genotypes. 80% (four of five) of the samples were characterized as genotype T4 and one sample (20%) as genotype T5. In another case, a 17-year-old male patient with GAE turned out to be infected with *Acanthamoeba* spp. genotype T5 (Lackner et al., submitted for publication). The authors suggest, that the immunocompetent patient might have been infected nosocomially with acanthamoebas during a surgically sinusitis treatment. The GAE treatment was successful but the patient showes severe consequential neuropsychological defects.
Additionally to cases of *Acanthamoeba* spp. infections in humans, several authors reported on the occurrence of *Acanthamoeba* genotype T4 in animals for instance in squirrels (Lorenzo-Morales, 2007) and in a keel-billed toucan (*Visvesvara et al., 2007*). Walochnik et al. (1999) published the detection of *Acanthamoeba* spp. genotype T4 from a lizard. Another study demonstrated the isolation of *Acanthamoeba triangularis* and *Acanthamoeba polyphaga* from the gut of lizards (Madriagal Sesma et al., 1988). Moreover, *Acanthamoeba polyphaga* was detected in faeces samples of an *Iguana iguana* (lizard) (Hassl and Benyr, 2003).
Although, genotype T5 is the second most isolated *Acanthamoeba* genotype from environmental samples the detection of this genotype in clinical samples is low. Until

now, the reason for this big discrepancy between the number of isolates from the environment and clinical samples is not clear (Spanakos et al., 2006).

5.5.2 *B. mandrillaris*

For the establishment of the *B. mandrillaris* specific PCR during this study it was necessary to cultivate these amoebas on Vero cells. Interestingly, in our study the growth of *Balamuthia mandrillaris* on Vero cells was much weaker than reported by Visvesvara et al. (1993). In our study, it took 14 days until *Balamuthia,* which had been stored at -80°C before, destroyed the Vero cells after inoculation in contrast to the study of Visvesvara et al. (1993). In that case, *B. mandrillaris* destructed the green monkey kidney cell sheet during five to seven days

Primer sensitivity testing demonstrated the detection limit of 0.30 amoebae in one ml for the primer pair Bal18F/ Bal18rev compared with 0.20 amoebae amplified with the primer pair 5'Balspec16S and 3'Balspec16S as published by Yagi et al. (2005). The development of a multiplex real-time PCR assay for the detection of *Acanthamoeba* spp, *B. mandrillaris* and *N. fowleri* in CSF from clinical samples was published by Qvarnstrom et al. (2006). In this study, the detection limit of one amoeba in 50 µl was determined and the author suggested that the centrifugation of CSF to achieve a cell pellet would increases the sensitivity of this PCR. With this technique, at least a single amoeba can be detected in clinical samples.

5.5.3 *E. invadens*

The reptile group most affected by *E. invadens* were the snakes (15 of 17) followed by the chelonians (14 of 20). Lizards' samples turned out to be *E. invadens* negative in both sample drawings. Although, altogether 53.7% (29 of 54) of the sampled animals showed positive signals in the *E. invadens*-specific PCR, no entamoebas were detected in culture.

Bradford et al. (2008) also observed a high discrepancy between *Entamoeba* spp. PCR results and direct microscopy. The author thus assumed that *Entamoeba* occurs more frequently in gastrointestinal tracts of reptiles than previously found before molecular biological techniques were available.

The first medium used to detect entamoebas during this study was TYS-I 33 (Clark and Diamond, 2002), a standard medium for cultivation of *Entamoeba* spp.
Despite the addition of antibiotics to the liquid medium for the isolation of *Entamoeba invadens* from swabs, co-isolated bacteria and fungi overgrew the culture flask within only a few hours. First, the medium became cloudy and finally gas formation led to leaky caps of the culture flasks. For that reason, another medium was tested. This medium namely LE-medium (Clark and Diamond, 2002) was then used. Although, LE-medium showed less bacteria growth than TYS-I 33 medium, also here all samples were negative. However, lizards were also negative in PCR, thus, unfortunately it remains unclear whether this medium was better than the other.

The establishment of two different *E. invadens* PCR detection systems was uncomplicated because of the fast growth and easy cultivation of entamoebas in axenic culture with TYS-I 33 medium. We chose two different target genes for the establishment of the PCR, first the 18S rRNA gene, that is highly conserved and exists in a high number of copies. The second gene of choice was the 5.8S rRNA gene, between the ITS1 (internal transcribed spacer) and the ITS2 region, where the primer binding sites were located. Som et al. (2000) showed that the sequences of ITS1 region are almost identical between the species in *E. histolytica* and *E. dispar*, but quite variable between the species *E. moshkovskii* and *E. invadens*.
Both primer pairs were tested in parallel setups using the standard program GastB52°C. Gel electrophoresis demonstrated strong signals on agarose gels under UV exposure without any unspecific amplicons. Moreover, after demonstrating the usability of the Inv18sf/ Inv18sf and Inv5.8f/ Inv5.8r primers the sensitivities of these two primer pairs were tested with *E. invadens* DNA isolated from axenic culture. A dilution series demonstrated a detection limit of 0.05 amoebas per ml for the primer pair Inv18sf and Inv18sr in contrast to the primer pair Inv5.8f and Inv5.8r, which has a detection limit of 93.75 amoebas per ml. Because of this result, the primer pair of choice was Inv18sf/ Inv18sr.
During this study, Bradford et al. (2008) published a PCR detection system for *E. invadens*, which requires a cell number between 308 and 616 to show positive *results* by amplification of the 18S rRNA gene.

5.6 Medical relevance

5.6.1 Medical relevance for animals

In our study, we investigated animals under quarantine or animals that had been abandoned. All these animals had been in close contact with other individuals and partly even with animals of several different taxa. Therefore, the introduction of new animals infected with entamoebas into the collection might be the reason for the high detection rate for *E. invadens*. Unfortunately, we have no information on the frequency or number of introduced reptiles that were under quarantine.

Several authors suggest the introduction of new reptiles into a zoological collection might be a source of infection. The reptile collection in this study was subject to a frequent change of the livestock due to newly incoming animals and transfer of reptiles to the exhibition. This frequent change led to new arrangements of the animal population in one room and within one enclosure respectively. This increases the chance of acquiring an infection. Interestingly, only one of 17 (5.8%) snakes (*Python regius*) showed negative results for *E. invadens* in both sample drawings. A reason for this result could be, that the *Python regius* was the last snake introduced to the collection and that this snake had never been kept in one enclosure with other snakes. Moreover, the *Python regius* was kept exclusively in the basement of the reptile and amphibian house of the zoo and was never part of the exhibition. The missing contact to other animals particularly other snakes might be the reason for no acquirement of the *E. invadens* infection, generally being common within in this reptile collection.

Kojimoto et al. (2001) reported on an *Entamoeba* sp. outbreak in a zoological collection in Japan where four ball pythons showed symptoms of a severe colitis and amoebosis. The snakes were kept in one enclosure and the source of infection might have been the introduction of turtles into the zoo just before the outbreak of infections with *Entamoeba* sp. Examinations revealed multifocal necrosis in the brains and furthermore a damage of the lungs and the intestine.
As mentioned above, MacNeill et al. (2002) reported a case of 40 dead hatchling wood turtles due to *Entamoeba* sp. In that study, no new animals had been introduced to the collection in the past year. Therefore, the authors excluded new introduced animals as source of infection.

However, not only the risk of acquiring an infection with amoebae is an important aspect in zoological collections, also other pathogens constitute a risk for reptiles. In case of *Salmonella* spp. infections can be transmitted from one lizard to another by eating contaminated faeces by hatchlings (Troyer K., 1982 in Mermin et al., 1997) or in case of chelonians also by transovarial transmission (Austin and Wilkin, 1998 in Ebani and Fratini, 2005).

Several studies on the infection rate of *Salmonella* spp. in reptiles have been published, for example a study on the occurrence of *Salmonella* spp. in captive reptiles in Trinidad. That project demonstrated that 9% of the investigated chelonians were *Salmonella*-positive although the animals were apparently healthy. Furthermore, 14% of the snakes and 50% of the lizards were infected by the bacteria without clinical signs (Gopee et al., 2000).
Moreover, a study from the zoo at Seoul Grand Park in Korea demonstrated that the rate of *Salmonella* spp. infection in reptiles is 30.4% (Jang et al., 2008). 46 reptiles (27 snakes, 11 chelonians and eight lizards) were investigated for the occurrence of *Salmonella* spp. in cloaca swab samples resulting in the detection of 14 positive samples. Reptiles infected with *Salmonella* spp. usually show no clinical signs. Nevertheless, *Salmonella enterica* may cause severe symptoms like granuloma, pneumonia, septicemia and death (Ebani and Fratini, 2005).

Cases of disease caused by mycobacteria in cold-blooded animals are rare because these organisms are opportunistic organisms in reptiles, nevertheless they can be pathogenic for humans (Frye, 1991 in Ebani and Fratini, 2005). Generally, these bacteria can cause symptoms like lung disease, cutaneous infections and soft tissue lesions in reptiles. Soldati et al. (2004) investigated tissue samples of 48 snakes, 27 chelonians and 15 lizards from private households, shops or zoological collections for the occurrence of mycobacteria and chlamydiae. The author demonstrated that 25.56% (23 of 90) of the investigated reptiles (14 snakes, six chelonians and three lizards) were infected with mycobacteria.

Chlamydiae are only sporadically detected in snakes, chelonians and lizards. Ebani and Fratini (2005) suggested, that the infection rate with *Chlamydophila* spp. is higher than reported due to missing clinical signs. Generally, diagnosis is made at necropsy, with typical lesions in the heart, lungs, liver, spleen and small intestine. As mentioned above, Soldati et al. (2004) investigated 90 reptiles for the occurrence of chlamydiae and

detected 58 (64.5%) positive samples. In detail, 49 reptile samples were positive for "Chlamydia-like" isolates and 9 (10%) samples for *C. pneumoniae*, which causes pneumonia in humans.

5.6.2 Medical relevance for humans

Acquiring an infection from reptiles is possible in case of close contact with infected animals, e.g. in case of having a pet or visiting a petting zoo.

Besides amoebae, also numerous bacteria that can also cause disease in humans are pathogens in reptiles. Several authors (Soldati et al., 2004, Mermin et al., 1997, Sanyal et al., 1997, Jang et al., 2008) have reported the detection of medically relevant organisms like *Salmonella* spp. *Mycobacteria* spp. and *Chlamydia* spp. in reptiles.

It is well known, that particularly children can be infected by *Salmonella* spp. from reptilian pets in their household (Sanyal et al., 1997; Mermin et al., 1997). In the United States, the ban of housing turtles of small size in private households led to a decrease of *Salmonella* spp. infections acquired from pet turtles (Lamm et al., 1972 in Mermin et al., 1997; Cohen et al., 1980 in Mermin et al., 1997). Moreover, handling with infected reptiles is a risk for employees in a pet shop as well as for animal keepers in a zoo.

In addition, humans can acquire a mycobacteria infection from reptiles. Atypical mycobacteria are the causatives of mycobacterial infections in reptiles as reported by Sodati et al. (2004). Contact between reptiles and *Mycobacterium* spp. in their natural environment as well as in zoological collections is most likely due to the fact that these bacteria occur in water, soil and dust. Because reptiles are resistant against mycobacteria, which is an opportunistic organism in cold-blooded animals, cases of diseases are rare (Frye, 1991 in Ebani and Fratini, 2005). In case of an infection, typical findings in necropsy are grayish-white nodules in the subcutis and many organs (Soldati et al., 2004). In difference to reptiles, atypical mycobacteria are potentially pathogen for humans and cause dermal symptoms similar to those in cold-blooded animals. Hassl et al. (2004) reported a case of a mycobacterial infection acquired from a reptile pet. In this case, an immunocompetent young man had an enlarged lymph node in the left inguinal region caused by *Mycobacterium fortuitum*, which was characterized by PCR. Clinical examination of his snakes (two *Python morulus*) showed dermal changes especially on the mouth of one individual. Close physical contact between the patient, which used to sleep with his pet snakes in one bed might have been the route of disease transmission.

An example for getting infected with pathogens even without direct contact to infected animals was reported by Friedman et al. 1998. In that case, 39 children acquired a *Salmonella* spp. infection without even touching the reptile, but by touching the wooden barrier of the enclosure while attending a Komodo dragon exhibit at a Zoo in Denver, USA. Moreover, a study from the Antwerp Zoo demonstrated that the environment of the reptile department is contaminated with *Salmonella* spp. The bacteria were isolated from 47% of the environmental samples collected at the kitchen where the food for the reptiles was prepared, from floors, window benches and railings in public space as well as from cages (Bauwens et al., 2006). These results demonstrate the role of reptiles as contaminators of the environment with pathogens.
However, to our knowledge, there are no reports on environmental contamination with entamoebas or the occurrence of FLA in public areas in zoos.

5.6.3 Prophylaxis

The findings of the studies on *Salmonella* spp. demonstrated the zoos of Antwerp (Bauwens et al., 2006) and Denver (Friedman et al., 1998) that a high hygienic standard and prevention efforts in a zoo are important on behalf of visitors and employees, even if no information on the health status or medical history of the animals is available. For example in our study, some snakes, chelonians and lizards were under quarantine after having been confiscated at the airport or found being abandoned. Because of this, there was no information on the origin of the animal, medical history or previous treatment.

If new animals are introduced to a resident collection, quarantine is essential in order to be sure, that no pathogen might be transmitted. In case of some viruses transmitted by air, like paramyxovirus it is even necessary to place the new animal into a separate room.
Moreover, standard hygienic measures like wearing disposable gloves and disinfection of the hands after handling every single animal can avoid the transmission of pathogens from one animal to another. The same hygienic standards should be given after handling instruments like snake hooks which should not be used for animals under quarantine and animals in a resident collection at the same time. It is also important that newly introduced animals should be handled after the resident ones.
Disinfection of the cages, to kill cysts and trophozoites of *E. invadens* and FLAs should be performed regularly. Several authors reported the efficiency of different disinfectants

like, 4.chlor-M.cresol, 5% dilution of sodium hypochlorite and 5% ammonium solution (Eckert et al., 2005 in Pasmans et al., 2008 and Cranfield et al., 1999 in Pasmans et al., 2008). Cursons et al. (1980) investigated the effect of disinfectants on FLAs. In that study, the authors demonstrated that four disinfectants (chlorine, chlorine dioxide, deciquam 222 and ozone) can be used to for the disinfection of waters contaminated with FLAs. Moreover, *Naegleria* spp. turned out to be more sensitive to chlorine and chlorine dioxide than acanthamoebas, but there were no differences between ozone or deciquam 222.

To avoid possible transmission of *E. invadens* from one animal to another it is important to ensure, that lizards or snakes are not kept together with chelonians or crocodilians. Due to the fact that entamoebas commonly occur in chelonians and crocodilians without causing disease a transmission of *E. invadens* may occur when the reptiles are kept in the same room. Moreover, reptiles from different genera should not be mixed and housed separated from each other by geographic regions (Pasmans et al., 2008).

5.7 Pelodera strongyloides

Additionally to the samples drawn at the zoo Schönbrunn, we received faeces samples for the detection of FLAs and *E. invadens* of a cobra from the "Vienna Aquarium" that died during this study. In these samples, a nematode was detected. The cultivation of this nematode was very easy on NN agar plates coated with *E. coli* as a food source and the fast growth and reproduction of these worms was observed via microscope. This nematode was identified as *Pelodera strongyloides* (Figure 57) by PCR and sequencing.

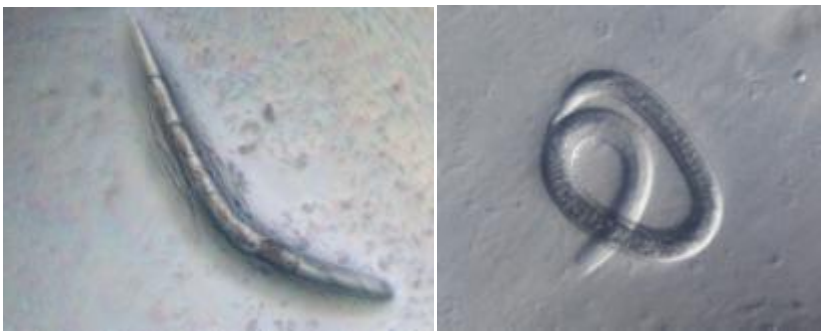


Figure 57: *Pelodera strongyloides* (orig.)

P. strongyloides (Schneider, 1860) is a facultatively pathogenic, free-living soil nematode that shows a direct life cycle. It lives in organic matter and can cause dermatitis after contact with contaminated material by invasion of the skin by third-stage larvae. Typical symptoms of *Pelodera* dermatitis are erythema, papulocrustous skin lesions, alopecia

and pruritus. Unfortunately, we have no information on a possible skin disease from this dead snake. Yeruham et al. (2005) reported on a case of *Pelodera* dermatitis in a dairy cattle herd and suggested, that moistly and dirty environmental conditions as well as a temperature of 21°C to 28°C facilitate the appearance of *P. strongyloides*. The cobra in this project was housed in a terrarium filled with soil and plants and a temperature of about 30°C.

Several cases of *P. strongyloides* infections in animals, like in voles (Klimpel et al., 2007), cattle (Yeruham, 2005) dogs (Saari and Nikander, 2006) and a black bear (Fitzgerald et al., 2008) have been reported. Rashmir-Raven et al., (2000), reported on a case of *Pelodera* dermatitis in a Tennessee walking horse. The stallion, which refused to move, was presented with a severe dermatitis involving three limbs. The surgical removal of the lesions and the treatment with oxytetracycline led to recovery within five weeks.

Furthermore, there are also reports on dermatitis in humans due to this nematode (Caumes et al., 2002 and Veraldi et al., 2005). The patients presented folliculitis and creeping eruptions after returning from voyages to the tropics (Martinique, Guadeloupe, Mexico, Brazil and Thailand). Treatment with ivermectin and albendazole for eight to 38 days led to complete recovery from *Pelodera* dermatitis.

To our knowledge, there are no reports on the occurrence of *P. strongyloides* neither in snakes nor in reptiles on the whole.

6 Glossary

***Acanthamoeba*:** Free-living ubiquitous soil amoebae without flagellate stage. Some strains of this genus cause infections in humans and animals at different sites (eye, bone, brain and lungs)

Aerobic: This term refers to oxygen requiring cellular respiration and verbatim means "with air". Opposite: anaerobic.

Agarose: This polysaccharide is obtained by the purification of agar. It forms a gel that is used for different types of electrophoresis and immunodiffusion (agarbiose). An agarose gel is made by heating and cooling a TAE-buffer and agarose (powder) solution, typically with a concentration of 1- 2 % agarose.

Agar plate: is a growth medium (agar plus nutrient) containing sterile Petri dish for the culture of microorganisms or aerobic amoebae.

AIDS: This disease is caused by a retrovirus called human immunodeficiency virus (HIV-1, HIV-2) that leads to immune system failure and debilitation. AIDS is brought forward by direct contact with body fluids.

Anaerobic: This term literally means "without air". An anaerobic organism can not grow and may die in presents of oxygen. Obligate anaerobic organisms will die in presents of atmospheric levels of oxygen; facultative anaerobic organisms are enabled to use oxygen in present of it.

Axenic culture: An axenic culture describes a culture with only one microbial species, free of "contaminating" organisms.

Balamuthia mandrillaris: a free-living amoeba known to cause granulomatous amoebic encephalitis in immunocompetent and immunocompromised humans and animals.

Binary fission: Division of a cell and its DNA without Mitosis into two genetically identical daughter cells.

Cerebrospinal fluid (CSF) Liquor cerebrospinalis, is a clear bodily fluid that occupies the subarachnoid space and the ventricular system around and inside the brain.

Chronic: This term is used for a slow progressive and continuous disease of long duration.

Cornea: Is the transparent five layers (epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium) consisting front part of the eye.

Corneal abrasion: Rubbing off of the surface of the cornea in the course of a therapy

Cyst: The resistant duration stage of many protozoan organisms.

Cytoplasm: is the cell-part which is enclosed within the cell and contains organelles like mitochondria in eukaryotic cells. Most cellular activities, like metabolic pathways, occur in the cytoplasm.

DNA Polymerase: An enzyme necessary for the replication of DNA.

DNA (Deoxyribonucleic acid): is the carrier of the genetic information in organisms and some viruses and is build by two in a double helix twisted chains of nucleotides. The nucleotides are joined by hydrogen bonds, two bonds in case of the complementary bases adenine and thymine and three bonds in case of guanine and cytosine. Each DNA strand contains many units composed by phosphate, 5 carbon sugar and a nitrogen containing carbon base (Adenine, Thymine, Guanine or Cytosine).

Ectocyst: Outer cyst wall.

Electrophoresis: A technique to separate nucleic acids, proteins or other compounds based on the movement toward a positive or negative electrical pole in an electric field. The molecules embedded

in a gel migrate a relative distance within an electric field due to the size of the molecules. Afterwards, the staining of the gel is necessary to observe any patterns of banding.

ELISA (Enzyme-linked immunosorbent assay): A technique to detect antibodies or antigens in a sample used in medical diagnosis.

Encephalitis: Inflammation of the brain.

Encystment: Cyst stage formation

Endocyst: Inner cyst wall.

Endophthalmitis: An inflammation of the internal parts of the eye that may lead to loss of vision or enucleation.

Entamoeba: a genus of anaerobic internal pathogenic amoebas that can also be found as commensal.

Enucleation: surgery removal of the eye, leaving the eye muscles intact.

Enzyme: a biochemical catalyst produced by living organisms.

Epidemiology: the study of health and illness affecting factors in populations.

Eukaryotic: A cell with a true nucleus, surrounded by an envelope. This nucleus contains the DNA. Cell organelles like mitochondria and chloroplasts are membrane bound.

Flagellum: is a tail like structure of prokaryotic and eukaryotic cells for locomotion.

Folliculitis is the inflammation of hair follicles in different number that may occur anywhere on the skin.

GAE: a disease of the CNS (central nervous system) caused by *Balamuthia mandrillaris* or *Acanthamoeba* spp.

GenBank: Nucleic acid and protein sequence database at the National Library of Medicine in the USA.

Gene: Genes are formed from DNA and build the units of inheritance, which are located on a specific locus on a chromosome.

Genome: The entirety of genes in a eukaryotic cell including nuclear genes and genes of DNA containing organelles.

Granulomatous: nodular granulation tissue formed of immune cells at sites of inflammation to wall off foreign substances, which cannot be eliminated.

Ig (immunoglobulins or antibodies): components of the immune system to identify and eliminate foreign objects circulating in blood or body fluids.

Immunocompetence: normal activity, efficacy and immune response of the immune system.

Immunosuppression: Reduction of the efficacy and activity of the immune system.

Infection: is the invasion and reproduction of a pathogen in a host organism.

Inflammation: a reaction of vascular tissues to a stimuli with the function to erase the trigger, which can be a pathogen, irritants or damaged cells.

Isolate: A single organism or a strain separated from clinical or environmental samples. This term is also used for a pure culture produced from an isolate.

Keratitis: This term is used for an inflammation of the cornea without ulceration caused by injury.

Lumbar puncture In medicine, a lumbar puncture (colloquially known as a spinal tap) is a diagnostic and at times therapeutic procedure that is performed in order to collect a sample of cerebrospinal fluid (CSF) for biochemical, microbiological, and cytological analysis, or occasionally as a treatment ("therapeutic lumbar puncture") to relieve increased intracranial pressure.

Meninges (singular meninx): The meninges are protecting together with the cerebrospinal fluid, the central nervous system. The meninges are organized into three layers: the dura mater, the arachnoid mater and the pia mater.

Mitochondrion: is a cell organelle in eukaryotic cells that generates most of the ATP (adenosine triphosphate) for the production of chemical energy. Furthermore, mitochondria are part of many other processes like signaling, cell death and the cell cycle control.

Meiosis: In difference to Mitosis, the number of chromosomes changes from diploid to haploid during Meiosis. Usually this comes along with a recombination of maternal and paternal genetic material. Moreover, Meiosis performs two divisions (Meiosis I and II).

Mitosis: During this process, the chromosomes within the nucleus of a eukaryotic cell are separated into two daughter nuclei followed by a cytokinesis. This process results in the production of two identical daughter cells.

Naegleria fowleri: is a thermophilic free-living amoeba that shows a life cycle with three stages (trophozoite, flagellate and cyst). *N. fowleri* causes PAME.

Non-nutrient Agar Plates: is a growth medium (agar without nutrient) containing sterile Petri dish for the culture of microorganisms or aerobic amoebae.

Nucleic acid: DNA (Deoxyribonucleic acid) serves as the genetic material of organisms and many viruses. It consists of tow long polymers with a sugar (desoxyribose) and phosphate backbone and one of four bases attached to each sugar. These bases are called adenine, guanine, cytosine and thymine.
RNA (ribonucleic acid) contains uracil instead of thymine and uses the sugar ribose instead of desoxyribose. The RNA serves as the genetic material for some viruses and has a number of functions related to the protein synthesis.

Nucleolus: Is a "sub-organelle" of the cell nucleus and surrounded by chromatin without being separated by membranes from the nucleoplasm. The main function is the assembly of ribosome components.

Operculum: In biology, operculum (Latin for "little lid") has been used to describe several different anatomical features, in animals, in humans and even in plants

Opportunistic: opportunistic pathogens do not cause disease in individual with a healthy immune system, but in immunosuppressed individuals.

Organelles: are membrane-enclosed compartments within the cell with specific functions.

Parasite: a parasite is an organism that obtains nutrition from a host organism. The parasite can live inside or outside of the host.

Pathogen: is an infectious agent that causes disease to its host.

Pathogenic: The ability to cause diseases.

Pathogenicity: The ability of a pathogen to damage the host.

Phagocytosis: is the engulfment of solid particles by the cell membrane of protists. The food particle is enclosed by food vacuoles. The engulfment of liquid nourishment is called pinocytosis.

PHMB (polyhexamethylene biguanide): is a disinfectant for the use on skin and in contact lens solutions.

Phylogeny: studies the relatedness of different groups of organisms. Nowadays, phylogenetic studies base on molecular sequencing and morphological features of the organisms.

Pleocytosis: This term is used to describe an increased cell count {Gk. pleion more}, in the most cases an increase in white blood cell count in fluid from the body like cerebrospinal fluid (CSF).
A Pleocytosis is an indicator of an infectious, inflammatory, or malignant condition.

Polymerase chain reaction (PCR): Is a very sensitive and specific technique to amplify DNA by using a heat stable polymerase, two oligonucleotide primers flanking the target sequence and continuous cycles of primer annealing, elongation and dissociation.

Polymerase: is an enzyme, that polymerizes new RNA and DNA by using a DNA or RNA template as matrix. The polymerase is a essential component of replication and transcription process.

Primary amoebic meningoencephalitis (PAME): a disease of the CNS, caused by the free-living amoeba *N. fowleri*.

Protozoa: are unicellular heterotrophic protists, like amoebas.

Pseudopodium: are temporary emerging structures from eukaryotic cells and typical for amoeboid movement.

RFLP (restriction fragment length polymorphism):
Is a technique for detection of sequence variation in the DNA by breaking the DNA into pieces with restriction enzymes. Fragments are analyzed by gel electrophoresis.

TAE- buffer: TAE buffer is the abbreviation of Tris- acetat- EDTA- buffer, named by its components TRIS (Tris-(hydroxymethyl)-aminomethan), Acetat (salt of CH3COOH) and EDTA (Ethylendiamintetraessigsäure). This is a buffer solution which is used in agarose gelelectrophoresis.

Taq Polymerase: A thermo stable enzyme used in PCR. This enzyme is named after *Thermus aquaticus*, a thermophilic bacterium.

Taxonomy: Classification of organisms in biology.

Thermophil: tolerant to heat

Trophozoite: The actively feeding and multiplying stage of a protozoan.

Unikont: A eukaryotic cell featuring a single flagellum.

Vacuole: A membrane bound organelle usually filled with water.

7 Literature:

Aichelburg AC, Walochnik J, Assadian O, Prosch H, Steuer A, Perneczky G, Visvesvara GS, Aspöck H, Vetter N. Successful treatment of disseminated *Acanthamoeba* sp. infection with miltefosine. Emerg Infect Dis. 2008 Nov;14(11):1743-6.

Amaral Zettler LA, Nerad TA, O'Kelly CJ, Peglar MT, Gillevet PM, Silberman JD, Sogin ML. A molecular reassessment of the Leptomyxid amoebae. Protist. 2000 Oct;151(3):275-82.

Anzil AP, Rao C, Wrzolek MA, Visvesvara GS, Sher JH, Kozlowski PB. Amebic meningoencephalitis in a patient with AIDS caused by a newly recognized opportunistic pathogen. Leptomyxid ameba. Arch Pathol Lab Med. 1991 Jan;115(1):21-5.

Bakardjiev A, Azimi PH, Ashouri N, Ascher DP, Janner D, Schuster FL, Visvesvara GS, Glaser C. Amebic encephalitis caused by *Balamuthia mandrillaris*: report of four cases. Pediatr Infect Dis J. 2003 May;22(5):447-53.

Bauer RW, Harrison LR, Watson CW, Styer EL, Chapman WL Jr. Isolation of *Acanthamoeba* sp. from a greyhound with pneumonia and granulomatous amebic encephalitis. J Vet Diagn Invest. 1993 Jul;5(3):386-91.

Bauwens L, Vercammen F, Bertrand S, Collard JM, De Ceuster S. Isolation of Salmonella from environmental samples collected in the reptile department of Antwerp Zoo using different selective methods. J Appl Microbiol. 2006 Aug;101(2):284-9.

Bodi I, Dutt N, Hampton T, Akbar N.Fatal granulomatous amoebic meningoencephalitis due to *Balamuthia mandrillaris*. Pathol Res Pract. 2008;204(12):925-8.

Booton GC, Carmichael JR, Visvesvara GS, Byers TJ, Fuerst PA. Identification of *Balamuthia mandrillaris* by PCR assay using the mitochondrial 16S rRNA gene as a target. J Clin Microbiol. 2003a Jan;41(1):453-5.

Booton GC, Carmichael JR, Visvesvara GS, Byers TJ, Fuerst PA., Genotyping of *Balamuthia mandrillaris* based on nuclear 18S and mitochondrial 16S rRNA genes. Am J Trop Med Hyg. 2003b Jan;68(1):65-9.

Booton GC, Visvesvara GS, Byers TJ, Kelly DJ, Fuerst PA. Identification and distribution of *Acanthamoeba* species genotypes associated with nonkeratitis infections. J Clin Microbiol. 2005 Apr;43(4):1689-93.

Bosch, I., Deichsel, G. Morphological study of pathogenic and potentially pathogenic amoebae of the types "*Entamoeba*" and "*Hartmannella-Acanthamoeba*" from reptiles Morphologische Untersuchungen an pathogenen und potentiell pathogenen Amoeben der Typen "*Entamoeba*" und "*Hartmannella-Acanthamoeba*" aus Reptilien, Zeitschrift fur Parasitenkunde 40 (2), pp. 107-129, 1972

Bradford CM, Denver MC, Cranfield MR. Development of a polymerase chain reaction test for *Entamoeba invadens*. J Zoo Wildl Med. 2008 Jun;39(2):201-7.

Byers J, Faigle W, Eichinger D. Colonic short-chain fatty acids inhibit encystation of *Entamoeba invadens*. Cell Microbiol. 2005 Feb;7(2):269-79.

Canfield PJ, Vogelnest L, Cunningham MI, Visvesvara GS. Amoebic meningoencephalitis caused by *Balamuthia mandrillaris* in an orang utan. Aust Vet J. 1997 Feb;75(2):97-100.

Calsamiglia M, Pijoan C, Trigo A. Application of a nested polymerase chain reaction assay to detect *Mycoplasma hyponeumoniae* from nasal swabs. *Journal of Veterinary Diagnostic Investigation* 11 (3), pp. 246-251,1999

Caumes E, Ly F, Bricaire F. Cutaneous larva migrans with folliculitis: report of seven cases and review of the literature. Br J Dermatol. 2002 Feb;146(2):314-6. Review.

Cavalier-Smith T. Only six kingdoms of life. Proc Biol Sci. 2004 Jun 22;271(1545):1251-62.

Cerva L, Novăk K. Amoebic meningoencephalitis: 16 fatalities. Science. 1968 Apr 5;160(823):92.

Clark CG. The evolution of *Entamoeba*, a cautionary tale. Res Microbiol. 2000 Oct;151(8):599-603.

Clark CG, Diamond LS. Methods for cultivation of luminal parasitic protists of clinical importance. Clin Microbiol Rev. 2002 Jul;15(3):329-41.

Claerhout I, Goegebuer A, Van Den Broecke C, Kestelyn P. Delay in diagnosis and outcome of *Acanthamoeba* keratitis. Graefes Arch Clin Exp Ophthalmol. 2004;242:648-53.

Cogo PE, Scagli M, Gatti S, Rossetti F, Alaggio R, Laverda AM, Zhou L, Xiao L, Visvesvara GS. Fatal *Naegleria fowleri* meningoencephalitis, Italy. Emerg Infect Dis. 2004 Oct;10(10):1835-7.

Cursons RT, Brown TJ, Keys EA. Effect of disinfectants on pathogenic free-living amoebae: in axenic conditions. Appl Environ Microbiol. 1980 Jul;40(1):62-6.

Dacks JB, Walker G, Field MC. Implications of the new eukaryotic systematics for parasitologists. Parasitol Int. 2008 Jun;57(2):97-104. Epub 2007 Dec 4. Review.

Daft BM, Visvesvara GS, Read DH, Kinde H, Uzal FA, Manzer MD. Seasonal meningoencephalitis in Holstein cattle caused by *Naegleria fowleri.* J Vet Diagn Invest. 2005 Nov;17(6):605-9.

De Jonckheere JF. A Century of Reserach on the Amoeboflagellate Genus *Naegleria*. Acta Protozool. 2002, 41: 309-342

De Jonckheere JF. Molecular definition and the ubiquity of species in the genus *Naegleria*. Protist. 2004 Mar;155(1):89-103.

Deetz TR, Sawyer MH, Billman G, Schuster FL, Visvesvara GS. Successful treatment of *Balamuthia* amoebic encephalitis: presentation of 2 cases. Clin Infect Dis. 2003 Nov 15;37(10):1304-12. Epub 2003 Oct 17.

Denver, M.C., Cranfield, M.R., Graczyk, T.K., Blank, P., Wisnieski, A., Poole, V. A review of reptilian amebiasis and current research on the diagnosis and treatment of amebiasis at the Baltimore Zoo. Proc. Am. Assoc. Zoo Vet. 1999 1, pp. 1-15

Dunnebacke TH, Schuster FL, Yagi S, Booton GC. *Balamuthia mandrillaris* from soil samples. Microbiology. 2004 Sep;150(Pt 9):2837-42.

Dunnebacke TH. The ameba *Balamuthia mandrillaris* feeds by entering into mammalian cells in culture. J Eukaryot Microbiol. 2007 Sep-Oct;54(5):452-64.

Ebani Valentina V., Fratini Filippo, *Bacterial zoonoses among domestic reptiles.* Annali della Facoltà di Medicina veterinaria, LVIII/2005. pp. 85-91. ISSN 0365-4729

Fahrni JF, Bolivar I, Berney C, Nassonova E, Smirnov A, Pawlowski J. Phylogeny of lobose amoebae based on actin and small-subunit ribosomal RNA genes. Mol Biol Evol. 2003 Nov;20(11):1881-6. Epub 2003 Aug 29.

Finnin PJ, Visvesvara GS, Campbell BE, Fry DR, Gasser RB. Multifocal *Balamuthia mandrillaris* infection in a dog in Australia. Parasitol Res. 2007 Jan;100(2):423-6. Epub 2006 Oct 11.

Fitzgerald SD, Cooley TM, Cosgrove MK. Sarcoptic mange and Pelodera dermatitis in an American black bear (*Ursus americanus*). J Zoo Wildl Med. 2008 Jun;39(2):257-9.

Foreman O, Sykes J, Ball L, Yang N, De Cock H. Disseminated infection with *Balamuthia mandrillaris* in a dog. Vet Pathol. 2004 Sep;41(5):506-10.

Frank, W., Bosch, I. Isolation of amoebae of the type "*Hartmannella-Acanthamoeba*" and "*Naegleria*" from cold-blooded vertebrates | [Isolierung von Amoeben des Typs "*Hartmannella-Acanthamoeba*" und "*Naegleria*" aus Kaltblütern]. Zeitschrift fur Parasitenkunde 40 (2), pp. 139-150, 1972

French SS, Matt KS, Moore MC, The effects of stress on wound healing in male tree lizards (*Urosaurus ornatus*). Gen Comp Endocrinol. 2006 Jan 15;145(2):128-32. Epub 2005 Sep 26.

Friedman CR, Torigian C, Shillam PJ, Hoffman RE, Heltzel D, Beebe JL, Malcolm G, DeWitt WE, Hutwagner L, Griffin PM. An outbreak of salmonellosis among children attending a reptile exhibit at a zoo. J Pediatr. 1998 May;132(5):802-7.

Gopee NV, Adesiyun AA, Caesar K. Retrospective and longitudinal study of salmonellosis in captive wildlife in Trinidad. J Wildl Dis. 2000 Apr;36(2):284-93.

Hammersmith KM. Diagnosis and management of Acanthamoeba keratitis. Curr Opin Ophthalmol. 2006 Aug;17(4):327-31.

Hassl A, Benyr G, Appelt S, Freilebende Amöben als opportunistische Darmparasiten von Reptilien. Mitt Österr Ges Tropenmed Parasitol, 2000

Hassl A, Benyr G. Hygienic evaluation of terraria inhabited by amphibians and reptiles: cryptosporidia, free-living amebas, salmonella. Wien Klin Wochenschr. 2003;115 Suppl 3:68-71.

Hassl A., Armbruster C., Filip T.: Mycobacterium-infections in reptiles: a cause of zoonosis? 7th International Symposium of Pathology and Medicine of Reptiles and Amphibians, Berlin 2004

Herbst R, Ott C, Jacobs T, Marti T, Marciano-Cabral F, Leippe M. Pore-forming polypeptides of the pathogenic protozoon Naegleria fowleri. J Biol Chem. 2002 Jun 21;277(25):22353-60.

Hiti K, Walochnik J, Faschinger C, Haller-Schober EM, Aspöck H.One- and two-step hydrogen peroxide contact lens disinfection solutions against *Acanthamoeba*: how effective are they? Eye. 2005;19:1301-5.

Innis C, Papich M, Young D. Pharmacokinetics of metronidazole in the red-eared slider turtle (Trachemys scripta elegans) after single intracoelomic injection. J Vet Pharmacol Ther. 2007 Apr;30(2):168-71.

Jang YH, Lee SJ, Lim JG, Lee HS, Kim TJ, Park JH, Chung BH, Choe NH. The rate of *Salmonella* spp. infection in zoo animals at Seoul Grand Park, Korea.
J Vet Sci. 2008 Jun;9(2):177-81.

Jakob W, Wesemeier HH. Intestinal inflammation associated with flagellates in snakes. J Comp Pathol. 1995 May;112(4):417-21.

Kinde H, Visvesvara GS, Barr BC, Nordhausen RW, Chiu PH. Amebic meningoencephalitis caused by *Balamuthia mandrillaris* (leptomyxid ameba) in a horse. J Vet Diagn Invest. 1998 Oct;10(4):378-81.

Klimpel S, Förster M, Schmahl G. Parasite fauna of the bank vole (*Clethrionomys glareolus*) in an urban region of Germany: reservoir host of zoonotic metazoan parasites? Parasitol Res. 2007 Dec;102(1):69-75. Epub 2007 Sep 12.

Kodet R, Nohýnková E, Tichý M, Soukup J, Visvesvara GS. Amebic encephalitis caused by *Balamuthia mandrillaris* in a Czech child: description of the first case from Europe. Pathol Res Pract. 1998;194(6):423-9.

Kojimoto A, Uchida K, Horii Y, Okumura S, Yamaguch R, Tateyama S. Amebiasis in four ball pythons, *Python reginus*. J Vet Med Sci. 2001 Dec;63(12):1365-8.

Lesh FA. Massive development of amebas in the large intestine. Fedor Aleksandrovich Lesh (Lösch). Am J Trop Med Hyg. 1975 May;24(3):383-92.

Lorenzo-Morales J, López-Darias M, Martínez-Carretero E, Valladares B. Isolation of potentially pathogenic strains of *Acanthamoeba* in wild squirrels from the Canary Islands and Morocco. Exp Parasitol. 2007 Sep;117(1):74-9. Epub 2007 Mar 27.

Lozano- Alarcón F, Bradley GA, Houser BS, Visvesvara GS. Primary amebic meningoencephalitis due to *Naegleria fowleri* in a South American tapir. Vet Pathol. 1997 May;34(3):239-43.

Maciver SK. The threat from *Balamuthia mandrillaris*. J Med Microbiol. 2007 Jan;56(Pt 1):1-3.

MacNeill AL, Uhl EW, Kolenda-Roberts H, Jacobson E. Mortality in a wood turtle (*Clemmys insculpta*) collection. Vet Clin Pathol. 2002;31(3):133-6.

Madrigal Sesma MJ, Chamorro Garcia L, Guillen LP (1988) Free- living amoebas and cold-blooded animals. J Parasitol 74: 883- 884

Marciano-Cabral F, Cabral G. *Acanthamoeba* spp. as agents of disease in humans. Clin Microbiol Rev. 2003a Apr;16(2):273-307.

Marciano-Cabral F, MacLean R, Mensah A, LaPat-Polasko L. Identification of *Naegleria fowleri* in domestic water sources by nested PCR. Appl Environ Microbiol.2003b Oct;69(10):5864-9.

Marciano-Cabral F, Cabral GA. The immune response to *Naegleria fowleri* amebae and pathogenesis of infection. FEMS Immunol Med Microbiol. 2007 Nov;51(2):243-59. Epub 2007 Sep 21. Review.

Matin A, Jeong SR, Faull J, Rivas AO, Khan NA. Evaluation of prokaryotic and eukaryotic cells as food source for *Balamuthia mandrillaris.* Arch Microbiol. 2006 Oct;186(4):261-71. Epub 2006 Jul 18.

Meerovitch E. Thermal Barrier to the Infectivity of *Entamœba invadens* in Snakes. Nature, Volume 185, Issue 4713, pp. 631 (1960).

Meersseman W, Lagrou K, Sciot R, de Jonckheere J, Haberler C, Walochnik J, Peetermans WE, van Wijngaerden E. Rapidly fatal *Acanthamoeba* encephalitis and treatment of cryoglobulinemia. Emerg Infect Dis. 2007 Mar;13(3):469-71.

Mermin J, Hoar B, Angulo FJ. Iguanas and *Salmonella* marina infection in children: a reflection of the increasing incidence of reptile-associated salmonellosis in the United States.

Pediatrics. 1997 Mar;99(3):399-402.

Miller R. Eric, Zoo and wild animal medicine: current therapy, Edition: 6, illustrated Elsevier Health Sciences, 2007, pp 156f

Morgan, K.N., Tromborg, C.T., Sources of stress in captivity**,** *Applied Animal Behaviour Science* 102 (3-4), pp. 262-302, 2007

Neumeister B, heinrich K, Geiss R, Braun W, Kimmig P, Mikrobiologische Diagnostik, 2009 2., vollst. überarb. Aufl., 1180 S., 570 Abb., geb. Georg Thieme Verlag 3-13-743602-8

Nikolaev SI, Berney C, Petrov NB, Mylnikov AP, Fahrni JF, Pawlowski J. Phylogenetic position of Multicilia marina and the evolution of Amoebozoa. Int J Syst Evol Microbiol. 2006 Jun;56(Pt 6):1449-58.

Nicholas KB, Nicholas HB Jr., Deerfiled DW II. (1997): GeneDoc: Analysis and Visualization of Genetic Variation. Embnew News 4:14.

Niyyati M, Lorenzo-Morales J, Rezaeian M, Martin-Navarro CM, Haghi AM, Maciver SK, Valladares B., Isolation of *Balamuthia mandrillaris* from urban dust, free of known infectious involvement. Parasitol Res. 2009 Aug 15.

Ozaki K, Matsuo K, Tanaka O, Narama I. Amoebosis in the flat-shelled spider tortoise (*Acinixys planicauda*). J Comp Pathol. 2000 Nov;123(4):299-301.

Pasmans F, Blahak S, Martel A, Pantchev N., Introducing reptiles into a captive collection: the role of the veterinarian. Vet J. 2008 Jan;175(1):53-68. Epub 2007 Mar 8.

Pélandakis M, Serre S, Pernin P. Analysis of the 5.8S rRNA gene and the internal transcribed spacers in *Naegleria* spp. and in *N. fowleri*. J Eukaryot Microbiol. 2000 Mar-Apr;47(2):116-21.

Petry F, Torzewski M, Bohl J, Wilhelm-Schwenkmezger T, Scheid P, Walochnik J, Michel R, Zöller L, Werhahn KJ, Bhakdi S, Lackner KJ. Early diagnosis of *Acanthamoeba* infection during routine cytological examination of cerebrospinal fluid. J Clin Microbiol. 2006 May;44(5):1903-4.

Pawlowski J, Burki F. Untangling the phylogeny of amoeboid protists. J Eukaryot Microbiol. 2009 Jan-Feb;56(1):16-25.

Pussard M, Pons R. (1977) Morphologie de la paroi kystique et taxonomie du genre *Acanthamoeba* (Protozoa, Amoebida). Protistologica. 13:557-598.

Qvarnstrom Y, Visvesvara GS, Sriram R, da Silva AJ., Multiplex real-time PCR assay for simultaneous detection of *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri*. J Clin Microbiol. 2006 Oct;44(10):3589-95.

Rashmir-Raven AM, Black SS, Rickard LG, Akin M. Papillomatous pastern dermatitis with spirochetes and *Pelodera strongyloides* in a Tennessee Walking Horse. J Vet Diagn Invest. 2000 May;12(3):287-91.

Richter B, Kübber-Heiss A, Weissenböck H, Schmidt P. (2008a) Detection of *Cryptosporidium* spp., *Entamoeba* spp. and *Monocercomonas* spp. in the gastrointestinal tract of snakes by in-situ hybridization. J Comp Pathol. 2008a Feb-Apr;138(2-3):63-71. Epub 2008 Mar 4.

Richter B, Kübber-Heiss A, Weissenböck H. Diphtheroid colitis in a *Boa constrictor* infected with amphibian *Entamoeba* sp. Vet Parasitol. 2008b May 6;153(1-2):164-7. Epub 2008 Jan 17.

Rideout BA, Gardiner CH, Stalis IH, Zuba JR, Hadfield T, Visvesvara GS. Fatal infections with *Balamuthia mandrillaris* (a free-living amoeba) in gorillas and other Old World primates. Vet Pathol. 1997 Jan;34(1):15-22.

Rosenberg AS, Morgan MB. Disseminated acanthamoebiasis presenting as lobular panniculitis with necrotizing vasculitis in a patient with AIDS. J Cutan Pathol. 2001 Jul;28(6):307-13.

Saari SA, Nikander SE. *Pelodera* (syn. *Rhabditis*) *strongyloides* as a cause of dermatitis--a report of 11 dogs from Finland. Acta Vet Scand. 2006 Sep 5;48:18.

Sangwan VS, Burman S, Tejwani S, Mahesh SP, Murthy R. Amniotic membrane transplantation: a review of current indications in the management of ophthalmic disorders. Indian J Ophthalmol. 2007 Jul-Aug;55(4):251-60. Review.

Sanyal D, Douglas T, Roberts R. *Salmonella* infection acquired from reptilian pets. Arch Dis Child. 1997 Oct;77(4):345-6.

Schroeder JM, Booton GC, Hay J, Niszl IA, Seal DV, Markus MB, Fuerst PA, Byers TJ. Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of acanthamoebae from humans with keratitis and from sewage sludge. J Clin Microbiol. 2001 May;39(5):1903-11.

Schuster FL. Cultivation of pathogenic and opportunistic free-living amebas. Clin Microbiol Rev. 2002 Jul;15(3):342-54. Review.

Schuster FL, Dunnebacke TH, Booton GC, Yagi S, Kohlmeier CK, Glaser C, Vugia D, Bakardjiev A, Azimi P, Maddux-Gonzalez M, Martinez AJ, Visvesvara GS. Environmental isolation of *Balamuthia mandrillaris* associated with a case of amebic encephalitis. J Clin Microbiol. 2003 Jul;41(7):3175-80.

Schuster FL, Visvesvara GS. Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. Int J Parasitol. 2004a Aug;34(9):1001-27. Review.

Schuster FL, Glaser C, Honarmand S, Maguire JH, Visvesvara GS. *Balamuthia* amebic encephalitis risk, Hispanic Americans. Emerg Infect Dis. 2004b Aug;10(8):1510-2. No abstract available.

Seal DV. *Acanthamoeba* keratitis update-incidence, molecular epidemiology and new drugs for treatment. Eye. 2003 Nov;17(8):893-905.

Seijo Martinez M, Gonzalez-Mediero G, Santiago P, Rodriguez De Lope A, Diz J, Conde C, Visvesvara GS. Granulomatous amebic encephalitis in a patient with AIDS: isolation of *Acanthamoeba* sp. Group II from brain tissue and successful treatment with sulfadiazine and fluconazole. J Clin Microbiol. 2000 Oct;38(10):3892-5.

Shi W, Liu M, Gao H, Li S, Xie L. Perioperative treatment and prognostic factors for penetrating keratoplasty in *Acanthamoeba* keratitis unresponsive to medical treatment. Graefes Arch Clin Exp Ophthalmol. 2009 May 9.

Shrestha NK, Khanal B, Sharma SK, Dhakal SS, Kanungo R., Primary amoebic meningoencephalitis in a patient with systemic lupus erythematosus.; Scand J Infect Dis. 2003;35(8):514-6.

Silberman JD, Clark CG, Diamond LS, Sogin ML. Phylogeny of the genera Entamoeba and Endolimax as deduced from small-subunit ribosomal RNA sequences. Mol Biol Evol. 1999 Dec;16(12):1740-51.

Singhal T, Bajpai A, Kalra V, Kabra SK, Samantaray JC, Satpathy G, Gupta AK.

Successful treatment of *Acanthamoeba* meningitis with combination oral antimicrobials. Pediatr Infect Dis J. 2001 Jun;20(6):623-7.

Soldati G, Lu ZH, Vaughan L, Polkinghorne A, Zimmermann DR, Huder JB, Pospischil A. Detection of mycobacteria and chlamydiae in granulomatous inflammation of reptiles: a retrospective study. Vet Pathol. 2004 Jul;41(4):388-97.

Som I, Azam A, Bhattacharya A, Bhattacharya S. Inter- and intra-strain variation in the 5.8S ribosomal RNA and internal transcribed spacer sequences of *Entamoeba histolytica* and comparison with *Entamoeba dispar*, *Entamoeba moshkovski*i and *Entamoeba invadens*. Int J Parasitol. 2000 May;30(6):723-8.

Spanakos G, Tzanetou K, Miltsakakis D, Patsoula E, Malamou-Lada E, Vakalis NC. Genotyping of pathogenic Acanthamoebae isolated from clinical samples in Greece--report of a clinical isolate presenting T5 genotype. Parasitol Int. 2006 Jun;55(2):147-9.

Stechmann A, Cavalier-Smith T. Rooting the eukaryote tree by using a derived gene fusion. Science. 2002 Jul 5;297(5578):89-91.

Stothard DR, Schroeder-Diedrich JM, Awwad MH, Gast RJ, Ledee DR, Rodriguez-Zaragoza S, Dean CL, Fuerst PA, Byers TJ. The evolutionary history of the genus *Acanthamoeba* and the identification of eight new 18S rRNA gene sequence types. J Eukaryot Microbiol. 1998 Jan-Feb;45(1):45-54.

Tavares M, Correia da Costa JM, Carpenter SS, Santos LA, Afonso C, Aguiar A, Pereira J, Cardoso AI, Schuster FL, Yagi S, Sriram R, Visvesvara GS. Diagnosis of first case of *Balamuthia* amoebic encephalitis in Portugal by immunofluorescence and PCR. J Clin Microbiol. 2006 Jul;44(7):2660-3.

Tawari B, Ali IK, Scott C, Quail MA, Berriman M, Hall N, Clark CG. Patterns of evolution in the unique tRNA gene arrays of the genus Entamoeba. Mol Biol Evol. 2008 Jan;25(1):187-98. Epub 2007 Nov 1.

Thebpatiphat N, Hammersmith KM, Rocha FN, Rapuano CJ, Ayres BD, Laibson PR, Eagle RC Jr, Cohen *Acanthamoeba* keratitis: a parasite on the rise. EJ.Cornea. 2007 Jul;26(6):701-6.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgings DG. (1997): The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleid Acids Res. 24:4876-4882.

Veraldi S, Bottini S, Carrera C, Gianotti R. Cutaneous larva migrans with folliculitis: a new clinical presentation of this infestation. J Eur Acad Dermatol Venereol. 2005 Sep;19(5):628-30.

Vilcek S, Elvander M, Ballagi-Pordány A, Belák S. Development of nested PCR assays for detection of bovine respiratory syncytial virus in clinical samples. J Clin Microbiol. 1994 Sep;32(9):2225-31. Erratum in: J Clin Microbiol 1994 Nov;32(11):2887.

Visvesvara GS, Balamuth W. Comparative studies on related free-living and pathogenic amebae with special reference to *Acanthamoeba*. J Protozool. 1975 May;22(2):245-56.

Visvesvara GS, Martinez AJ, Schuster FL, Leitch GJ, Wallace SV, Sawyer TK, Anderson M. Leptomyxid ameba, a new agent of amebic meningoencephalitis in humans and animals. J Clin Microbiol. 1990 Dec;28(12):2750-6. Review.

Visvesvara GS, Stehr- Green JK Epidemiology of free- living amoeba infection. J. Protozool 1990, 37: 25S- 33S.

Visvesvara GS, Schuster FL, Martinez AJ. *Balamuthia mandrillaris*, N. G., N. Sp., agent of amebic meningoencephalitis in humans and other animals. J Eukaryot Microbiol. 1993 Jul-Aug;40(4):504-14.

Visvesvara GS, De Jonckheere JF, Sriram R, Daft B. Isolation and molecular typing of *Naegleria fowleri* from the brain of a cow that died of primary amebic meningoencephalitis. J Clin Microbiol. 2005 Aug;43(8):4203-4.

Visvesvara GS, De Jonckheere JF, Marciano-Cabral F, Schuster FL. Morphologic and molecular identification of *Naegleria dunnebackei* n. sp. isolated from a water sample. J Eukaryot Microbiol. 2005 Nov-Dec;52(6):523-31.

Visvesvara GS, Booton GC, Kelley DJ, Fuerst P, Sriram R, Finkelstein A, Garner MM. In vitro culture, serologic and molecular analysis of *Acanthamoeba* isolated from the liver of a keel-billed toucan (*Ramphastos sulfuratus*). Vet Parasitol. 2007 Jan 19;143(1):74-8. Epub 2006 Sep 8.

Visvesvara GS, Moura H, Schuster FL. Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. FEMS Immunol Med Microbiol. 2007 Jun;50(1):1-26. Epub 2007 Apr 11. Review.

Walia R, Montoya JG, Visvesvera GS, Booton GC, Doyle RL. A case of successful treatment of cutaneous *Acanthamoeba* infection in a lung transplant recipient. Transpl Infect Dis. 2007 Mar;9(1):51-4.

Walochnik J, Hassl A, Simon K, Benyr G, Aspöck H. Isolation and identification by partial sequencing of the 18S ribosomal gene of free-living amoebae from necrotic tissue of *Basiliscus plumifrons* (Sauria: Iguanidae). Parasitol Res. 1999 Jul;85(7):601-3.

Walochnik J, Haller-Schober E, Kölli H, Picher O, Obwaller A, Aspöck H. Discrimination between clinically relevant and nonrelevant Acanthamoeba strains isolated from contact lens-wearing keratitis patients in Austria. J Clin Microbiol. 2000 Nov;38(11):3932-6.

Walochnik J., Aspöck H., Amöben, Bandwürmer, Zecken...Parasiten und parasitäre Erkrankungen des Menschen in Mitteleuropa. – Denisia **6**, und Kat.OÖ. Landesmuseums, N.F.**186**, 600 pp. (2002)

Walochnik J, Aspöck H., Die Diagnostik von Infektionen mit freilebenden Amöben (FLA) Diagnostics of Infections with free-living amoebae (FLA). LaboratoriumsMedizin. Band 29, Heft 6, Seiten 446–456, (2005)

Walochnik J, Aspöck H, Die Parasiten des Menschen aus der Sicht der Koevolution - Denisia 20, zugleich Kataloge der Oberösterreichischen Landesmuseen, pp. 323ff (2007)

Walochnik J, Aichelburg A, Assadian O, Steuer A, Visvesvara G, Vetter N, Aspöck H. Granulomatous amoebic encephalitis caused by *Acanthamoeba* amoebae of genotype T2 in a human immunodeficiency virus-negative patient. J Clin Microbiol. 2008 Jan;46(1):338-40.

Westmoreland SV, Rosen J, MacKey J, Romsey C, Xia DL, Visvesvera GS, Mansfield KG. Necrotizing meningoencephalitis and pneumonitis in a simian immunodeficiency virus-infected rhesus macaque due to *Acanthamoeba*. Vet Pathol. 2004 Jul;41(4):398-404.

Yagi S, Booton GC, Visvesvara GS, Schuster FL. Detection of *Balamuthia* mitochondrial 16S rRNA gene DNA in clinical specimens by PCR. J Clin Microbiol. 2005 Jul;43(7):3192-7.

Yeruham I, Perl S. Dermatitis in a dairy herd caused by *Pelodera strongyloides* (Nematoda: Rhabditidae). J Vet Med B Infect Dis Vet Public Health. 2005 May;52(4):197-8.

Zhou L, Sriram R, Visvesvara GS, Xiao L. Genetic variations in the internal transcribed spacer and mitochondrial small subunit rRNA gene of *Naegleria* spp.
J Eukaryot Microbiol. 2003;50 Suppl:522-6.

8 APPENDIX

8.1 Abstract

Molekularbiologischer Nachweis humanpathogener Amöben in Reptilien

Reptilien können verschiedene Amöbenspezies beherbergen, die als potentielle Pathogene auftreten können. Im Menschen können diese Protozoen mitunter tödliche Erkrankungen verursachen. Das Ziel dieser Studie war, gesunde Tiere des Reptilienbestand eines Tiergartens auf *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri* sowie *Entamoeba invadens* zu untersuchen.

Acanthamoeba spp*., B. mandrillaris* und *N. fowleri* gehören zu den frei lebenden Amöben (FLA). Unter diesem Begriff werden Organismen zusammengefasst, die zwar nicht näher miteinander verwandt sind, die aber durchwegs potentiell pathogen sind, obwohl sie nicht auf einen Wirt angewiesen sind.

Zu den Erkrankungen, die von Akanthamöben verursacht werden, zählen die *Acanthamoeba*-Keratitis, sowie die in Immunsupprimierten auftretende Granulomatöse Amöbenenzephalitis (GAE). Auch die Infektion mit *B. mandrillaris* führt zu einer GAE, allerdings sind hier auch immunkompetenten Personen betroffen. *N. fowleri*-Infektionen betreffen vor allem Kinder und die Infektion erfolgt meist beim Baden. Die von den Näglerien hervorgerufene Primäre Amöbenmeningoenzephalitis (PAME) führt unbehandelt innerhalb weniger Tage zum Tod. Auch Tiere können von Infektionen mit FLA betroffen sein und mitunter schwer bzw. tödlich erkranken. Aus der Literatur ist eine ganze Reihe von Infektionen bei Tieren bekannt.

Entamoeba invadens ist ein anaerober Einzeller, der mitunter tödliche Amöbosen in Reptilien verursachen kann. Aus der Literatur bekannte Berichte von *E. invadens*-Infektionen in Reptilien beziehen sich vorwiegend auf kranke bzw. verstorbene Tiere, deren Infektionen häufig erst bei der Sektion der Tiere festgestellt wurden. Die Durchseuchungsrate lebender und vor allem asymptomatischer Reptilien ist allerdings weitgehend unbekannt.

Im Zuge von sechs Beprobungen wurden Maul- und Kloakenabstriche von insgesamt 54 Reptilien (17 Schlangen, 20 Schildkröten und 17 Echsen) genommen. Die Auswertung der Proben erfolgte parallel in Kultur (aerobe auf Agarplatten/ anaerobe in Flüssigkultur) und mittels PCR. Zusätzlich wurden vereinzelt Stuhl- und Gewebeproben auf das Vorkommen von Amöben untersucht.
Außerdem wurde im Rahmen dieses Projektes ein schon bestehendes PCR Protokoll zum Nachweis von *Acanthamoeba* spp. zu einer Nested-PCR modifiziert, sowie zwei neue PCR-Systeme zum Nachweis von *B. mandrillaris* und *E. invadens* etabliert.

Insgesamt waren 64,8 % der Reptilien positiv auf *Acanthamoeba* spp. und auch im Fall von *E. invadens* konnte mittels PCR eine hohe Befallsrate bestimmt werden, da sich 53,7 % der Reptilien als positiv erwiesen. Mit Ausnahme einer Schlange ("Boa Ziehharmonika-Krankheit") waren alle Reptilien völlig asymptomatisch. In der Kultur erwiesen sich 20,3% der beprobten Reptilien als *Acanthamoeba* spp. positiv. Im Zuge dieser Studie konnte weder *B. mandrillaris* noch *N. fowleri* mittels Kultur oder PCR nachgewiesen werden. Auch im Fall von *E. invadens* war die Kultur negativ. Dies stimmt mit den Ergebnissen aus der Literatur überein, da Infektionen mit diesen Organismen in Tieren und Menschen überaus selten sind. Die hohe Infektionsrate mit *Acanthamoeba* spp. war aufgrund der ubiquitären Verbreitung von *Acanthamoeba* spp. zu erwarten, im Gegensatz zu der hohen Infektionsrate mit *E. invadens,* da dieser Organismus vorwiegend in erkrankten Tieren und nicht in asymptomatischen Tieren vorkommt.

Die ubiquitäre Verbreitung von *Acanthamoeba* spp. stellt ein potentielles Infektionsrisiko vor allem für immunsupprimierten Menschen dar, während *E. invadens* ein gesundheitliches Risiko für Reptilien darstellt. Dies betrifft vor allem Tiere die Stresssituationen ausgesetzt sind, wie sie zum Beispiel in einem Zoo gegeben sind. Die Ergebnisse dieser Studie zeigen, dass ein regelmäßiges Screening des Tierbestandes sinnvoll wäre, um das Infektionsrisiko für Tiere, Zooangestellte und Zoobesucher zu minimieren.

Abstract

Amoebae of medical relevance in reptiles

Establishment of a PCR detection system for parasitic amoebae

Reptiles have been reported to harbor several potentially pathogenic amoebae, which can be of medical relevance for humans and animals.

Acanthamoeba spp., *B. mandrillaris* and *N. fowleri* are summarized within the term "free-living amoebas" (FLA), which comprises amoebae that are not necessarily closely related to each other: However, they all are potentially pathogenic, although they not depend on a host. Infections caused by *Acanthamoeba* spp. are *Acanthamoeba* keratitis on the one hand and Granulomatous amoebic encephalitis (GAE), a severe disease in immunosuppressed humans on the other hand. Also *B. mandrillaris* can cause GAE, but these cases are usually independent from the immune status of the patient. Primary amoebic meningoencephalitis (PAME), which leads to death within a few days without treatment, is caused by *N. fowleri* and generally affects children with a history of swimming. Additionally, several authors reported that FLAs can also cause severe and deadly diseases in animals.

Entamoeba invadens is an anaerobic organism that can cause severe and often fatal amoebosis in reptiles. Several *E. invadens* infections in cold-blooded animals have been reported to date, but usually these cases were described from diseased or dead animals. Therefore, these amoebosis were detected during the dissection of the affected animals. However, the infection rates in living and asymptomatic reptiles are largely unknown.

The aim of this study was the screening of asymptomatic reptiles of a zoological collection for the occurrence of FLA *Acanthamoeba*,spp., *B. mandrillaris* and *Neagleria. fowleri* and *Entamoeba invadens*.

During this study, in the course of six sample drawings, mouth and cloacal swabs from 54 reptiles (17 snakes, 20 chelonians and 17 lizards) were collected. These samples were investigated both by culture (aerobic culture on agar plates / anaerobic culture in

liquid medium) and PCR for the occurrence of FLA and *E. invadens*. Additionally, in a few cases, stool and tissue samples were investigated. For molecular analysis a standard PCR protocol for the detection of *Acanthamoeba* spp. was modified to a nested PCR and two new PCR protocols for the detection of *B. mandrillaris* and *E. invadens* were established.

Our results showed that 64.8% of the investigated reptiles turned out to be *Acanthamoeba* spp. positive and 53.7% *E. invadens* positive in PCR. Cultivation of swab samples led to the isolation of *Acanthamoeba* spp. from 20.3 % of the investigated reptiles. Neither *B. mandrillaris* nor *N. fowleri* was detected and isolated, which is concordant with the low number of cases in humans and animals, which are rare, corroborates this. Furthermore, no *E. invadens* was isolated from mouth and cloacal swabs. With the exception of one snake (*Boa* accordion disease") all reptiles appeared be healthy and without any clinical symptoms.
The high infection rates with *Acanthamoeba* spp. can be explained by the high ubiquity of acanthamoebae. In contrast, the high infection rates with *E. invadens* were unexpected, because this organism occurs predominantly in diseased animals instead of apparently healthy and asymptomatic animals.

The high occurrence of *Acanthamoeba* spp. constitutes a potential risk for humans, particularly immunocompromised people, while high numbers of *E. invadens* pose a serious threat to reptiles, particularly under stress as in case of captivity. Our results clear indicate that a periodical screening of the livestock in zoological collections would be meaningful to minimize the health risk for animals, keepers and visitors.

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8.3 CURRICULUM VITAE

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Attendance at national congresses:

5. June 2009: "Parasitological expert discussions" at the Department of Medical Parasitology, Clinical Institute of Hygiene and Medical Microbiology, Medical University of Vienna.

20.- 22 Nov. 2008: XXXXII Conference of the ÖGTP in Innsbruck/ Austria*

22.- 24. Nov. 2007: XXXXI Conference of the ÖGTP in Vienna/ Austria

24 May 2006: "Helminthological expert discussions" at the Natural History Museum in Vienna/ Austria.

*With oral presentation of current studies