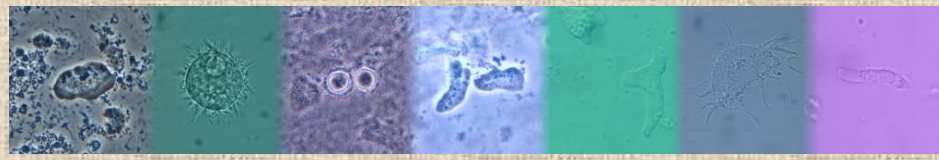

# 15th International Meeting on the Biology and Pathogenicity of Free-Living Amoebae

# Abstract booklet

**July 14th to 19th, 2013**

Vienna, Austria

## Main Organisers

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Fiona Henriquez – Glasgow, UK
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Albrecht Kiderlen – Berlin, Germany
Jacob Lorenzo-Morales – La Laguna, Spain
Sutherland McCiver – Edinburgh, UK
Norbert Müller – Berne, Switzerland
Craig Roberts – Glasgow, UK

# Contents

# Program

| Monday | Tuesday | Wednesday | Thursday | Friday | Saturday | Sunday |
|---|---|---|---|---|---|---|
| | | | | | | 14.7.2013 |
| | | | | | | 19:30<br>welcome at the NHM |
| 15.7.2013 | 16.7.2013 | 17.7.2013 | 18.7.2013 | 19.7.2013 | | |
| 9:00-10:30<br>**key note lecture I**<br>Brendan Loftus:<br>„The genome of Acanthamoeba“ | 8:20-18:00<br>**excursion** | 9:00-10:30<br>**key note lecture II**<br>Johan De Jonckheere: “What do we know by now about Naegleria?” | 9:00-10:30<br>**key note lecture III**<br>Alexey Smirnov:<br>“Diversity, phylogeny and systematics of lobose amoebae” | 9:00-10:30<br>molecular biology and immunology | | |
| | | **coffee break** | | | | |
| 11:00-12:30<br>ecology & distribution | | 11:00-12:30<br>diagnostics & disinfection | 11:00-12:30<br>cell biology | 11:00-12:30<br>history & closing session | | |
| | | **lunch break** | | | | |
| 14:00-15:30<br>interactions with bacteria | | 14:00-15:00<br>poster session I<br>**A-L** | 14:00-15:00<br>poster session II<br>**M-Z** | | | |
| | | **coffee break** | | | | |
| 16:00-17:30<br>interactions with other cells | | 15:30-17:30<br>therapy | 15:30-17:30<br>virulence factors | | | |
| | | | 19:00<br>roof-top party at the hotel | | | |

# PROGRAM DETAILS

## Monday July 15th

### *9:00-10:30 Keynote lecture I*

**The genome of Acanthamoeba**

Brendan Loftus

## Coffee break

### *11:00-12:30 Ecology & Distribution*

(chair: Sutherland McCiver)

**Soil amoebae: Identification of new species and evaluation of the community structure**

Stefan Geisen, Johan F. De Jonckheere, Alexander Kudryavtsev, Alexey Smirnov, Cédric Berney, David Bass, Michael Bonkowski

**Ecology and distribution free-living amoebae in artificial swimming pools**

Katarína Trnková, Cyril Klement

**Determination of the origin of contamination of the thermal recreational waters in Guadeloupe**

Mirna Moussa, Océane Tissot, Jérôme Guerlotte, Johan F. De Jonckheere and Antoine Talarmin

**Isolation of seven strains of Balamuthia mandrillaris recovered from an artificial lagoon and one from a soil sample in Sonora, Mexico**

Luis Fernando Lares-Jiménez,Gregory C. Booton, Fernando Lares-Villa, Carlos Arturo Velázquez-Contreras, Paul A. Fuerst

**Acanthamoeba in Thailand: I. Prevalence and Genotype Distribution of Isolates from Freshwater Fish Gills**

Chaturong Putaporntip, Warisa Nuprasert, Somchai Jongwutiwes

**Acanthamoeba in Thailand: II. Ambiguity in Genotyping Some Isolates from Freshwater Sources and Fish Gills**

Somchai Jongwutiwes, Warisa Nuprasert, Chaturong Putaporntip

# Lunch break

## *14:00-15:30 Interactions with Bacteria*

(chair: Matthias Horn)

**Predator versus Aliens: Bacteria interactions with Acanthamoeba.**

Ruqaiyyah Siddiqui, Naveed Ahmed Khan, Junaid Iqbal, Mehwish Sagheer

**Metagenomic survey of amoebae and intracellular bacteria in drinking water**

Yann Héchard, Vincent Delafont, Laurent Moulin, Amélie Brouke, Didier Bouchon

**Free-living Amoebae as training grounds for legionellae - the establishment of models for testing the infectious potential of VBNC-legionellae**

Elisabeth Dietersdorfer, Alexander Kirschner, Alexander Indra, Barbara Schrammel, Ute Scheikl, Julia Walochnik

**Infection cycle and genomic features of the Acanthamoeba symbionts Amoebophilus asiaticus and Procabacter acanthamoebae**

Han-Fei Allen Tsao, Thomas Weinmaier, Thomas Penz, Thomas Rattei, Matthias Horn

**Interaction of Acanthamoeba with Arcobacter butzleri**

Muhammad Asif, Sutherland K Maciver, Bruce Ward

# Coffee break

## *16:00-17:30 Interactions with Other Cells*

(chair: Albrecht Kiderlen)

**Isolation and characterisation of various amoebophagous fungi and evaluation of their prey spectrum among free-living amoebae**

Rolf Michel, Julia Walochnik , Patrick Scheid (presenting author)

**The Balamuthia amebas and their association with UNEX, an unexpected, previously unknown fungal cell**

T. Dunnebacke, S. Yagi, C. Glaser, M. Vollmer, D Lee, and C. Chiu

**Continuous culture of Balamuthia mandrillaris fed with Trypanosoma cruzi epimastigotes**

José Luis Tapia, Cudberto Contreras Pérez; Sergio Pasten Sánchez; Govinda S. Visvesvara

**Balamuthia mandrillaris: In Vitro Interactions with Selected Protozoa and Algae (Video)**

José Luis Tapia, Benjamín N. Torres & Govinda S. Visvesvara

**Infectivity, resistance to environmental hazards and seroprevalence of Acanthamoeba polyphaga mimivirus**

Albrecht F. Kiderlen, Lars Möller, Bettina Wedekind, Andreas Nitsche

# Wednesday July 17th

## *9:00-10:30 Keynote lecture II*

**What do we know by now about the genus Naegleria?**

Johan F. De Jonckheere

# Coffee break

## *11:00-12:30 Diagnostics & Disinfection*

(chair: Naveed Khan)

### Extracorneal amoebic spread in Acanthamoeba keratitis

Jacob Lorenzo-Morales , Francisco Arnalich-Montiel , Laia Jaumandreu , Marina Leal , Carmen Mª Martín-Navarro, Atteneri López-Arencibia , María Reyes-Batllé , Alfonso Martín Cabello Vílchez , Rogelio López-Vélez , Basilio Valladares , Enrique Martínez-Carretero , José E. Piñero

### Clinical and parasitological evaluation of sever keratitis cases suspected of amoebic aetiology in contact lens wearers

Padzik M. , Chomicz L. , Szaflik J. P. , Izdebska J. , Oledzka G. , Szaflik J.

### Genotypic analysis of Acanthamoeba isolates from clinical samples in Italy

David Di Cave, Rossella D Alfonso, Carlo D Orazi, K.A. Dussey Comlavi, Rosa Monno, Federica Berrilli

### Free-living amoeba diagnosis performances and pitfalls

Pablo Goldschmidt, Christine Chaumeil

### Disinfectants, are they effective against microorganisms?

Mihaela Cardas, Selwa Alsam

### Effectiveness of three commonly used drinking water treatments on the inactivation of Acanthamoeba sp.

Silvia Cervero-Aragó, Sara Rodríguez-Martínez, Regina Sommer, Rosa M. Araujo

# Lunch break

## *14:00-15:00 Poster session I*

(chair: Julia Walochnik)

**POSTERS A-L** (Session in lecture hall)

# Coffee break

## *15:30-17:30 Therapy*

(chairs: Jacob Lorenzo-Morales & Craig Roberts)

### In vitro efficacy of clinically available drugs against growth and viability of Acanthamoeba castellanii keratitis isolate belonging to the T4 genotype

Naveed Ahmed Khan, Abdul Mannan Baig, Junaid Iqbal, Huma Kulsoom, Ruqaiyyah Siddiqui

### In vitro activity of commercially available moxifloxacin and voriconazole eye-drops against clinical strains of Acanthamoeba

Atteneri López-Arencibia , Carmen Mª Martín-Navarro, Francisco Arnalich-Montiel, María Reyes-Batllé, Alfonso Martín Cabello Vílchez, Basilio Valladares, José E. Piñero, Jacob Lorenzo-Morales

### Eye drops commonly used to treat bacterial keratitis have active ingredients and excipients capable of inhibiting Acanthamoeba

Jeehan Alestad, Craig W. Roberts, L. Henriquez and Kanna Ramesh

### Assessment of Tunisian olive leaf extracts against Acanthamoeba spp.

Ines Sifaoui , Atteneri López-Arencibia , Carmen Mª Martín-Navarro, Nadia Chammem, Mondher Mejri, Jacob Lorenzo-Morales, Manef Abderabba and José E. Piñero

### Enhanced siRNA-based therapy using Immunostimulating Complexes (ISCOM): Evidences of improved in vitro therapeutic effects against clinical strains of Acanthamoeba

Jacob Lorenzo-Morales, Carmen Mª Martín-Navarro, Teresa Cruz-Bustos, Atteneri López-Arencibia, María Reyes-Batllé, Julia Morales-Sanfrutos, Francisco Satoyo-González, José E. Piñero, Enrique Martínez-Carretero, Sutherland K Maciver, Basilio Valladares and Antonio Osuna

### Statins: A Novel Effective Therapeutic Approach against Acanthamoeba Infections validated using siRNAs

Carmen Mª Martín-Navarro, Jacob Lorenzo-Morales, Rubén P. Machin, Atteneri López-Arencibia, María Reyes-Batllé, Alfonso Martín Cabello Vílchez, José Manuel García-Castellano , Isabel de Fuentes , Brendan Loftus , Sutherland K. Maciver , Basilio Valladares , José E. Piñero

### Targeting Lysine and Histidine biosynthesis in Acanthamoeba species as targets novel inhibitor design

Christopher A. Rice, Craig W. Roberts, Fiona L. Henriquez

### Antimicrobial effect of 5-aminolevulinc acid mediated photodynamic therapy against methicillin resistant Staphylococcus aureus, Acanthamoeba and internalised bacteria

Mihaela Cardas, Selwa Alsam

## Thursday July 18th

## *9:00-10:30 Keynote lecture III*

### Diversity, phylogeny and systematics of lobose amoebae

Alexey Smirnov

## Coffee break

## *11:00-12:30 Cell Biology*

(chair: Yann Héchard)

### A Journey to the Nucleus

Frederik Schulz, Florian Wascher, Thomas Weinmaier, Rok Kostanjsek, Matthias Horn

### Ultrastructural study of encystment and excystment of Hartmannella vermiformis

Emilie Fouque, Nathalie Quellard, Béatrice Fernandez, Marie-Cécile Trouilhe and Yann Hechard

### A glimpse into the energy metabolism in free-living and parasitic amoebae

Michael Duchêne

### Biochemical characterization of the programmed cell death in Naegleria fowleri and Naegleria gruberi

Roberto Cardenas Zuñiga, Angelica Silva Olivares, Jesus Serrano Luna, Víctor Tsutsumi, Mineko Shibayama

### Emergence of amoebicidal resistance and routes to cell death in Acathamoeba castellanii

David Lloyd , A Denver Russell , James R Furr , Antoine C Hann , Neil A Turner , W Khunkitti , Simon V Avery , Christine Connell

## Lunch break

## *14:00-15:00 Poster session II*

(chair: Jacob Lorenzo-Morales)

**POSTERS M-Z** (Session in lunch hall)

## Coffee break

## *15:30-17:30 Virulence factors*

(chair: Norber Müller)

### Genomic, transcriptomic and proteomic identification of pathogenicity factors from Naegleria fowleri

Denise Corinne Zysset-Burri, Norbert Müller , Bruno Gottstein , Nadia Schürch, Matthias Wittwer

### Structure and function of acanthaporin, a pore-forming protein of pathogenic acanthamoebae

Matthias Leippe, Matthias Michalek , Frank Soennichsen , Joachim Grötzinger

### Two distinct major lysozymes in Acanthamoeba castellanii

Christoph Gelhaus, Matthias Leippe

### Re-evaluating the role of Acanthamoeba proteases in tissue invasion: observation of cytopathogenic mechanisms on MDCK cell monolayers and hamster corneal cells

Maritza Omaña-Molina, Arturo González-Robles, Lizbeth Iliana Salazar-Villatoro, Jacob Lorenzo-Morales, Ana Ruth Cristóbal-Ramos, Verónica Ivonne Hernández-Ramírez, Patricia Talamás-Rohana, René Méndez Cruz, Adolfo Martínez-Palomo

### Evidence for MyD88-dependent, TRIF-independent activation of Toll-Like Receptors by Acanthamoeba castellanii using human and murine systems

Antonella Cano, Fiona L. Henriquez, Manuela Sanna, James Alexander, Craig W. Roberts, Antonella Mattana

## *19:00 Roof-top party at the hotel*

# Friday July 19th

## *9:00-10:30 Molecular biology and Immunology*

(chair: Fiona Henriquez)

### The phylogenetic structure of genus Acanthamoeba based on sequences of the nuclear small subunit ribosomal RNA gene – an update to 2013

Paul A. Fuerst

### Protein profiles and immunoreactivities of different Acanthamoeba genotypes

Wilawan Pumidonming, Martina Koehsler, David Leitsch, Julia Walochnik

### Vaccination with lentiviral vector expressing nfa1 gene confers protective immune response to mice infected with Naegleria fowleri

Jong-Hyun Kim, Hae-Jin Sohn, Jinyoung Lee, Hee-Jong Yang, Moon-Hee Shim, Hee-Kyoung Kang, Ho-Joon Shin

**Naegleria fowleri: Production of MUC5AC and pro-inflammatory cytokines in mucoepithelial cells via TLR2 and TLR4**

Moises Martinez-Castillo, Karla Gil-Becerril, Jesus Serrano-Luna, Victor Tsutsumi, Mineko Shibayama

# Coffee break

## *11:00-12:30 History & Closing session*

**Old and new amoebae**

Julia Walochnik

## *12:30 End of Meeting*

# Abstracts

## Keynote lectures

(in alphabetical order)

**Notes:**

## What do we know by now about the genus Naegleria?

**Johan F. De Jonckheere**
de Duve Institute & Scientific Institute of Public Health, Brussels, Belgium

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The genus Naegleria was established in 1912, a century ago. Although it was an interesting organism because it can transform very rapidly from an amoeba into a flagellate form, N. gruberi was studied in only a few laboratories for many decades.
The genus attracted much more attention when it was found in 1968 that a Naegleria species could provoke a deadly disease in man, primary amoebic meningoencephalitis. As a result the new species N. fowleri was described in 1970. Because Naegleria is a free-living organism, also the pathogenic one, efforts were made to isolate N. fowleri from the environment, in order to know where people could become infected. Apart from N. fowleri, many Naegleria strains were isolated which were not the pathogenic species, and for convenience all these nonpathogenic strains were originally put into the gruberi species. It became clear very soon that N. gruberi was a species complex, with many cryptic species. Due to molecular techniques it became possible to differentiate them easily and at this moment the genus Naegleria consists of 47 different species. They are all characterized by a typical internal transcribed spacer (ITS) length and sequence. There are a few other Naegleria spp. which are pathogenic in experimental animals, but no human cases caused by these have been detected yet.
The ITS1 and 5.8S rDNA sequences also showed that N. fowleri is not a homogeneous species and 8 different types can be discerned. These types have an uneven distribution in the world and a possible evolution and dispersal route has been proposed.
Recently the total genome of N. gruberi has been determined and there are some efforts to obtain the total genome sequence of N. fowleri. But it would be more worthwhile to compare the total genome of N. fowleri to that of N. lovaniensis, its closest relative, in order to understand what makes N. fowleri pathogenic, while N. lovaniensis remains harmless to man. From the total genome a number of deductions have been made on the biochemistry of N. gruberi, some of which seem to contradict what we know about this amoeboflagellate. Some of these predictions are now being tested in the laboratory.

**Notes:**

## The genome of Acanthamoeba

**Brendan Loftus**
School Of Medicine & Medical Science, Conway Institute, Belfield, Dublin

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Acanthamoeba spp has long occupied a position of significant interest for both biomedical and evolutionary biologists existing at the intersection between human pathogen and environmental host. Interrogation of the genome sequence of Acanthamoeba castellanii offers clues as to the molecular basis underlying this dichotomy and offers an opportunity to study the effect of environmental pressures on the evolution of some intracellular pathogens. The A. castellanii genome is the first from a free-living solitary member of the Amoebozoa, one of the major subdivisions of the eukaryote. Comparison with the multicellular Dictyostelids highlights the emergence of certain features associated with metazoan multi-cellularity.

**Notes:**

## Diversity, phylogeny and systematics of lobose amoebae

**Alexey Smirnov**
Saint Petersburg State University

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Under the term “a lobose amoeba” we commonly understand an amoeboid protist, forming pseudopodia of “lobose” type and possessing predominantly acto-myosin cytoskeleton. Naked lobose amoebae are relatively small but very diverse group of protists(no more than 300 well-known species); however these organisms do not form a monophyletic group in the phylogenetic tree, being distributed among several phyloge¬netic branches of Amoebozoa. Those are probably monophyletic Tubulinea and Variosea and paraphyletic (or polyphyl¬etic) Discosea, unifying a number of relatively independent lineages. Modern system of amoebae is based on the combination of morphological and molecular characters and considerably differs from solely morphology - based systems; many morphological characters were re-evaluated or shown to be taxonomically non-significant with the development of molecular systematics. Morphospecies of amoebae most probably are widely distributed and represent complexes of genotypes showing relatively high polymorphism of studied genes even within a single genome. This complicates molecular species identification and in many cases requires complex studies of isolates to assign them reliably to a known species or recognize them as new ones. Gene polymorphism embarrasses molecular ecological studies of amoebae and makes difficult interpretation of phylogenetic trees based on (or including many of) environmental DNA sequences. Primary tar¬gets in amoebae studies remain (1) isolation, iden¬tification and sequencing of amoeba species belonging to the clades, poorly sampled or missing in the phylogenetic trees; (2) studies of the genetic structure of amoebae morphospecies and related problem of DNA barcoding of amoeba species and (3) obtaining reliable data on the amoebae species distribution patterns in local- and global- scale.

# Abstracts

## Oral Presentations

(in alphabetical order)

**Notes:**

## Eye drops commonly used to treat bacterial keratitis have active ingredients and excipients capable of inhibiting Acanthamoeba

**Jeehan Alestad (1), Fiona L. Henriquez (2), David Lockington (3), Kanna Ramesh (3) and Craig W. Roberts (1)**
(1) Strathclyde Institute of Pharmacy & Biomedical Sciences, University of Strathclyde, (2) Institute of Biomedical and Environmental Health Research, School of Science, University of the West of Scotland, (3) Tennent Institute of Ophthalmology, Gartnavel General Hospital, Glasgow

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The early symptoms of Acanthamoeba keratitis are similar to bacterial, fungal and viral keratitis. As Acanthamoeba keratitis is relatively rare and treatment is arduous, patients presenting with keratitis are normally treated with eye drops designed to treat the more common causes, such as bacteria. The potential of some of the agents used in these antibacterial formulations to affect Acanthamoeba has not been extensively studied. Herein, we demonstrate that some of the commonly used excipients and active ingredients of eye drops generally prescribed for suspected bacterial keratitis are active against Acanthamoeba castellanii. These findings have significant implications for clinical practice and the epidemiology of Acanthamoeba keratitis. Thus, the results suggest that the specific formulations of antibacterial eye drops that have some efficacy against Acanthamoeba should be preferred over other formulations, where the etiology of keratitis is not definitive and could be due to Acanthamoeba. Furthermore, the relatively common use of these formulations, with the potential to inadvertently treat Acanthamoeba keratitis, raises the possibility that the incidence of Acanthamoeba keratitis might be higher than previously reported.

**Notes:**

## Interaction of Acanthamoeba with Arcobacter butzleri

**Muhammad Asif, Sutherland K Maciver, Bruce Ward**
University of Edinburgh

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The soil amoebae of the genus Acanthamoebae are abundant and have a worldwide distribution. Some bacteria have evolved mechanisms to evade killing by Acanthamoeba using strategies like toxins secretion and avoiding lysosomal degradation. The human pathogen Legionella have ability to proliferate and eventually kill the Acanthamoeba cell. Free-living Acanthamoeba serve as training grounds for the environmental bacteria resulting in their better survival in the environment, resistance to antibacterial substances and increased virulence. The purpose of our research is to study the interaction of Acanthamoeba with Arcobacter butzleri. The interaction was monitored in a variety of experimental conditions. These included co-culture experiments (on agar plates with overlaid dead/live bacteria and in axenic cultures), gentamicin protection assay (both in plates and suspension), plaque assay, interaction in more natural condition using Acanthamoeba (Neff) adapted to feed on bacteria instead of axenically grown, direct visualization using time-lapse microscopy technique and interaction with other isolates of Acanthamoeba. The results of the experiments showed that A. butzleri are quickly internalized and can be seen inside the vacuoles within minutes after bacteria are added to the Acanthamoeba. The uptake of A. butzleri is in general following a kind of “capping” phenomenon whereby bacteria aggregate the Acanthamoeba surface at the uroid. A. butzleri seem to infect the Acanthamoeba successfully and even proliferate during the initial few hours with eventual killing and rupture of some of the host cells. However, they are unable to maintain this for longer and start declining until 24-48h after which although they have been detected to persist for up to seven days but at a very low number. Re-infection experiments are under way whereby bacteria recovered from one infection are used for the second infection. Initial results suggest that these bacteria have increased virulence.

**Notes:**

## Evidence for MyD88-dependent, TRIF-independent activation of Toll-Like Receptors by Acanthamoeba castellanii using human and murine systems

**Antonella Cano (1,2), Fiona L. Henriquez (3), Manuela Sanna (2), James Alexander (1), Craig W. Roberts(1), Antonella Mattana (2)**
(1) Strathclyde Institute of Pharmacy & Biomedical Sciences, University of Strathclyde Glasgow UK, (2) Department of Biomedical Sciences, University of Sassari, Sassari Italy, (3) School of Science, University of the West of Scotland Paisley UK

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Acanthamoeba spp. are opportunistic, facultative parasites that can cause a severe potentially blinding keratitis and a fatal encephalitis in humans. Toll-Like Receptors (TLRs) are pattern recognition receptors (PRR) expressed on innate immune cells, including macrophages that are known to participate in the immune response to Acanthamoeba. The aim of our study was to elucidate the role of TLRs on monocytes/macrophages, in the recognition and response to Acanthamoeba castellanii. For this purpose, both human and murine models have been used, combining the advantages of these two different systems. Results demonstrate that the release of pro-inflammatory cytokines by murine macrophages after challenge with Acanthamoeba is MyD88 dependent and TRIF independent. In human monocytes cytokine production stimulated by trophozoites is ablated by blocking TLR4. In contrast, blocking TLR2 ablates cytokine production to molecules released by Acanthamoeba. Taken together our study indicates that TLRs and their associated signalling pathways play important roles in Acanthamoeba infections, in both human and murine systems.

**Notes:**

## Disinfectants, are they effective against microorganisms?

**Mihaela Cardas, Selwa Alsam**
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Staphylococcus aureus is a leading cause of nosocomial infections which can lead to serious life-threatening diseases. The genus Acanthamoeba, represent an important reservoir for a range of pathogenic bacterial strains, particularly when in the encysted state, as the cyst architecture can protect the internalised bacteria from biocidal activity. Consequently, this study aimed to investigate the effectiveness of five disinfectants currently in use in UK hospitals, namely, 10% actichlor, 70% ethanol, 1% virkon, 5% biocleanse and hand sanitizer, on methicillin-sensitive S. aureus (MSSA), methicillin-resistant S. aureus (MRSA) and S. epidermidis (SE) internalised within trophozoites and cysts of the T4 pathogenic Acanthamoeba castellanii isolate and the non-pathogenic T7 Acanthamoeba astronyxis isolate. Internalised MRSA, MSSA and SE were incubated for different time intervals in the presence of the named disinfectants. The findings revealed that T4 pathogenic trophozoites and cysts protect MRSA, MSSA and SE against disinfectants more effectively than the non-pathogenic T7. In addition, the intracellular, antibiotic, multi-resistant MRSA withstands exposure to these disinfectants more readily than either the intracellular, antibiotic-sensitive MSSA, or the avirulent SE. Interestingly, 10% actichlor, 5% biocleanse and 1% virkon inactivated intracellular bacteria after 10 minutes of exposure, compared with 70% ethanol and hand sanitizer that were only effective against intracellular bacteria after 5 h of exposure. Given that Acanthamoeba cysts are ubiquitous in the environment and protect internalised bacteria against the activity of disinfectants, our findings underline the capacity of Acanthamoeba cysts to equally act as “Trojan horse” in spreading MRSA and MSSA to susceptible human hosts.

**Notes:**

## Antimicrobial effect of 5-aminolevulinc acid mediated photodynamic therapy against methicillin resistant Staphylococcus aureus, Acanthamoeba and internalised bacteria

**Mihaela Cardas, Selwa Alsam**
University of Essex

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Due to the rise in the number of antibiotic multi-resistant pathogens, there is increasing interest into the potential of photodynamic therapy (PDT) as a novel method for treating microbial infections. PDT involves the activation of photosensitizers, following exposure to specific light waves. This activation leads to the generation of singlet oxygen and free radicals that cause lethal oxidative damage to strategic biological targets. Therefore, the aim of this study was to evaluate the effectiveness of 5-ALA mediated PDT on methicillin resistant S. aureus (MRSA), Acanthamoeba trophozoites, and amoebae containing internalised bacteria. MRSA, Acanthamoeba trophozoites and amoebae with internalised bacteria, were incubated with various concentrations of 5-ALA, followed by exposure to light for 30 min. The results showed that 5-ALA mediated PDT reduces Acanthamoeba trophozoites viability by 60% and MRSA viability by 20%, as compared with controls, which were maintained in the dark. Interestingly, in percentage terms, the number of MRSA that survived within trophozoites, mirrored the percentage determined for MRSA cultured under axenic conditions. Overall, the data suggest that PDT may well be a viable therapeutic option to combat infections caused by Staphylococcus and Acanthamoeba.

**Notes:**

## BIOCHEMICAL CHARACTERIZATION OF THE PROGRAMMED CELL DEATH IN Naegleria fowleri AND Naegleria gruberi

**Roberto Cardenas Zuñiga (1), Angelica Silva Olivares (1), Jesus Serrano Luna (2), Víctor Tsutsumi (1), Mineko Shibayama (1)**
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Naegleria fowleri is an amoeboflagellate that belongs to the group of free-living amoebae and is the etiologic agent of a fulminant infection of the central nervous system called primary amoebic meningoencephalitis (PAM). Presently, non- specific drug is available for the treatment of this disease, although amphotericin B has been frequently used. However, in most cases people infected with this microorganism die in about 7 days. Therefore, in order to obtain new and more specific therapeutic drugs for the treatment of PAM, we first analyzed some of the biological machinery that could be affected during the process of cell death. The programmed cell death (PCD) is a physiologic phenomenon described in several microorganisms and is characterized by triggering a sequence of morphological and biochemical changes that start by a wide number of stimuli, like infection, membrane receptors and some drugs. The mechanisms of PCD in N. fowleri and N. gruberi trophozoites have not been described. In the present work, we analyzed in N. fowleri and N. gruberi the typical parameters described as the morphological and biochemical hallmarks of PCD. Our results showed that amphotericin B at 10µg/ml was able to induce PCD, in both free living amoebae. This was characterized by the disappearance of the typical cell morphology, with rounding and decrement in cell size, similarly to the shrinkage observed in other cell in process of PCD. The cell viability in both amoebae was decreased. We evaluated also the intracellular production of ROS, showing an increment in them. However, potassium (K+i) concentration diminished. Phosphatidylserine externalization was present. Based in these results we can conclude that AmB is able to induce morphological and biochemical changes in N. fowleri and N. gruberi that are similar to those reported during PCD in other eukaryotic cells. This work was supported by grant SEP-CONACYT number 128317.

**Notes:**

## Genotypic analysis of Acanthamoeba isolates from clinical samples in Italy

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Acanthamoeba keratitis (AK) is a corneal disease caused by members of a genus of free-living amoebae and is associated predominantly with contact lens (CL) use. This study reports 55 cases of culture-proven AK diagnosed in Italy. Genotype identification was carried out with a PCR assay based on sequence analysis of the 18S rRNA gene, and sensitivity and specificity were evaluated in comparison with traditional parasitological techniques. A 405 bp region of the 18S rRNA gene (ASA.S1) was amplified using the genus specific primers JDP1 and JDP2. Genotype assignment was based on phenetic analysis of the ASA.S1 subset of the nuclear small-subunit rRNA gene sequence excluding the highly variable DF3 region. Phylogenetic analysis was also performed on the sequences obtained.
Tha materials has been obtained from three hospitals from the city of Rome for a total of 21 isolates and from the University Hospital of Bari (34 isolates). Twelwe out of the 55 genetically characterized isolates were assigned to the T4 genotype. Nine isolates were identified as belonging to the T15 genotype. This finding represents a confirm to the first association between the T15 genotype and human amoebic keratitis previously described in the same area (Di Cave et al. 2009). We underline the occurrence of the genotype T3 (13/55) between the isolates coming from the south of the country recovered for the first time in Italy.

**Notes:**

## Effectiveness of three commonly used drinking water treatments on the inactivation of Acanthamoeba sp.

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During the last decade, the presence of Free-living amoebas (FLA) within drinking water systems as well as on faucets from domestic water systems has been reported. Although some FLA species have an intrinsic pathogenicity, its role as a vector for pathogenic bacteria like Legionella became an increased concern of health authorities. Even multiple barriers and treatments applied along the drinking water supply system resulted insufficient for the control of FLA, due to its biological characteristics. The fact that under unfavourable environmental conditions cells are able to change from trophozoite stage into a resistance form, the cysts, confer them a higher resistance towards biocides compared to other microorganisms.
The aim of this work was to study the effect of three commonly used drinking water disinfection procedures, heat treatment, chlorination and UV irradiation on two Acanthamoeba strains treating its life stages, trophozoites and cysts, separately. Amoeba inactivation was quantified by using an adapted Most Probable Number method (MPN).
The results showed that trophozoites were, as expected, more sensitive than cysts at most of the treatments conditions applied. However, differences appeared between life stages depended on the kind of treatment and strain used. When comparing the inactivation of the two Acanthamoeba strains some significant differences were also found. Thus, when studying the effectiveness of drinking water treatments, not only differences between species but also differences between strains should be taken into account.

**Notes:**

## Free-living Amoebae as training grounds for legionellae - the establishment of models for testing the infectious potential of VBNC - legionellae

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Freeliving amoebae are known to be reservoirs and vehicles for many important human pathogens like Legionella pneumophila.
Under poor nutrient conditions, L. pneumophila is not able to grow in the absence of amoebae. In some cases, like after disinfection, they can even enter the viable but non culturable (VBNC) state. If a formerly nonculturable legionella strain enters a host cell, which is normally an amoebae, it may become resuscitated and culturable again. Furthermore it has been shown, that pathogenic legionellae become more invasive for human macrophages after intracellular replication in amoebae.
Two amoeba models and one macrophage – model are being installed. The amoeba models are used as “training grounds” for legionellae.
Using these modelsystems the viability of legionellae in amoebae and the infectivity for human macrophages of VBNC legionellae (laboratory-induced and environmental) before and after passage through amoebae will be assessed.
For setting up the amoeba models the amoebae were cultured axenically and infected with Legionella pneumophila at different bacteria:amoeba ratios ranging from 10:1-200:1. The incubation was carried out under different environmental conditions and the progression of the infection was observed at different time points (6 – 48 hours) by using an inverted microscope and a phase contrast microscope. The presence of intracellular bacteria was assessed by FISH – analysis.

**Notes:**

## A glimpse into the energy metabolism in free-living and parasitic amoebae

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Earlier this year the report on the genome project of Acanthamoeba castellanii (Neff strain) was published (Clarke et al., 2013), and the new data allow to analyse genes coding for the enzymes of metabolic pathways. Here a preliminary comparison was performed between the energy metabolism of A. castellanii and Entamoeba histolytica (Loftus et al., 2005). Whereas the acanthamoebae possess mitochondria and can generate ATP molecules by oxidative phosphorylation, the entamoebae are microaerophilic organisms devoid of classical mitochondria who generate ATP mainly by glycolysis. Since glycolysis is common to both amoebae, the annotated A. castellanii protein sequences were retrieved and examined in comparison to the E. histolytica genes. It appears that both amoebae use tricks to save energy investments, A. castellanii has an ADP-dependent glucokinase that uses lower-grade ADP to phosphorylate glucose, in addition, both organisms possess a pyrophosphate-dependent phosphofructokinase. A. castellanii, not E. histolytica, also has a 2,6-phosphofructokinase with possible regulatory function.

Since both A. castellanii and E. histolytica belong to the eukaryotic supergroup Amoebozoa (Watkins and Gray, 2008), it was expected that simple protein Blast analysis would reveal similarities between the two organisms. Whereas some glycolytic enzymes had close similarity to those from Dictyostelium spp., none of them had any similarity to the E. histolytica glycolysis genes. The A. castellanii genes were sometimes grouping with bacterial, plant, mammalian, fungal or even fish genes rather than with E. histolytica. This colourful picture will certainly allow to derive potential targets for chemotherapy among the metabolic enzymes encoded in the A. castellanii genome. Moreover, it will be very interesting in the close future to obtain genome data from other Acanthamoeba spp. to see if the pathways found there are grouping more closely or whether each species represents a new world of its own.

**Notes:**

## The Balamuthia amebas and their association with UNEX, an unexpected, previously unknown fungal cell

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An unexpected, novel, fungal organism, provisionally named UNEX, as well as the ameba, Balamuthia mandrillaris, were recovered from a survivor of the usually fatal amebic encephalitis. Frozen sections prepared during the operation for the suspected brain tumor were seen to contain sheets of protozoan organisms. Investigators at both the California Encephalitis Project and CDC confirmed the amebic presence by IFA, ELISA, and PC. Recovery of the amebas was complicated by the early growth of a mass of the unusual (UNEX) cells on mammalian cultures masking their presence. The ameba and the UNEX, now separated, are cultured on feeder cells as well as in axenic media. Genomic analysis, including 18S and SRS (small ribosomal subunit) sequencing of rRNA, placed the UNEX phylogenetically within the fungal kingdom, likely in the phylum Ascomycota. However, unlike the Fungi, the UNEX cells move independently and display active pseudopodia-like processes that appear beneath their semi rigid, rounded body. Interactions between the ameba and UNEX in axenic media include ameba wrapping their pseudopodia around a quiescent UNEX crushing, then releasing it. Within the same culture, active UNEX move onto an ameba, soon joined by other UNEX where they stay until all ameba movement, and, eventually, the ameba disappear. The UNEX are the survivors. To determine whether they were actually in the patientâ€™s tissues along with the amebas, or were they a contaminant of the samples, we are evaluating an antibody produced to the UNEX cells. While still ongoing; the initial results indicate that an early product reacts specifically with the UNEX and that they are co-located with the amebas in the patient tissue. It is reasonable, at this time, to indicate that the UNEX played a role in the survival and well being of the patient who continues to be doing well.

**Notes:**

## Ultrastructural study of encystment and excystment of Hartmannella vermiformis

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Hartmannella vermiformis is a free-living amoeba which is widely distributed in the environment. It is known to colonize hot water networks and to be the reservoir of pathogenic bacteria such as Legionella pneumophila. The control of H. vermiformis in hot water networks represents an important health issue, but there is few data on H. vermiformis in the literature. It has two developmental stages: a vegetative form called trophozoite and a resistance form called cyst. In the cystic form, H. vermiformis is more resistant to disinfection treatments, so a better understanding of encystment is necessary. We investigated the encystment by light and electron microscopy on two strains of H. vermiformis, a reference strain (ATCC 50237) and an environmental strain. Kinetics were performed in Neff s encystment medium. Trophozoites had an irregular shape and had one or more pseudopodia; they contained a large contractile vacuole and numerous digestive vacuoles. During the first stage of encystment (3h), the cells rounded, becoming denser and the number of vacuoles decreased. In the second stage of encystment (6h), synthesis of cyst wall was observed and it was made of a fibrillar material. After 9h, cystic wall synthesis was completed; it was composed of two layers: the endocyst and the ectocyst. The cyst wall was smooth and there were no visible ostioles, contrary to the genera Acanthamoeba and Naegleria. Then, we investigated the process of excystment. Kinetics of excystment were performed by incubation of mature cysts in the PYNFH modified nutritive medium. During excystment, the appearance of a large autophagic vacuole was observed. Finally, trophozoites exited from a frangible zone where the cyst wall was disrupted.
This work helped to better understand the processes of differentiation in H. vermiformis, a free-living amoeba poorly described, despite its high prevalence in water networks. Besides, we performed studies to assess encystment regulation and cyst resistance to treatments.

**Notes:**

## The phylogenetic structure of genus Acanthamoeba based on sequences of the nuclear small subunit ribosomal RNA gene – an update to 2013

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The amoebae in the genus Acanthamoeba were discovered by Castellani and described by Volkonsky in 1930. Prior to biochemical or molecular approaches to systematic classification, species were described using morphology (primarily cyst structure), or cytology of nuclear division (used by Pussard and Pons, 1977). Over time, 20+ putative species were proposed based on such criteria. Molecular approaches were introduced in the 1970's (isozyme patterns), followed by DNA based RFLP analysis of mitochondrial DNA in the 1980's. Sequence information primarily from the nuclear small subunit ribosomal RNA gene (Rns) was accumulated in the
1990's to refine patterns of phylogenetic relationships. Our lab contributed seminal papers (Gast et al. 1994 and Stothard et al. 1998), setting the framework for Rns genotype groups (sequence types). Our studies identified 12 sequence types within Acanthamoeba by 1998, based on a definition describing different types when isolates showed >5% pairwise divergence between Rns sequences. In 2013, data on full or partial Rns sequences had been reported for over 1730 isolates of Acanthamoeba, representing a remarkable increase of almost 500 sequences in two years. Information from "almost complete" Rns sequences (> 2000 bases) is available for over 290 isolates, with 216 sequences classified within genotype T4 (74%), a percentage slightly greater than that of isolates with partial sequences (71%). T4 is the most common sequence type in both environmental and clinical studies. The only other sequence types with numbers exceeding 5% of the total are types T3 and T5. Since 1998, when 12 sequence types were defined, additional reports have increased the number of types to as many as 20+. However, no new type represents more than 5% of isolates except sequence type T15 (A. jacobsi). Details of
sequence types and numbers will be discussed. Variation within sequence types and the relationship between species names and sequence types will be considered in a second presentation.

**Notes:**

## Soil amoebae: Identification of new species and evaluation of the community structure

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Protozoa and especially amoebae are the major consumers of bacterial production in soil, forming the base of the heterotrophic eukaryotic food web that channels the energy flow via bacteria to higher trophic levels in the soil food web (i.e. the bacterial energy channel).
As part of the EU-project EcoFINDERS, we have been combining microscopic and molecular high-throughput sequencing techniques and determined the diversity of amoebae in different soils across Europe and in high altitude soils in Tibet. This information is essential to identify the functions of the major amoeboid taxa in soils.
Cultivation and subsequent morphological and molecular description of amoeba strains from diverse soils indicated a high level of species diversity and probable existence of new lineages corresponding to genera and even higher taxa. A detailed analysis of soils from high altitudes in Tibet revealed 25 distinct sequences within the supergroup Amoebozoa belonging to 15 species which could be distinguished morphologically. Combined morphological and phylogenetic information revealed that at least11 clones represent previously non-described species. We found a high diversity of taxa and even new genera especially in the class Variosea. Similarly, amoebae from the class Heterolobosea within the supergroup Excavata were species-rich. We could describe several new species within the genus Allovahlkampfia and even a new genus currently only known by environmental sequences.
We also investigated the diversity of soil amoebae in a 454 high-throughput sequence study with newly designed Acanthamoeba-specific primers. Within this study, ten independent soil replicates from two land-use intensities were analysed which were located at six distinct sites across Europe. On average, we obtained 1000 sequences with a large diversity of different Acanthamoeba-specific sequences at each site. These included many currently unknown ones suggesting an enormous hidden diversity even within a comparably well-described genus of amoebae.

**Notes:**

## Two distinct major lysozymes in Acanthamoeba castellanii

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Apart from the pathogenic properties of Acanthamoeba, free-living protozoa have to emerge microbial infection and must be able to feed on microbiota in soil or water. Protein extracts of cultured A. castellanii were tested for lysozymes exerting bacteriolytic activity. We isolated two different protein species with lysozyme activity by liquid chromatography, yielding homogeneous material with an apparent molecular mass of 13 kDa and 21 kDa. Both enzymes were found to be active at a mildly acidic pH by degrading bacterial cell walls of Gram-positive Micrococcus luteus. Identification of the purified proteins using mass spectrometry, followed by sequence alignment and homology modeling revealed that the major lysozymes of Acanthamoeba belong to two distinct lysozyme classes, i.e. the c-type lysozyme, mostly found in vertebrates and arthropods, and the Entamoeba-type lysozyme that represents a class of lysozymes primarily found in Entamoeba histolytica. As Acanthamoebae can be considered as relatively primitive eucaryotes the lysozymes presented here might belong to an ancient armamentarium for cellular defense and nutrition.

**Notes:**

## Free-living amoeba diagnosis performances and pitfalls

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Free-living amoeba can provoke severe multi-organ, eye, skin, and central nervous system infections. They can be spread through contact lens use, cuts or skin wounds or by being inhaled. For keratitis, the prompt and appropriate management to ensure the best visual outcome is vital: protozoa may lead to blindness without adequate treatment. However, the clinical diagnosis does not give an unequivocal indication of the causative organisms. A wide range of agents can produce similar clinical pictures and therefore, the laboratory plays here a key role. Direct microscopic examination of stained corneal smears (Giemsa pH: 7.4, Gram or fluorescence) by may confirm circa 50% of positive cases. NAATs (Nucleic Acid Amplification-based Techniques) did dramatically improve the rate of positive diagnosis. We did identify several diagnosis pitfalls most of which are related to:

**A- Quality of samples**. The material sent to the laboratory should represent the topography of protozoa replication-sites or their persistent/latent sanctuaries. Laboratory tests lack of negative predictive value if specimens contain inappropriate (reduced) amount of material. For keratitis diagnosis the samples should be obtained by deep scraping in the edge of the lesions and of the infiltrates after assessment of corneal-depth by pakimetry. Negative results from epithelial cell swabbing or tears lack of negative predictive value.

**B- Laboratory staff training**. Direct diagnosis is fastidious and the very few square-millimeters stained smears may not always be pathognomonic of the agent infecting a tissue. Because positive smears are rare in most settings, collections of positive and negative panel slides should be available for training and for further periodical diagnosis ability evaluations.

**C- Inappropriate rinsing** of fluorescein used routinely by ophthalmologists for eye surface (corneal-epithelium) integrity assessment and/or topical anesthetics introduced with the samples. Fluorophore or anesthetic (traces) introduced into the tubes with corneal samples inhibit Taq-polymerase and produce false negative PCR conclusions.

**D- Heparin or its derivatives**, antibiotics, biocides, disinfectants or preserved eye-drops. These agents may produce false negative PCRs or delay culture positivity.

**E- Inappropriate treatment of specimens**. The inadequate enzymatic or non-enzymatic cyst-lysis that may not release the DNA from the rigid cyst structures, inducing false negative conclusions.

**F- NAATs** with sub optimal specificity detecting signals triggered by other targets (false positive) or NAATs unable to detect all the variants (false negative).

**G- Stability of fluorogenic** (Taqman) labeled probes, which should be periodically validated.

**H- Primer-dimerization** or polymerization during the amplification process (unpredictable).

I- Lack of eluted DNAs stability after magnetic bead extraction (even kept at –80°C).

**J- SYBR-Green real-time PCR melting assay interpretation**. SYBR-Green, an inexpensive tool does not require probes, should produce a single integrated melt curve peak. However, nonspecific products are coamplified and many overlapping peaks may simultaneously appear. Quenching or erasing nonspecific peaks may be possible if the Tms for protozoa-target primers are artificially increased by Locked Nucleic Acids, LNA (analogues with bicyclic furanose unit locked).

In conclusion, especially for eye infections, the quality of molecular diagnosis, in addition to DNA carry-over and cross-contamination risk management, should consider and solve numerous technical pitfalls, that are responsible for false negative results.

**Notes:**

## Metagenomic survey of amoebae and intracellular bacteria in drinking water

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Free-living amoebae (FLA) feed on bacteria by phagocytosis. However, some bacterial species are able to resist phagocytosis, to survive and even sometimes to multiply within FLA. These bacteria might be pathogenic for humans mainly because they are also able to resist macrophages phagocytosis. For example, the interaction between FLA and Legionella pneumophila has been thoroughly documented. Consequently, FLA are responsible for survival and dissemination of many bacteria. In water, presence of FLA and pathogenic bacteria could lead to health concerns. However, no global study has been published to describe FLA and their intracellular bacteria in drinking water. Our goal is to describe, using a targeted metagenomic approach, FLA and associated bacteria in drinking water networks.
In our study, we have filtered water from drinking water networks and placed filters on Petri dishes allowing growth of FLA. FLA were collected at migration front and total DNA (i.e. from FLA and their intracellular bacteria) was extracted. Both amaeboal (18S rDNA) and bacterial (16s rDNA) DNA were amplified by PCR and amplicons were sequenced using pyrosequencing. The resulting sequences were analyzed to identify corresponding FLA and bacteria mainly at the genus level. Hartmannella is, by far, the most represented FLA genus. Several FLA were isolated and are currently characterized. Many bacteria were identified belonging to forty different genera. Some of them were already described but almost 50% belongs to eighteen genera which were never associated to FLA. We are now focusing our study on mycobacterium diversity.
Our results confirm that FLA are reservoirs for many bacteria, among which are pathogens or emerging pathogens. Therefore, it is important to depict this bacterial diversity by using global tools. Our approach might be used to thoroughly describe FLA and associated bacteria, in order to better understand their interactions and to evaluate their potential impact on human health.

**Notes:**

## Acanthamoeba in Thailand: II. Ambiguity in Genotyping Some Isolates from Freshwater Sources and Fish Gills

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Our previous survey of acanthamoebae from freshwater sources in diverse regions of Thailand has shown a prevalence of ~13.4% (145 positives/1085 samples examined) whereas ~20% (212 postives/1062 samples) of freshwater fish gills harbored this free-living amoeba. Analysis of the small subunit ribosomal RNA (SSU rRNA) sequences reveals that T4 is the most common genotype identified, accounting for 71% and 80% of positive samples from freshwater sources and from fish gills, respectively. Other genotypes have been detected with low frequency. Importantly, several of these isolates could not be definitely assigned to known genotypes reported so far. Based on cyst structure, these unassigned isolates belong to Pussard and Ponds' morphological group II. Meanwhile, phylogenetic analysis inferred from the SSU rRNA sequences of these unassigned genotypes spanning 2 kb has placed them into 3 distinct clusters relating with T1, T2 and T11 genotypes. However, sequence alignment of these unassigned genotypes comparing with their closely related known genotypes has shown nucleotide differences approaching or slightly greater than 5%, suggesting that they may belong to novel genotypes or may be subgenotypes within these related genotypes. Thermotolerant study based on in vitro cultivation in 1.5% non-nutrient agar overlaid with heat-inactivated Escherichia coli has shown that some of these novel isolates could grow relatively well at 40-42ºC, one of the characteristics of virulence in Acanthamoeba. Undoubtedly, acanthamoebae in environmental sources exhibit extensive genetic diversity and some of them may not be unambiguously assigned to known genotypes.

**Notes:**

## In vitro efficacy of clinically available drugs against growth and viability of Acanthamoeba castellanii keratitis isolate belonging to the T4 genotype

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The effects of clinically available drugs targeting cholinergic, muscarinic, alpha-adrenergic, dopamine, and serotonin receptors, intracellular calcium levels and/or function of calcium-dependent biochemical pathways, the ion channels and cellular pumps were tested against a keratitis isolate of Acanthamoeba castellanii belonging to the T4 genotype. In vitro growth inhibition (amoebistatic) assays were performed by incubating A. castellanii with various concentrations of drugs in the growth medium for 48 h at 30C. To determine amoebicidal effects, amoebae were incubated with drugs in phosphate buffered saline for 24 h and viability was determined using Trypan blue exclusion staining. For controls, amoebae were incubated with the solvent alone. Of the eight drugs tested, amlodipine, prochlorperazine and loperamide showed potent amoebicidal effects as no viable trophozoites were observed ( 95% kill rate), while amiodarone, digoxin, and apomorphine exhibited up to 50% amoebicidal effects. In contrast, haloperidol and procyclidine did not affect viability but inhibited A. castellanii growth. Importantly, amlodipine, prochlorperazine and loperamide showed compelling cysticidal effects. Cysticidal effects were irreversible, as cysts treated with aforementioned drugs did not re-emerge as viable amoebae upon inoculation in the growth medium. Except apomorphine and haloperidol, all tested drugs blocked trophozoites differentiation into cysts in encystation assays. Given the limited availability of effective drugs to treat amoebal infections, clinical available drugs tested in this study offer potential agents in managing keratitis and granulomatous amoebic encephalitis caused by Acanthamoeba spp. and possibly against other meningo-encephalitis-causing amoebae, such as Balamuthia mandrillaris, and Naegleria fowleri.

**Notes:**

## Infectivity, resistance to environmental hazards and seroprevalence of Acanthamoeba polyphaga mimivirus

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The Acanthamoeba polyphaga mimivirus (ApMV), a large DNA virus of the Mimiviridae family that replicates in Acanthamoeba, seems to be somehow associated with community- and hospital-acquired pneumonia. While ApMV has only rarely been detected in clinical specimens, serological studies in France and Canada indicate that patients suffering from pneumonia display a significantly increased seroprevalence for ApMV compared to controls. Comparable data from Germany are not available so far.
In an ongoing study, a fluorescence-based diagnostic method was established for determining the seroprevalence of ApMV in healthy individuals and in clinically defined risk groups. Among 350 healthy blood donors, 2.3% revealed significant levels of antibodies against ApMV.
Furthermore, biological characteristics such as infectivity and pathogenicity for different amoebae and resistance to environmental and chemical hazards were studied. While ApMV was pathogenic (lysing host cells within 3-4 days) for all Acanthamoeba species tested, it was harmless to Balamuthia mandrillaris, despite electron microscopical evidence that intact virus had been incorporated by the amoeba. We found no evidence for uptake of ApMV by mammalian cells, nor of pathogenicity.
ApMV was resistant to desiccation as well as repeated freeze-thawing and remained infectious for Acanthamoeba after more than 2 years in saline at 5°C. It was inactivated by heat >70°C and UV irradiation of 0.1 J cm-2, provided it was in a translucent environment (e.g. PBS and not PYG medium). Standard laboratory disinfectants were effective at 3 min exposure time.

Notes:

## Vaccination with lentiviral vector expressing nfa1 gene confers protective immune response to mice infected with Naegleria fowleri

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Naegleria fowleri, a pathogenic free-living amoeba, causes fatal primary amoebic meningoencephalitis (PAM) in humans and animals. The nfa1 gene (360 bp) cloned from a cDNA library of N. fowleri produces a 13.1 kDa recombinant protein, which is located on pseudopodia, particularly food-cup structure. The nfa1 gene plays an important role in the pathogenesis of N. fowleri infection. To examine the effect of nfa1 DNA vaccination against N. fowleri infection, we constructed lentiviral vector (pCDH) expressing nfa1 gene. The expression of nfa1 gene in CHO cell and human primary nasal epithelial cell transfected with the pCDH/egfp-nfa1 vector was observed by fluorescent microscopy and Western blotting analysis. For in vivo mouse study, BALB/c mice were intranasally vaccinated with viral particles of viral vector expressing nfa1 gene. To evaluate the effect of vaccination and immune responses of mice, we analysed IgG levels (IgG, IgG1, and IgG2a), cytokine induction (IL-4 and IFN-γ), and survival rate of PAM-developed mice. Both levels of IgG and IgG subclass (IgG1 and IgG2a) in vaccinated mice were significantly increased. The cytokine analysis show that vaccinated mice elicited stronger IL-4 and IFN-γ production than other control groups, suggesting a Th1/Th2 mixed type immune response. In vaccinated mice, high levels of Nfa1-specific IgG antibody were continued until 12 weeks post-vaccination. The mice vaccinated with viral vector expressing nfa1 gene also exhibited significantly higher survival rate (90%) after challenged with N. fowleri trophozoites. Finally, the nfa1 vaccination effectively induces protective immunity in N. fowleri-infected mice. These results suggest that DNA vaccination using viral vector may be potential method against N. fowleri infection.

**Notes:**

## Isolation of seven strains of Balamuthia mandrillaris recovered from an artificial lagoon and one from a soil sample in Sonora, Mexico

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Since Balamuthia mandrillaris was first reported to be a causative agent of Granulomatous amoebic encephalitis in humans, it has been thought that its environmental niche is restricted to soil and dust. In an earlier study from Mexico, we reported the first isolation of an environmental sample of B. mandrillaris from a water sample. Here, we report the isolation of an additional 8 strains of B. mandrillaris from Mexico. These are all new environmental isolates, 7 from water samples and one from soil. Isolations were obtained, with some variations, using protocols previously published. Confirmation of the identity of each isolate was based on results from successful PCR amplification using with the species specific primer set 5'Balspec16S (5′-CGCATGTATGAAGAAGACCA-3′) and 3'Balspec16S (5′-TTACCTATATAATTGTC GATACCA-3′), which amplifies the mitochondrial 16S-like ribosomal RNA gene from B. mandrillaris. Success in amplification was determined using comparisons of amplifications of DNA from the strain CDC-V039 and the water strain (ITSON-BM1) as positive controls. The soil sample was isolated from soil taken from a plant shop, which is similar to the source of one of the first environmental isolations, one that was obtained from soil of a potted plant. This similarity suggests that such a nutrient rich environment is necessary to facilitate growth of B. mandrillaris. The success in obtaining seven new isolates from water confirms the presence of B. mandrillaris in aquatic habitats, expanding the spectrum of its ubiquity. The similarity among these new isolates, and their relationship with previous clinical and environmental isolates of B. mandrillaris was examined by using biochemical and immunological studies. These analyses showed very high homogeneity of total protein products (assessed using electrophoresis) and similar antigenic moiety (when tested by Western Blot) among the eight new isolates and two controls. Genetic comparisons of these strains will be presented in a separate abstract.

**Notes:**

## Structure and function of acanthaporin, a pore-forming protein of pathogenic acanthamoebae

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Pore-forming proteins of many pathogens are considered to play a major role in mediating diseases and several of these have been comprehensively analyzed also at the structural level. From eukaryotic parasites, our group was the first to molecularly characterize up to the level of the three-dimensional structure a protein toxin, the pore-forming protein of the enteric human pathogen Entamoeba histolytica, namely amoebapore A. We were interested in characterizing the molecular armamentarium of the potentially highly pathogenic species Acanthamoeba culbertsoni that is involved in the elimination of phagocytozed bacteria, and, particularly, in the killing of human host cells. We have identified in amoebic extracts a polypeptide that formes pores in membranes that we termed acanthaporin. Comprehensive functional and structural studies have been conducted that included isolation of the protein from its natural source, molecular cloning of the gene of its precursor, recombinant expression of the protein in bacteria and monitoring of its biological activity. The elucidation of the tertiary structure by heteronuclear multidimensional NMR spectroscopy revealed that the toxin of A. culbertsoni shows no similarity to other pore-forming proteins such as amoebapores from E. histolytica and naegleriapores from Naegleria fowleri. Notably, the three-dimensional structure of acanthaporin represents a yet unknown protein fold and may become the founding member of a new protein superfamily. Mechanistically, we learned from the determination of three-dimensional structures from active and inactive species of the protein and from chemical cross-linking experiments on membranes that amoebaporin turns in a pH-dependent fashion from a masked dimer into an potent monomer that then may assemble into an oligomeric pore that eventually perforates the target cell membranes.

**Notes:**

## Emergence of amoebicidal resistance and routes to cell death in Acathamoeba castellanii

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Synchronous encystment enables progression of resistance of Acanthamoeba castellanii (Neff), to biocides to be charted.
Trophozoite viability was measured by ability to form plaques on lawns of Escherichia coli. Confocal laser scanning microscopy using either Calcofluor white, Congo Red or the anionic oxonol dye, DiBaC4(3), or flow cytometry with propidium iodide plus fluorescein diacetate, or by using oxonol provides rapid quantification, but for cysts, plaque formation is most definite. Emerging resistance during encystation during short-term exposure to the minimum amoebicidal concentrations of each biocide during the first 36 h of differentiation was tested using HCl and moist heat as possible resistance markers. Development of the acid-insoluble, protein-containing ectocyst and the cellulosic endocyst walls were followed by the acid - and alkali-insoluble residues of cell samples. Resistance to polyhexamethylene biguanide (PHMB), benzalkonium chloride, propamidine isethionate, pentamidine isethionate, dibromopropamidine isethionate, hydrogen peroxide) and to moist heat was seen to occur between 14 and 24 h after trophozoites were inoculated into the encystment medium. HCl resistance developed at between 0 and 2 h, and to chlorhexidine diacetate (CHX) between 24 and 36 h. Acid-insoluble residues increased after 8 h and alkali-insoluble residues (cellulose) were detected after 16 h and coincided with the emergence of resistance to all the agents tested (except HCl). These results suggest that resistance to the biocides tested probably results largely from permeability barriers of the cyst envelope rather than from site-specific inhibition. For CHX, PHMB or myristamido-propyldimethylamine treatments, apoptosis was not detectable using the annexin flow cytometric assessment, and a necrotic rather than autophagic avenue to cell death is implicated. Despite the clear requirements for controlled intracellular autophagic activities as well as involvement of specific factors and signals in structural and functional reorganization (indicated by transcriptomics, proteomics, and metabolomics), in situ elimination of intra-corneal cyst formation remains problematic.

**Notes:**

## In vitro activity of commercially available moxifloxacin and voriconazole eye-drops against clinical strains of Acanthamoeba

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Acanthamoeba is an opportunistic pathogen which is the causal agent of a sight-threatening ulceration of the cornea known as “Acanthamoeba Keratitis” (AK) and, more rarely, an infection of the central nervous system called “Granulomatous Amoebic Encephalitis” (GAE). Furthermore, current therapeutic measures against AK are arduous and show limited efficacy against the cyst stage of Acanthamoeba. Moxifloxacin, a fourth generation fluoroquinolone, has been used with other drugs to treat GAE, but its efficacy as a treatment for AK is not known. Voriconazole has been used to treat AK; however its cysticidal efficacy is not known. Both drugs are commercially available as eye-drops.
The aim of this study was to evaluate the in vitro activity of these eye-drops against Acanthamoeba compared to two reference drugs (chlorhexidine and amphotericin B) which are currently used to treat AK and GAE. The sensitivity of two clinical and one type strain of Acanthamoeba to the commercial concentrations of the four drugs was evaluated with a colorimetric assay. Mature cysts were incubated with voriconazole to determine their sensitivity to this drug. The effects on cell proliferation and cell toxicity were determined using standard procedures with commercial kits. The four tested compounds were active against the Acanthamoeba strains in this study. Although it prevented encystation, moxifloxacin’s amoebicidal activity was low. Voriconazole activity was greater than that of the other drugs, even at a concentration lower than in commercial eye drops. It was effective against cysts and decreased cell proliferation, with low cellular cytotoxicity. Voriconazole could be used against AK as a first line treatment or in combination. Moxifloxacin is an interesting adjuvant to consider as it is effectively prevents encystation of the amoeba which often complicates infection resolution. In addition, moxifloxacin is effective in preventing secondary bacterial infections.

Notes:

## Extracorneal amoebic spread in Acanthamoeba keratitis

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An Acanthamoeba keratitis case series is reviewed for documented extracorneal spread of the amoeba in Hospital Ramón Y Cajal, Madrid, Spain. Three patients with 4 instances of microbiologically confirmed extracorneal amoebic spread were identified. Patient 1 had a nodular scleritis following penetrating keratoplasty treated successfully with double freeze-thaw cryotherapy; patient 2 had an intraocular dissemination of the amoeba detected in a retrocorneal membrane; and patient 3 had both an intraocular dissemination of the amoeba following keratoplasty treated successfully with intraocular and systemic voriconazole and a nodular scleritis treated with double freeze-thaw cryotherapy and large diameter corneal graft to treat corneal recurrence.

The conclusion raised from this study allows us to confirm that Acanthamoeba can migrate to the sclera or the intraocular tissues in some instances, such as long-standing disease or penetrating keratoplasty. Prompt biopsy for microbiological analysis and early treatment is required if suspected. Voriconazole can be effective for intraocular invasion when used orally and intraocularly. Scleral involvement might require a surgical approach with double freeze-thaw cryotherapy to treat the localize disease.

**Notes:**

## Enhanced siRNA-based therapy using Immunostimulating Complexes (ISCOM): Evidences of improved in vitro therapeutic effects against clinical strains of Acanthamoeba

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RNAi-based approaches provide a promising therapeutic approach for the treatment of cancer and infectious diseases. Moreover, siRNA-based therapies are currently being reported as successful therapeutic and drug discovery approaches against many pathogens. However, application of siRNA-based therapeutics to the clinic is held up by several challenges associated with the cargo (siRNA) and the delivery system. Currently, liposome-, polymer- and peptide-based delivery systems as viral vectors are being used to avoid these issues have shown to elicit significant gene silencing and cell growth regression in preclinical studies. More recently, it was reported that the functionalization of immunostimulating complexes (ISCOM)-type nanocapsules with lipid vinyl sulfones could be of potential use for therapy and immunological techniques. The lipid vinyl sulfones anchor to the ISCOM via the hydrophobic zone of their structure and can be charged with pharmacologically active molecules. These functionalized nanocapsules can incorporate protein A and bind to G immunoglobulins (IgG) to make vehicles directed at the surface antigens of infectious agents or tumor cells and deliver the encapsulated molecules in a highly specific way. Therefore, functionalized ISCOMs should be evaluated for the delivery of siRNA as they could be used in order to avoid all the mentioned post-siRNA delivery challenges.
In this case, Acanthamoeba was chosen as an in vitro model for the validation. siRNA-based therapeutics have been successfully used against these pathogens for both direct therapy and antiamoebic drug discovery but are currently being held up by siRNA high toxicity side effects. The obtained results in this model, demonstrated that functionalized ISCOM charged with anti-Acanthamoeba IgG and specific siRNAs with previously confirmed in vitro therapeutic potential against Acanthamoeba were able to induce amoebic elimination and low cytotoxicity to eukaryotic cells in vitro.

**Notes:**

## Statins: A Novel Effective Therapeutic Approach against Acanthamoeba Infections validated using siRNAs

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Acanthamoeba is an opportunistic pathogen in humans, whose infections most commonly manifest as Acanthamoeba keratitis or, more rarely, granulomatous amoebic encephalitis. Although there are many therapeutic options for the treatment of Acanthamoeba, they are generally lengthy and/or have limited efficacy. Therefore, there is a requirement for the identification, validation, and development of novel therapeutic targets against these pathogens. Recently, RNA interference (RNAi) has been widely used for these validation purposes and has proven to be a powerful tool for Acanthamoeba therapeutics. Ergosterol is one of the major sterols in the membrane of Acanthamoeba. 3-Hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase is an enzyme that catalyzes the conversion of HMG-CoA to mevalonate, one of the precursors for the production of cholesterol in humans and ergosterol in plants, fungi, and protozoa. Statins are compounds which inhibit this enzyme and so are promising as chemotherapeutics. In order to validate whether this enzyme could be an interesting therapeutic target in Acanthamoeba, small interfering RNAs (siRNAs) against HMG-CoA were developed and used to evaluate the effects induced by the inhibition of Acanthamoeba HMG-CoA. It was found that HMG-CoA is a potential drug target in these pathogenic free-living amoebae, and various statins were evaluated in vitro against three clinical strains of Acanthamoeba by using a colorimetric assay, showing important activities against the tested strains. We conclude that the targeting of HMG-CoA and Acanthamoeba treatment using statins is a novel powerful treatment option against Acanthamoeba species in human disease that it is currently being considered by clinicians in Spain.

Notes:

## Naegleria fowleri: PRODUCTION OF MUC5AC AND PRO-INFLAMMATORY CYTOKINES IN MUCOEPITHELIAL CELLS VIA TLR2 AND TLR4

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Naegleria fowleri causes primary amoebic meningoencephalitis; this infectious disease is acquired by exposure to water bodies contaminated with the parasite. The amoebae invade the nostrils, penetrate the epithelium of the olfactory mucosa, and then migrate through the olfactory nerves by crossing the cribiform plate and eventually installing in the central nervous system. It is known that the activation of toll-like receptors (TLR s) by infectious parasites induces the production of pro-inflammatory cytokines, secretion of mucins and antimicrobial peptides. In the present study, we analyzed the production of MUC5AC, IL-8 and IL-1β in the mucoepithelial cells (NCI-H292) via TLR2 and TLR4 by interaction with N. fowleri trophozoites. NCI-H292 human cells were treated with specific inhibitors of TLR2 (OXPAPC; mAb against TLR2) and TLR4 (CLI-095). Then, the cell line was co-cultured with N. fowleri trophozoites in a 1:1 ratio during 1, 3, 6, 12, and 24 h. The effect of the trophozoites in the production of MUC5AC was evaluated by immunofluorescence, the synthesis and production of IL-1β, and IL-8 were determinated by RT-PCR and ELISA assays. The results showed that MUC5AC production and the expression and production of IL-1β, and IL-8 induced by N. fowleri was inhibited with TLR2 and TLR4 inhibitors. Finally, we found that TLR4 recognition is more efficient to recognize molecules presents in N. fowleri than TLR2. The present study suggests that innate immune response may be involved during the infection by N. fowleri.
This work was supported by SEP-CONACYT 128317grant.

**Notes:**

## Isolation and characterisation of various amoebophagous fungi and evaluation of their prey spectrum among free-living amoebae

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Amoebophagous fungi can be found ubiquitous in soil and aquatic habitats. As endoparasitic fungi they invade free living amoebae (FLA) forming huge thalli inside their hosts (e.g. Cochlonema sp.) As predacious fungi they capture amoebae by adhesive hyphae, respectively by specialized organs to trap, destroy and feed on amoebal trophozoites. Various species and strains of amoebophagous fungi were isolated from different sources and determined morphologically by identifying the species-specific conidia according to Drechsler (1935 – 1945). The isolated strains were members of the genera Acaulopage and Stylopage. To study their predator-prey relationship these fungi were cocultivated with different FLA in vitro. The amoebophagous fungi used FLA with and without cyst-forming capacity as prey. In general the amoebal trophozoites succeeded in escaping their predators by forming cysts. In order to obtain pure conidia suspensions, suitable for further investigations, the fungi were associated with cystless amoebae such as Vannella sp. or Saccamoeba sp. – strains. The results of the prey spectrum analysis will be presented. It could be shown that not only various FLA with divergent taxonomic positions are suitable as prey organisms but several members of cellular and acellular slime moulds as well. These studies emphasize the important ecological role of those amoebophagous fungi by controlling the prevalence and abundance of FLA and amoebal stages of certain slime moulds in natural environment.

**Notes:**

## Determination of the origin of contamination of the thermal recreational waters in Guadeloupe.

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The distribution of free-living amoebae in most of the thermal recreational waters in Guadeloupe revealed that the pathogenic Naegleria fowleri is the most frequently encountered thermophilic species, followed by N. lovaniensis. In most of the thermal recreational waters in Guadeloupe, the concentration of N. fowleri is rather low in most water samples, ranging from 0 to 22 per liter (Moussa et al. 2013), but its presence is regular at the most popular sites. We observed that the baths, which are regularly cleaned, are not always the least contaminated ones. Therefore, we undertook a study to better understand the origin of contamination of the main thermal recreational waters. Two origins were possible, original contamination of the springs or contamination of the streams by soil, especially during rainy season. The sites were analysed at different points of the flow, at the hot spring source and at the water supply of the main baths (Bain de Dolé, Bain des Amours, Bains Jaunes, Matouba). A filtration concentration method and a PCR technique for detection were used.
The results show that the flowing water supplies of Bain de Dole and Matouba are contaminated by N. fowleri, but that at the hot spring source no N. fowleri are observed. These results suggest that the contamination occurs after the emergence of the water from the hot spring. Therefore, a pipeline conducting the water directly from the spring to the bath and a protection of the bath from streaming water could avoid contamination. This system has been applied with success at a thermal bath in a clinic at Matouba. This study provides important data for the prevention of contamination of recreational baths in Guadeloupe.

**Notes:**

## Re-evaluating the role of Acanthamoeba proteases in tissue invasion: observation of cytopathogenic mechanisms on MDCK cell monolayers and hamster corneal cells

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The present study demonstrated biological differences among two isolates of Acanthamoeba (Acanthamoeba castellanii and Acanthamoeba polyphaga) genotype T4 isolated from contact lenses of patients with Acanthamoeba keratitis. Optimal culture conditions, amoebae virulence, morphological analysis of the cytopathic effect on MDCK cell monolayers and hamster cornea were evaluated in both strains. Moreover, qualitative and quantitative analyses of conditioned medium and proteases were evaluated and compared between both isolates. Further than highlighting the biological differences found between both strains, the most important observation in this study was the fact that proteases both in total extracts and in conditioned medium are apparently not determinant in tissue destruction. Through this study, we showed the movement of amoebae through its interaction with cultured cells and hamster cornea. An interestingly finding was that no lysis of corneal tissue was observed as it had previously been suggested in vitro studies. These results together with previous assays, allowed us to conclude that the invasion and disruption of corneal tissue are performed by the penetration of the amoebae through cell junctions, either by the action of proteases only promoting cellular separation but not their destruction and/or a mechanical effect exerted by amoebae, and by phagocytosis of the recently detached cells as those attached to the corneal epithelium, which leads to the modification of its architecture facilitating the migration and destruction of deeper layers of the corneal epithelium. Therefore, we suggest that contact-dependent mechanisms in Acanthamoeba pathogenesis are more relevant that previously considered.

**Notes:**

## Clinical and parasitological evaluation of sever keratitis cases suspected of amoebic aetiology in contact lens wearers

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Purpose. Sever keratitis cases suspected of amoebic aetiology in Polish patients using contact lenses and usefulness of in vitro diagnosis in proper therapeutic management were analyzed retrospectively.
Material and methods. Six female patients, 26–41 years old, contact lens wearers, all with severe eye pain, photophobia and inflammation, some previously treated unsuccessfully, were admitted to our hospital (2009-2012). The slit-lamp, in vivo confocal microscopy, in vitro parasitological examinations were performed. Corneal scrapings from all patients, initially examined in the light microscope were cultivated in BSC growth medium; genotype of some detected amoebae by PCR technique was determined. Status of protozoan populations was in vitro monitored.
Results. Acanthamoeba keratitis in 4 patients was diagnosed; mixed bacterial-fungal-amoebic infections were detected in 2 of the patients. Amoebic cysts were detected by in vivo confocal microscopy in 2 patients, while Acanthamoeba trophozoites developed in corneal scraping cultures of all of them. Prolonged pharmacotherapy in 4 cases was undertaken; surgical procedures were necessary in 2 cases (keratoplasty, corneal grafting). Amoebic strains were morphologically identified as belonging to Acanthamoeba castellanii species and by PCR as T4 genotype. Several amoebic strains detected differed in viability and in vitro surviving time.
Conclusions. Complex infective aetiology, late recognition of amoebic infections were the factors influencing therapeutic difficulties. Variability in symptom intensity, differences in resistance to pharmacotherapy and surgical management efficacy appearing in these keratitis incidences correlated with the surviving time of cultivated amoebae. Early proper diagnosis in Acanthamoeba keratitis confirmed by detection of live trophozoites in corneal scraping cultures are decisive for the treatment efficacy, particularly in contact lens wearers. In vitro monitoring of dynamics of Acanthamoeba strains isolated from eyes may be useful tool both for proper diagnosis and treatment prognosis.

**Notes:**

## Protein profiles and immunoreactivities of different Acanthamoeba genotypes

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Acanthamoeba is a free-living protozoan found in a wide variety of habitats. A classification of Acanthamoeba into currently sixteen genotypes (T1-T17) has been established, however, data on differences between genotypes on the protein level are scarce. The aim of this study was to compare protein and immunoreactivity profiles of Acanthamoeba genotypes. Thirteen strains, both clinical and non-clinical isolates, from genotypes T4, T5, T6, T7, T9, T11 and T12, representing all three morphological groups, were investigated for their protein profiles and IgG, IgM and IgA immunoreactivities. It was shown that protein and immunoreactivity profiles of Acanthamoeba genotypes T4, T5, T6, T7, T9, T11 and T12 are clearly distinct, but banding patterns do correlate to morphological groups. Normal human sera revealed anti-Acanthamoeba antibodies against isolates of all investigated genotypes, interestingly, however only very weak IgM and virtually no IgA immunoreactivity with T7 and T9, both representing morphological group I. The strongest IgG, IgM and IgA immunoreactivities were observed in genotypes T4, T5 and T6. Differences of both, protein and immunological patterns, between cytopathic and non-cytopathic strains, particularly within genotype T4, were not so much concerning banding patterns, but rather expression levels.

**Notes:**

## Acanthamoeba in Thailand: I. Prevalence and Genotype Distribution of Isolates from Freshwater Fish Gills

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Freshwater fish are abundant and serve as an important protein source in Thailand. Recently, a case of Acanthamoeba keratitis potentially acquired during freshwater fish farming has been identified in this country. Therefore, we have determined the prevalence and genotype distribution of Acanthamoeba isolated from freshwater fish from diverse areas of Thailand. In total, 1062 fish belonging to 47 different species were included in this study. After isolation of gills from each fish, they were subject to cultivation in 1.5% non-nutrient agar seeded with heat inactivated Escherichia coli and incubated at ambient temperature (25-30ºC). Characteristic trophozoites and cysts of Acanthamoeba were detected from 212 fish (19.96%). Acanthamoebae were isolated from gills of herbivorous fish significantly greater than those from carnivorous ($p = 0.0004$) and omnivorous fish ($p = 0.0367$) whereas no significant difference in positive rates between carnivorous and omnivorous fish ($p = 0.0890$) was observed. Fish that frequently harbored acanthamoebae on their gills ( 25% positive rate) belong to snakeskin gourami (Trichogaster pectoralis, Regan 1910), red cheek barb (Puntius orphoides, Valenciennes 1842), pygmy gourami (Trichopsis sp.,Canestrini 1860), three spot gourami (Trichogaster trichopterus, Pallas, 1770), squaretail mullet (Liza vaigiensis, Quoy & Gaimard 1825), Asian freshwater needlefish (Xenentodon canciloides, Bleeker 1853), spotted scat (Scatophagus argus, Linnaeus 1766) and Nile tilapia (Oreochromis niloticus, Linnaeus 1758). Based on the diagnostic fragment 3 (DF3) sequences, 7 known genotypes (T3, T4, T5, T9, T12, T13 and T17) were identified among these isolates. It is noteworthy that genotype 4 was the most common type identified, accounting for ~80% of all positive samples. Importantly, some isolates possess novel DF3 sequences that could not be assigned to any known genotypes. Therefore, wide distribution of several genotypes of acanthamoebae occurs among freshwater fish in Thailand. Identification of novel DF3 sequences of Acanthamoeba in this study highlights extensive genetic diversity of this genus in nature.

**Notes:**

## Targeting Lysine and Histidine biosynthesis in Acanthamoeba species as targets novel inhibitor design

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Acanthamoeba species are free-living protozoa that can become opportunistic parasites. They are found ubiquitously in the environment. Acanthamoeba species are emerging pathogens they can cause a severe corneal infection called Acanthamoeba keratitis (AK) in immunocompetent individuals, which may result in blindness. Many drugs used to treat Acanthamoeba infections are ineffective because they can induce encystation, so more reliable and effective therapies need to be found quickly to treat the rising occurrence of AK. Targeting essential amino acid biosynthesis is an exploitable avenue, since humans cannot synthesise essential amino acids, which Acanthamoeba can. This also limits the side effects caused by inhibitors to eukaryotic microorganisms. Herein, we discuss the exploitation of metabolic differences between Acanthamoeba and the human host, in order to develop effective antimicrobials with minimal toxicity. We analyse both the Histidine and the Lysine biosynthesis pathways through bioinformatics, molecular, and biochemical techniques in several Acanthamoeba species. Targeting essential amino acid biosynthesis, RNAi has been demonstrated to affect the genes involved throughout these biosynthetic pathways.

**Notes:**

## A Journey to the Nucleus

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Amoebae serve as hosts for various intracellular bacteria. These bacteria are able to overcome amoebal defense mechanisms and successfully establish a niche for intracellular replication, which is usually the cytoplasm or a modified phagosome. Here we describe the Hartmannella sp. symbiont Nucleicultrix amoebiphila, which unexpectedly was located inside its host nucleus, as demonstrated by fluorescence in situ hybridization and electron microscopy. N. amoebiphila shows an only low degree of 16S and 23S rRNA sequence similarity of about 90 % to its closest described relative, the paramecium symbiont Caedibacter caryophilus, and represents, together with Odyssella thessaloniciensis and Paracaedibacter sp., a novel monophyletic clade between the order Rickettsiales and Rhodospirillales. The analysis of the infection process and the developmental cycle of N. amoebiphila revealed that N. amoebiphila enters the nucleus within the first 6 hours post infection and initiates replication. Transmission occurs vertically upon host cell division as well as horizontally after host cell lysis. High infection levels were reached between 96 and 120 hours, at which time point the nucleus was pronouncedly enlarged and filled completely with bacteria. Sequencing of the 1.9 megabase genome of N. amoebiphila, provided first insights into genomic features underlying host-symbiont interactions. We found genes likely to be involved in attachment and entry into the host cell, escape of the phagosome, survival in the cytoplasm, and manipulation and exit from the host cell. FS-5 possesses three nucleotide transporters, indicating energy-parasitism. Furthermore genes for a putative type VI secretion system and a putative type IV secretion system are present. We also detected 31 flagellar genes which encode for a flagellum, which is a rare feature among the Rickettsiales. The discovery of N. amoebiphila in the nucleus of amoebae, an experimentally accessible model system, provides a unique possibility to further deepen our understanding of the mechanisms underlying endonuclear symbiosis.

**Notes:**

## Predator versus Aliens: Bacteria interactions with Acanthamoeba.

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Free-living amoebae play a versatile role in the environment and on human/animal health, due to their ability to capture prey such as bacteria and regulate microbial densities, act as a vector and/or reservoir for bacterial pathogens, and to cause serious human/animal infections. Here, we discuss multifaceted bacterial-amoebal interactions. In favorable environmental conditions, the interaction of Acanthamoeba with non-virulent bacteria results in lysis of the bacteria. However, the interaction with weak-virulent bacteria results in a symbiotic relationship or amoeba lysis may occur. The microbial survival of amoebae in harsh environments, ability to interact with bacteria, and their ability to aid transmission to susceptible hosts is of great concern to human, animal and ecosystem health. Here, we discuss the need and impact of Bacteria-Acanthamoeba interactions and debate the question: who is the beneficiary in Bacteria-Acanthamoeba interactions?

**Notes:**

## Assessment of Tunisian olive leaf extracts against Acanthamoeba spp.

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The olive tree (Olea europeaea, Oleaceae) has historically provided huge economic and dietetic benefits to the Mediterranean basin. In fact Olive Leaf Extracts (OLE) has also been used by native people of these areas in folk medicine to treat fever and other diseases such as malaria. Recently several studies focused the extraction of high-added value compounds from olive leaves, although, a few papers have been focus on the amoebicidal activity of the OLE. In the present communication, olives leaves extract by three different solvent of five different varieties were screened for their activity against Acanthamoeba castellanii Neff. The IC50/96 h was chosen as the appropriate and comparable data to give as previously described. The parasite have been inhibit by all extract with an IC50 ranged from 8.234 ± 1.703µg/ml for the alcoholic mixture Dhokkar extract to 33.661 ± 1.398 µg/ml for the methanolic extract of Toffehi variety. The correlation analysis between phenolic, flavonoids content, and the IC50 values of the amoebic ability of olive leaves extracts from different varieties, showed a non significant effect. In fact, this activity could be attributed to the triterpenic acid present in OLE such as Oleanolic and Maslinic acid.

**Notes:**

## Continuous culture of Balamuthia mandrillaris fed with Trypanosoma cruzi epimastigotes

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Balamuthia mandrillaris is a free-living soil ameba responsible of nasopharyngeal, cutaneous, and disseminated infections as well as granulomatous amebic encephalitis in humans, nonhuman primates and other animals. Since its first isolation from the brain of a dead pregnant mandrill baboon at the San Diego Wildlife Park, Balamuthia has been grown and maintained on several mammalian cell cultures (monkey kidney cells, human brain microvascular endothelial cells, human lung fibroblasts etc.). Furthermore, several Balamuthia isolates maintained on tissue cultures have been also adapted to grow in enriched cell free BM-3 culture medium. However, it is unknown why some isolates (CDC: V416, an Australian isolate) do not grow in it. During experimentation on the in vitro interactions of Balamuthia with several protozoa and algae, we observed that four Balamuthia isolates (the baboon isolate CDC:V039, the GA isolate CDC:V188, the NY isolate CDC:V451) including CDC: V416 (unable to grow in BM-3 medium) proliferated when T. cruzi epimastigotes were added to the culture at 28°C thus enabling a continuous culture of these isolates.
Material and Methods: Balamuthia mandrillaris cultures grown on Vero E6-monolayers were chilled on ice to dislodge the amebae, counted and 1x105 trophozoites inoculated into 25cm2 tissue flasks containing DMEM with 10% fetal bovine serum (FBS) and antibiotics. T. cruzi epimastigotes (5x104/mL) from brain heart infusion (BHI) culture were added to each flask and incubated at 28°C. Subsequently, the medium was changed once every 10-12 days with adding T. cruzi epimastigotes every time.
Results: All four isolates of B. mandrillaris ingested live T. cruzi epimastigotes and increased in number. CDC: V416 has been maintained in this way for more than one year. None of the isolates grew in the DMEM/FBS medium without added live epimastigotes. Balamuthia did not grow when inactivated (60°C/30min) epimastigotes were added instead of the live epimastigotes.
Conclusions: Balamuthia mandrillaris can be maintained indefinitely in continuous culture in DMEM/FBS medium containing live T. cruzi epimastigotes. Fastidious isolates like CDC: V416 and others which are unable to grow in the conventional BM-3 medium can be maintained in the commercially available DMEM/FBS medium with added live T. cruzi epimastigotes.

**Notes:**

## Balamuthia mandrillaris: In Vitro Interactions with Selected Protozoa and Algae (Video)

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Although Balamuthia mandrillaris was identified almost two decades ago as an agent of fatal granulomatous encephalitis in humans and other animals, little is known about its ecological niche, biological behavior in the environment, food preferences and predators, if any. According to recent reports, it is believed that Balamuthia mandrillaris feeds on small amebae that are present in its ecological niche, for instance Acanthamoeba. In order to test this hypothesis, we introduced different protozoa, one at a time, into culture medium containing Balamuthia to glean insights into how Balamuthia interacts with other organisms.
Material and Methods: B. mandrillaris (CDC:V416) grown in Vero-E6 monolayer culture were chilled on ice to dislodge the amebae, counted and adjusted to 104 trophozoites/mL and suspended in DMEM medium (10mL/10% FBS) with antibiotics at 37°C. Protozoa (N. fowleri, N. gruberi, Acanthamoeba spp., Trypanosoma cruzi epimastigotes Toxoplasma gondii tachyzoites, Giardia intestinalis, Paramecium sp.) or green algae were added to B. mandrillaris cultures and incubated at 37°C or at 28°C (Acanthamoeba spp., T. cruzi).The B. mandrillaris behavior against a potential prey, its ability to hunt and attack its food, the time required to fed and cause damage to the target cell by direct contact were videotaped.
Results: Balamuthia ingested trophozoites of N. fowleri, N. gruberi, Acanthamoeba spp., T. cruzi epimastigotes, T. gondii tachyzoites and Giardia. Balamuthia caused cytolysis of T. cruzi epimastigotes and T. gondii tachyzoites by direct contact. Balamuthia was never seen to feed on Acanthamoeba cysts or algae. Balamuthia trophozoites and cysts were eaten by Paramecium sp.
Conclusions: B. mandrillaris feeds on small amoebae in nature. Balamuthia may not find Giardia, T. cruzi epimastigotes and T. gondii tachyzoites in its ecosystem. However it is likely to come across free-living flagellates that cohabits balamuthia s niche. Paramecium occupies habitats such as fresh water and wet mud along with bacteria, algae, yeast and small protozoa including Acanthamoeba, Balamuthia and Naegleria. The interaction between Balamuthia and Paramecium leads us to think that Balamuthia in nature belongs to the food-chain of Paramecium.

**Notes:**

## ECOLOGY AND DISTRIBUTION FREE-LIVING AMOEBAE IN ARTIFICIAL SWIMMING POOLS

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Free living amoebae (FLA) are numerous and systematically heterogeneous protozoan group with a worldwide distribution. Some species of the FLA found in the environment are associated with human disease. Moreover, they constitute a potential reservoir for virulent bacteria, allowing the survival, the multiplication, and the dissemination of these pathogens in water systems. As the cases of human diseases caused by FLA were reported mainly from swimming pools, Regional Authority of Public Health in Slovakia provided a systematic monitoring since 2002. The investigation was focused on the study of FLA of artificial swimming pools in Central Slovakia.

The objectives of the study was i) to find data on taxonomic composition, occurrence and distribution of FLA ii) to analyze taxonomic structure of amoebic assemblages of different types of pools, iii) to find relationships between density and structure of assemblages and environmental variables, iv) to identify the proportion of potentially pathogenic taxa as the risk of amoebic infections.

Material was sampled from 58 sites (public indoor swimming pools, open-air swimming pools, therapeutic pools) in 2004 – 2007. A total of 471 samples were processed. Amoebae were isolated by culture in NNA medium with Enterobacter spp. at 20±2 ºC, 36±2 ºC, 44±2 ºC, respectively. Taxonomic identity was determined using diagnostic features discernible by light microscopy. Both univariate and multivariate statistics were used to determine the effects of environmental variables for density and structure of amoebae assemblages.

In total 23 taxa, including 13 species from classes Lobosea (subclass Gymnamoebia) and Heterolobosea belonging to four orders and eight subfamilies, including potentially pathogenic taxa were identified. The results suggested that both density and structure of amoebic assemblages were significantly affected by factors occurring mostly in the investigated therapeutic pools. If FLA themselves are opportunistic pathogens and vectors of pathogenic bacteria, our findings prove the multiplied public health risk.

**Notes:**

## Infection cycle and genomic features of the Acanthamoeba symbionts Amoebophilus asiaticus and Procabacter acanthamoebae

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Free-living amoebae feed on bacteria and other microorganisms, but they also serve as hosts for a variety of facultative and obligate intracellular bacteria, including human pathogens. All known obligate endosymbionts of Acanthamoeba are affiliated with either of four bacterial groups, the Chlamydiae, Bacteroidetes, Alpha- or Betaproteobacteria. Here we investigated the Acanthamoeba-endosymbiont relationship on the basis of two distinct endosymbionts, the Bacteroidetes symbiont Amoebophilus asiaticus and the Betaproteobacteria symbiont Procabacter acanthamoebae, respectively. Analysis of the infection cycle of both symbionts using fluorescence in situ hybridization revealed insights into attachment of the symbionts to their Acanthamoeba host cells, the uptake into the cytosol, intracellular survival and replication, and release at the end of the infection cycle. We observed that the extracellular stage of different A. asiaticus strains is significantly more infectious than the intracellular stage, suggesting a biphasic developmental cycle alternating between infectious and reproductive forms. The genome of A. asiaticus lacks most genes for de novo biosynthesis of co-factors, nucleotides and amino acids, which is a hallmark of obligate endosymbionts. In contrast, genome analysis of P. acanthamoebae revealed an unexpectedly broad metabolic potential, including the synthesis of a broad range of amino acids and co-factors. In addition, several genes encoding known virulence factors such as hemolysins, colicins, and patatin-like proteins were found, as well as four protein secretion systems. Our findings illustrate the diversity among obligate endosymbionts of Acanthamoeba and their interaction with their host cells.

**Notes:**

## Genomic, transcriptomic and proteomic identification of pathogenicity factors from Naegleria fowleri

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Naegleria fowleri is a free-living amoeba causing primary amoebic meningoencephalitis (PAM), an acute, fast progressing and mostly fatal disease of the central nervous system.
By comparison of N. fowleri with non-pathogenic Naegleria species several potental pathogenicity factors such as pore-forming polypeptides as well as different proteases could be identified. However, the cellular mechanisms and proteins accounting for the destructive nature of PAM are still poorly understood.
In a laboratory setting, the growth behavior and morphology as well as in vitro cytotoxicity and in vivo pathogenicity of the amoeba can be influenced by the composition of the axenic growth medium (1). While trophozoites in Nelson's medium are highly pathogenic, N. fowleri in PYNFH medium exhibit a low pathogenicity for mice1. Based on this high- versus low-pathogenicity model, the proteome of highly and weakly pathogenic N. fowleri was assessed by 2D gel electrophoresis combined with nano LC MS/MS. In order to identify the differentially expressed proteins of the two conditions, the as yet unknown genome and transcriptome of N. fowleri was de novo sequenced by Illumina technologies. Assembly, identification of open reading frames and annotation is in progress and will be presented at the meeting.
(1) D.C. Burri et al.: Development of a high- versus low-pathogenicity model of the free-living amoeba Naegleria fowleri. Microbiology (2012), 158, 2652-2660

# Abstracts

## Posters

(in alphabetical order)

Poster

## Strengthening surveillance of recreational waters Metropolitan Regions (MR) and V, by detection of Acanthamoeba spp.

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Some species of Acanthamoeba can cause keratitis, granulomatous encephalitis and disseminated infection. In Chile, in 1981, this agent was isolated in water and in 1993 in a patient s contact lens wearer with keratitis. Objetive: To evaluate the quality of recreational waters. Material and Methods: In total there were 120 samples, of MR: 30 pools, 15 rivers and lakes. In Region V, 30 pools, 15 rivers and 30 lakes and sea. The samples were analyzed by Standard Methods for the Examination of Water and Wastewater, 9711C. In the pools were recorded temperature, pH and free chlorine. Took 1 liter of water, filtered and cultivated. The samples were classified according to the groups established by Pussard and Pond s with a calibrat ed standard. Results: 41/120 (34.1%) were positive morphologically for Acanthamoeba spp, 10/41 (24.4%) of pools and 8/41 (19.5%) rivers and lakes in the RM. In Region V, 9/41 (21.9.0%) of pools, 10/41 (24.4%) of rivers and lakes and 4/41 (9.8%) of sea. The according to Pussard and Pond s: only the group II 23/41 (56.1%), only the Group III 1/41 (2.4%). Cysts of both groups 17/41 (41.5%). No significant differences ($p = 0.430$) between the result and temperature, either between the result and the pH ($p = 0.781$), or between the output and the chlorine ($p = 0.132$). 37/41 (90.2%) were positive by PCR. Is currently underway genotypic analysis, genotypes found T3 and T4. The results allow us to determine the baseline of recreational water pollution, strengthen epidemiological surveillance and analyze possible changes in existing regulations. Funding: Project SA11I2270 Fonis.

Poster

## Potentially pathogenic Free-Living Amoebae isolated from Thermal Spring Recreation Area in Hidalgo, Mexico.

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Free-living amoebae (FLA) occur in a wide range of habitats and include some pathogens of man. The potentially pathogenic FLA are thermotolerant. In the state of Hidalgo, Mexico there are several thermal springs that feed swimming pools for recreational use, one of these, is the recreative center EcoAlberto in Hidalgo, Mexico, hence the importance to study the presence of FLA in those sites. Samplings in rain and dry season were made. Twelve sampling sites were selected including thermal springs, swimming pools, streamrivers and ponds located in the same recreative center. Twenty three water samples were analyzed. Water temperature, pH, dissolved oxygen, conductivity and residual free-chlorine in swimming pools were measured by standard methods. Samples were concentrated and cultured onto NNE medium and incubated at 25 and 37° C. The isolates were morphologically identified and pathogenicity test of Naegleria and Acanthamoeba isolates was made. Naegleria isolates were analyzed by PCR method.
A total of 87 isolates of FLA belonging to the genera Naegleria, Acanthamoeba, Guttulinopsis, Hartmannella, Mastigameba, Mayorella, Platyamoeba, Paratetramitus, Rosculus,Vannella and Vexillifera were identified. The highest number of isolates belongs to the genera Naegleria; from thermal springs and during rainy season. The diversity and number of FLA isolates in swimming pools were lower than springs probably due to, one day a week the swimming pools are cleaned and disinfected with chlorine, although water are constantly flowing. Naegleria isolates were non pathogenic and one Acanthamoeba isolate was invasive in mice. Most of isolations were associated with higher temperature and sites where water of spring arises. The presence of Naegleria and Acanthamoeba is relevant because they include potentially pathogenic species and should therefore be considered a potential health risk associated with human activities in thermal spring environments.

Poster

## Occurrence of Free living amoebae in natural aquatic environments of Mexico Valley watershed

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Free-living amoebae (FLA) play an important role in the cycling of nutrients in aquatic food chains. However, their distribution in natural aquatic environments is not well known. To know the presence and distribution of FLA and its relation with some physicochemical parameters in streamrivers of Mexico Valley Watershead (MVW) in Central Mexico, a study was made. Fourteen streamrivers of MVW were sampled. Temperature, pH, conductivity, dissolved oxigen (DO), total (TC) and fecal coliforms (FC) were determined by Standard Methods. For amoebae isolation, samples of 50 mL were centrifuged and the pellets were seeded onto NNE-media and incubated at 25 and 37 ºC. Identifications were made on the basis of morphological features and pathogenicity test in Naegleria and Acanthamoeba isolates was made. Twenty five species of FLA representing 15 genera were isolated and identified. The genera more frequent were Vannella and Rosculus. The frequency of Acanthamoeba and Naegleria was low and only one Acanthamoeba isolate was invasive in mice. The highest species diversity was found at “Xopachi” river and the lowest one at “Nacimiento Presa Iturbide”. Highest numbers of TC and FC were registered in “La Planta” site and the lowest one in “Organillos”. Temperature with a mean of 9.5 °C. DO levels between 8.0 and 12.5 mg L -1; pH with a mean of 6.7. DO, pH and conductivity were within the limits for the growth of most of FLA. The highest diversity and number of FLA were found at “Xopachi”, “Santa Rosa” and “La Cabañita” sites probably due to this streamrivers are close to small villages that discharge wastewater to this water bodies and for livestock and agriculture activities in that areas. The presence of Acanthamoeba and Naegleria is important because they include potentially pathogenic species, although they can proliferate better at water temperature up to 25°C.
This work was supported by DGAPA-PAPIIT-IN-211712

Poster

## Genotyping of potentially pathogenic Acanthamoeba strains isolated from nasal swabs of healthy individuals in Peru

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In this study, a survey was conducted in order to determine the presence and pathogenic potential of free-living amoebae of Acanthamoeba genus in nasal swabs from individuals in two regions of Peru. Identification of isolates was based on cyst morphology and PCR-sequencing of the diagnostic fragment 3 to identify strains at the genotype level. The pathogenic potential of the isolates was also assayed using temperature and osmotolerance assays and extracellular proteases zymograms. The data revealed the presence of genotype T4 as the most common one in the samples included in this study but also genotype T15 was identified and all the isolated strains exhibited pathogenic potential traits. To the best of our knowledge, this is the first study on the characterization of Acanthamoeba strains at the genotype level and the first report of genotype T4 and T15 in Peru.

Poster

## Endosymbiotic non-tuberculous mycobacteria in a Hartmannella vermiformis strain isolated from the nasal mucosa of an HIV patient in Lima, Peru

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The presence of intracellular mycobacteria in a Hartmannella vermiformis strain isolated after culturing nasal swabs from an HIV patient from Lima, Peru is reported in this study. It is important to mention that the patient was previously diagnosed with pulmonary tuberculosis. The amoebae were isolated from the nasal swab using non-nutrient agar plates and were cloned by dilution until axenification and characterized at the species level after sequencing the 18S rDNA gene. Amoebic DNA sequencing confirmed the amoebae to belong to Hartmannella vermiformis species. Moreover, the diagnosis of the bacteria was based on microscopic observation of bacterial aggregations in axenic culture. The presence of nontuberculous mycobacteria was confirmed after performing specific PCR and sequencing for the identification of Mycobacterium species. To the best of our knowledge, this is the first report on the identification of endosymbiotic nontuberculous mycobacteria of Hartmannella vermiformis isolated from a clinical sample. Awareness within the clinicians and microbiologist should be raised in order to consider this way of infection at least in HIV patients.

Poster

## Acanthamoeba mauritaniensis: A BIOCHEMICAL PROTEASE CHARACTERIZATION

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Acanthamoeba spp. is a free-living amoeba with a wide distribution in nature and found in soil, salt and fresh water, domestic tap water, air conditioned filters, contact lens and lens cases. Occasionally these organisms can infect the human eye producing the amoebic keratitis (AK), granulomatous amoebic encephalitis (GAE), and in some cases skin infections. Both encephalitis and skin lesions are seen in immunocompromised patients, whereas AK is commonly seen in contact lens wearers with a deficient hygiene. The proteases have been considered as part of the mechanisms of damage in opportunistic microorganisms such as Acanthamoeba. In a previous work, using a model of AK in hamster we reported that A. castellanii and A. polyphaga were able to detach the cells of the corneal epithelium in early stages of infection. We also found that Acanthamoeba mauritaniensis isolated from the environment and co-incubated with MDCK monolayers is capable to produce an important cytophatic effect on these cultures. The objective of the present work was to analyze biochemically the proteases of this amoeba using SDS-PAGE gels co-polymerized with porcine gelatin and also using a chromogenic substrate (Azocoll). To determine the type of proteases present in A. mauritaniensis we used different inhibitors of the enzymes. The results showed that the zymogram gels presented different patterns of proteolytic activities when total amoeba extracts were used. When we added the protease inhibitors, aprotinin and PMSF, into the secretion products the protease activity was abolished. The chromogenic substrate assays confirmed that the main protease activity is the serine type, and PMSF was the main protease inhibitor that significantly decreased the activity. These results suggest that an environmental isolated strain A. mauritaniensis had a high protease activity and probably is capable to produce AK, similarly as other isolates. This work was supported by CONACyT 128317 grant.

Poster

## Stenotrophomonas maltophilia and free living amoebae

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Stenotrophomonas maltophilia is frequently involved in nosocomial infections and is know to be resistant to many antibiotics. This opportunistic bacteria can be recovered in hospital water networks. Free living amoebae (FLA) are also commonly found in these networks. The aim of this work was to study potential interactions between trophozoites of three FLA strains (Acanthamoeba castellanii, Acanthamoeba polyphaga, Hartmannella vermiformis) and two S. maltophilia strains isolated from hospital water.
In this aim, FLA (5.105/mL) - S. maltophilia (5.104/mL) co-incubations were carried out in PBS or in filtered tap water (0,22 µm) at 27°C for 4 days. After 48 and 96 h of incubation, bacterial colony forming units were numbered, and amoeba viability was determined by trypan blue staining. Electronic microscopy was also carried out after 2 and 48 h of coincubation to investigate the potential internalization of bacteria in amoebae. Moreover, after 24h of co-incubation, microorganisms were transferred in encystment medium and incubated at room temperature during 28 days to study the bacterial growth in this poor medium with or without FLA.
Whatever the co-incubation medium, the presence of amoebae supported the bacterial growth, whereas amoebae viability was not influenced by the bacteria. Using electronic microscopy, morphological differences between A.castellanii and H.vermiformis cocultures were evidenced with numerous bacteria inside H. vermiformis, whereas few bacteria were present inside A.castellanii. In encystment buffer, after 14 and 28 days of incubation, the presence of FLA allowed bacteria survival and growth.
In conclusion, this work shows that the presence of FLA supports S. maltophilia growth, in PBS or in tap water. A a consequence, in hospital water systems, a particular attention should be paid to the presence of FLA, which can promote S. maltophilia development, as described for other bacteria.

Poster

## A proposal for application of the molecular species concept to Acanthamoeba: The relationship between genotype classification of Acanthamoeba and species names

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The amoebae in the genus Acanthamoeba were discovered by Castellani and described by Volkonsky in 1930. Species of Acanthaoeba have been traditionally described using morphology (primarily cyst structure), or cytology of nuclear division (used by Pussard and Pons, 1977). Twenty-plus putative species were proposed based on such criteria. Morphology, however, is often plastic, dependent upon culture conditions. The lack of knowledge about sexual reproduction within Acanthamoeba, which negates use of the Biological Species Concept questionable, further obscures species identification. Molecular studies suggest that the relationship between phylogenetic relatedness and species names in Acanthamoeba is tenuous. Our lab identified 12 sequence types within Acanthamoeba by 1998 based on a definition describing isolates showing >5% pairwise divergence between Rns sequences. Sequence types usually included isolates assigned by morphology to multiple nominal species, and a nominal species often appeared in more than one sequence type. The
number of Rns sequences now available (>1700), together with extensive information about variation for two genes from the mitochondrial genome (the 16S-like RNA gene [rns] and the gene for cytochrome oxidase subunit I [COI]) allows a new consideration of whether species names can be assigned usefully to phylogenetic groups in Acanthamoeba identified using molecular methods. Existing species names are assigned to specific significant monophyletic phylogenetic clades identified using a multi-locus approach, and which differ by a minimum of 1% for a near-complete, verified sequence of the Rns gene. The most frequent (sub)type, T4-A, contains the greatest number of names, as well as isolates, but also includes the type strain of the genus described by Volkonsky (CCAP 1501/10 and ATCC 30011 or 50374), originally assigned the species name castelanii. Other clades are given preliminary assignments of species names based on the oldest name assigned to identified isolates from culture collections. Unique sequence motifs that can be used to identify each nominal species are presented.

Poster

## Continued low levels of genetic variability in newly isolated strains of Balamuthia mandrillaris

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Since the first report of Balamuthia mandrillaris as a causative agent of Granulomatous amoebic encephalitis in humans, the environmental niche of this amoeba was assumed to be restricted to soil and dust. In another abstract, we report the isolation of 8 new strains of B. mandrillaris from Mexico. This continues the pattern of an excess of isolates from North America, compared to other parts of the world. All of the new isolates are environmental isolates, 7 from water samples and one from soil. The only previous isolation from water was that of an independent water isolate from northern Mexico. We confirmed the identity of each isolate and examined genetic variation in these new isolates compared to older strains from clinical cases using the sequence of the mitochondrial 16S-like rRNA gene. Success in amplification was determined using comparisons of amplifications of DNA from the strain CDC-V039 and the water strain (ITSON-BM1) as positive controls. The similarity among these new isolates, and their relationship with previous clinical and environmental isolates of B. mandrillaris was examined. The results showed very high similarity of sequence among the eight new isolates and previous strains. This is consistent with low levels of variation in both the nuclear 18S rRNA gene and the mitochondrial 16S-like rRNA gene. In general, Balamuthia shows significantly lower levels of genetic variation as compared to other close members of the Amebozoa, such as Acanthamoeba, and Protacanthamoeba, as well as to other free-living amoeba such as Hartmanella, Vermaboeba, Vanella or Naegleria. The ecological niche occupied by Balamuthia, proposed to be an apex predator of the microscopic world, may be contributing to the low levels of genetic variation and lack of differentiation so far observed.

Poster (cartoons in a loop)

## The depiction of amoebae in comic art

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Amoebae have been depicted in comic/cartoon art for more than a century. In newspapers and books, the simple form of an amoeba or of a protist, and the simplicity required to draw such cells, seems to encourage their use to depict many aspects of human life. In many cases the representation may be of a generic “single-celled” organism, while in other cases the depiction is specifically of an amoeba or protist. A collection exceeding 700 single or multi-frame cartoons has been collected and characterized. These include predominantly cartoon art from newspapers in the United States or Britain. However, other sources are represented as well, but with fewer examples. Most occurrences appear as single time cartoons, but a few examples of a specific artists producing a string of related cartoons exists. The most prominent of these in published form are the representations of amoebae by Gary Larson in his cartoon strip “The Far Side” which appeared from 1980-1995. The existence of the internet has encouraged the appearance of additional examples of amoeba art,
especially in light of the simplicity of drawing an amoeba. The most outstanding examples of the presentation of amoeba art on the internet are the products of David Farley in “The Doctor Fun Page” which ran from 1993-2006. In many cases, amoeba art depicts biological processes. These tend to emphasize aspects of reproduction and sexual (or asexual) aspects of life. Processes of infection may also be represented. In other cartoons, the microbe appears as a surrogate, representing how a simpler life form would accomplish human tasks, or how it would deal with the vagaries of existence. A still further subset of cartoons represents the interaction of scientists with the subjects of their research. A representative group of
cartoons from the collection will be presented illustrating the variety of appearances of amoebae in cartoons.

Poster

## Cytotoxic activity of heterocyclic alkylphosphocholines against clinical isolates of Acanthamoeba spp.

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To date, no reliable therapy against Acanthamoeba infections was developed and presently used therapeutic agents are frequently ineffective and with many side effects. Because of the increasing number of cases, the development of new drugs continues intensively. At the present time the highest attention is focused on alkylphosphocholines (APCs) – phospholipid analogues which affect lytically on cell membranes of various parasitic protists. The aim of our study was to define cytotoxic effect of four new synthesised APCs with ammonium cation bounded in heterocycle: APC1 (IF16-P-4-Pip) and APC2 (IF16-P-2-MetPip) with piperidine, APC3 (IF16-P-Azep) with azepan and APC4 (IF16-P-Morf) with morpholine, which were tested in vitro on two clinical isolates of Acanthamoeba spp. of T4 genotype from AK cases. All APCs expressed significant inhibitive effect against isolates and the highest tested concentration (500 μM) caused total destruction of the cells. The lowest value of MTC was measured after application of the APC1 after 48 hours, when it reached the value of 31.25 μM for the strain of A. quina and 62.5 μM for the strain of A. lugdunensis. APC2 and APC3 manifested strong cytotoxic effects only on the strain of A. lugdunensis (MTC: 62.5 μM after 48 h), the lowest cytotoxic activity showed APC4 with effect similar to the reference APC which was miltefosine. The EC50 values determined by the linear regression showed higher sensitivity of A. lugdunensis strain for 3 of 4 compounds tested. After the application of all APCs pseudocyst-like forms were detected. APC1 elimination effect similar to the reference APC (miltefosine) following from the EC50 values indicates its possible therapeutic potential, although it is conditioned by further experiments both in vitro and in vivo.
The research was supported by grants VEGA 1/0600/11, VEGA 1/0796/12, UK/547/2013 and UK/161/2013.

Poster

## The Acanthamoeba shikimate pathway has a unique molecular arrangement and is essential for aromatic amino acid biosynthesis

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The shikimate pathway is the only known biosynthetic route for de novo synthesis of aromatic compounds. The pathway was once thought to be only present in prokaryotes, fungi and the chloroplasts of plants. However, it has now been identified in a number of diverse taxa, suggesting that it was an ancient eukaryotic innovation that has been retained in a subset of eukaryotes, replace in plants through the acquisition of the chloroplast, but lost in many including humans. We have recently demonstrated that A. castellanii is sensitive to inhibition by glyphosate a specific inhibitor of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase and thus the shikimate pathway. We now demonstrate that A. castellanii has a novel complement of shikimate pathway enzymes including unique gene fusions. It possesses a novel tetrafunctional gene fusion comprising of 3-dehydroquinate synthase, EPSP synthase, 3-dehydroquinate dehydratase and shikimate kinase and a novel trifunctional gene fusion comprising shikimate dehydrogenase, phosphoribosylanthranilate isomerase and indole-3-glycerol-phosphate synthase. It contains two Type I and one Type II DAHP synthases (for which molecular modelling predicts their likely sensitivities to feedback inhibition by phenylalanine, tyrosine and tryptophan) and a canonical chorismate synthase.

Poster

## Growth dynamic of Naegleria fowleri in a microbial freshwater biofilm

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The free-living amoebae are ubiquitous organisms that are encountered in natural and artificial environments. The occurrence in industrial cooling circuits of the pathogenic amoeba Naegleria fowleri is a health topic of interest. Biofilms are suspected to sustain the amoeba by providing their nutrients (bacteria). However, the behaviour of this thermotolerant amoeba within biofilms remains poorly documented.
This study is intended to assess the impact of temperature on the occurrence, development and survival of N. fowleri in complex freshwater biofilms. Independant well-controlled reactors (temperature, hydrodynamic conditions) equipped with glass coupons and fed with natural freshwater were performed at 22, 32 and 42°C. N. fowleri was experimentaly inoculated at day 1 in a single injection. The biofilms formed were then characterized regularly during 45 days. Analytical monitoring includes specific measurements of the N. fowleri densities in the biofilm, and of free-living amoebae. The number of sessile bacteria and other biofilms descriptors were also followed along the time.
Regardless of the temperatures, at 42°C, N. fowleri grew rapidly and reached high concentrations (100 amoeba cm-2). Whereas, even at 32°C, N. fowleri remained at low concentrations (10 amibes cm-2) during several weeks and disappeared quickly at 22°C. Moreover, the dynamic of the amoebic growth depends on the availability of nutrient (ratio bacteria/ amoebae).

Poster

## Is Flabellula polyphyletic?

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The members of Flabellulidae (Bovee, 1970) Page, 1987 reported to date form a relatively minor family of amoebae: it contains two similar genera, Flabellula Schaeffer, 1926 and Paraflabellula Page and Willumsen, 1983, with a handful of species each. They are typically rather flattened marine amoebae with conspicuous hyaloplasm, sometimes with a tendency to have more nuclei in their cells. Flabellulids (together with Gephyramoeba Goodey, 1914, Rhizamoeba Page, 1972 and Leptomyxa Goodey, 1914) belong among Amoebozoa, Tubulinea, Leptomyxida. Although leptomyxids form a rather polymorphic group, they behave well in phylogenetic analyses and are usually reconstructed as a monophylum.
During the past decade, we have isolated a number of flabellulid strains (cca 15). To confirm their morphology-based identification, we have also sequenced their SSU rRNA gene and conducted phylogenetic analyses. Approximately half of the flabellulid strains belonged among Leptomyxida and were closely related to other Flabellula / Paraflabellula strains (including F. citata, the type species). The rest of alleged flabellulids, however, formed a very long branch that occupied various positions in the tree (depending on dataset composition / model used), but never grouped with “true” leptomyxids. The type strain of F. trinovantica also belongs among these strange “flabellulids”. To further investigate this case of apparent polyphyly of Flabellula, we are sequencing other genes of the amoebae. Interestingly, analyses of actin gene sequences show monophyletic flabellulidae.

Poster

## Contamination of recreational water sources to Acanthamoeba spp in Tehran, Iran

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Acanthamoeba spp. is the ubiquitous potentially pathogenic free-living amoebae in nature such as water sources. Recently Acanthamoeba keratitis (Ak) continue to rise in Iran. Most of patients report a history of contact with water sources before the onset of disease. The main aim of the present study was to determine the occurrence of Acanthamoeba spp. in the recreational water sources of Tehran, Iran using morphological and molecular based tests. Overall, 55 samples were collected from recreational water sources including man- made and natural waters in Tehran province. Filtration and cultivation of samples were done using non-nutrient agar. Cloning of Acanthamoeba spp. was then performed to eliminate bacterial and fungi contamination. DNA extraction and PCR amplification were performed using JDP1-2 (genus-specific primer pair). Out of 55 water samples, 10 were positive for free living amoebae in which Acanthamoeba trophozoites and cysts were observed in 7 samples according to morphological criteria. Cloning of 7 isolates was done successfully. All of isolates present a 500 bp PCR band which is specific to Acanthamoeba genus. The positive water sources were mainly used for recreational purposes. Presence of Acanthamoeba in recreational water sources is of concern for high risk people. The present study is the second to identify Acanthamoeba in recreational water sources of parks in Tehran, Iran. Posting of alarming sign and education to high risk people is of utmost importance to prevent such Acanthamoeba related infections.

Poster

## Detection of Acanthamoeba in contact lenses from Madrid, Spain

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Acanthamoeba is a ubiquitous free-living amoeba which may produce a painful and potentially blinding corneal infection, Acanthamoeba keratitis that mainly occurs in contact lenses (CL) wearers with inadequate CL hygienic practices. In environmental studies carried out in Madrid, a high presence of Acanthamoeba in tap water has been shown (93.8%). The aim of the study was to investigate the presence of this protozoon in CL from health individuals. For this purpose, 177 healthy individuals participated by contributing with their CL and answering a hygiene habits questionnaire. DNA was extracted from the CL solution and a Taq man real time PCR was performed. Acanthamoeba viability was determined by culturing the CL on non-nutrient agar plates with inactivated Escherichia coli. Samples were considered negative in PCR when Ct value was over 38 and in culture if after 20 days no cyst or trophozoites were observed.
Acanthamoeba DNA was detected in 87 of samples (49.2%), while cultivable amoebae grew from only 1 sample (0.6%) The isolate was genotyped as T4, and it was isolated from an individual how reported rinsing CL with tap water, showering while wearing CL, and eye discomfort, as did 25.4% of the other individuals studied.
Thus, the results obtained suggest a high presence of Acanthamoeba spp. in CL from healthy wearers from Madrid; we might assume that the CL solutions are disinfecting appropriately the CL because only 1.1% of the positive PCR samples correspond to viable amoebae. Highlighting the importance of a good CL care to avoid AK acquisition as a close relationship between this protozoan and CL wearers was stated.
Project supported by PS09/1115(FISS); PC15/09(USP-CEU). T.S. Gomes was supported by Research Program EADS CASA-Brasil I and A. Magnet by Ministerio de Educación y Ciencia-Spain (FPU grant AP2009-0415)]

Poster

## Novel Acanthamoeba 18S RNA Gene Sequence Type from an Environmental Isolate

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Acanthamoeba genus comprises more than 20 species of free living amoeba widespread in very different habitats. Traditionally, species identification beyond this genus has been based on morphological characteristics of the cyst. However this classification has been shown as inconsistent because the correlation between binomial classification and molecular typing is, in most cases, not concordant. For this reason, a classification based in the 18S ribosomal RNA full gene sequence was proposed and to date 17 genotypes have been defined.
A new Acanthamoeba strain (USP-AWW-A68) was isolated from a water sample collected at the entrance of a Wastewater Treatment Plant in the central area of Spain. The concentrated water (IDEXX® Filta Max system) was inoculated to a non-nutrient agar plate seeded with heat inactivated Escherichia coli and subcultured until an axenic culture was obtained. The whole 18S RNA gene sequence was obtained clustering two fragments of approximately 1650 bp and 1100 bp respectively, overlapping in about 480 bp.
The sequence obtained was aligned with representatives of each Acanthameoba genotype and introns were removed for the analyses. Phylogenetic reconstructions comprised three tree-building methods: i) Neighbor-Joining (NJ) (gap/missing data treatment: pairwaise deletion, Kimura 2-parameters model for susbstitution); ii) Maximum Parsimony (MP) with Subtree-Pruning-Regrafting (SPR) (gap/missing data treatment: pairwaise deletion, with 10 initial trees and a maximum of 100) and iii) Maximum Likelihood (ML) (Gap/missing data treatment: partial deletion, Kimura 2-parameters model for substitution with very strong branch swap filter) with bootstrap test of 1000 in all trees.
Following the 5% dissimilarity criterion to assign new genotypes, USP-AWW-A68 is clearly differentiated, as dissimilarities respect the other genotypes vary from 7.7% for T16 to 33% for T7. USP-AWW-A68 emerges as an independent branch with any of the methods used for phylogenetic inference. This Acanthamoeba should therefore be considered as a new genotype, T18.
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Poster

## Naegleria fowleri: PRODUCTION OF MUC5AC AND PRO-INFLAMMATORY CYTOKINES IN MUCOEPITHELIAL CELLS VIA TLR2 AND TLR4

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Naegleria fowleri causes primary amoebic meningoencephalitis; this infectious disease is acquired by exposure to water bodies contaminated with the parasite. The amoebae invade the nostrils, penetrate the epithelium of the olfactory mucosa, and then migrate through the olfactory nerves by crossing the cribiform plate and eventually installing in the central nervous system. It is known that the activation of toll-like receptors (TLR s) by infectious parasites induce the production of pro-inflammatory cytokines, secretion of mucins and antimicrobial peptides. In the present study, we analyzed the production of MUC5AC, IL-8 and IL-1β in mucoepithelial cells (NCI-H292) via TLR2 and TLR4 by interaction with N. fowleri trophozoites. NCI-H292 human cells were treated with specific inhibitors of TLR2 (OXPAPC; mAb against TLR2) and TLR4 (CLI-095). Then the cell line was co-cultured with N. fowleri trophozoites in a 1:1 ratio during 1, 3, 6, 12, and 24 h. The effect of the trophozoites in the production of MUC5AC was evaluated by inmunofluorescence, the synthesis and production of IL-1β, and IL-8 were determinated by RT-PCR and ELISA assays. The results showed that MUC5AC production and the expression and production of IL-1β, and IL-8 induced by N. fowleri was inhibited with TLR2 and TLR4 inhibitors. Finally, we found that TLR4 recognition is more efficient to recognize molecules presents in N. fowleri than TLR2. This study overall provides information about the involvement of the innate immune response during the infection by N. fowleri.

Poster

## Comparison of counting methods for Naegleria fowleri in water

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The distribution of free-living amoebae in most of the thermal recreational waters in Guadeloupe revealed that the pathogenic Naegleria fowleri is the most frequently encountered thermophilic species, followed by N. lovaniensis. The concentration of N. fowleri is rather low in most water samples, ranging from 0 to 22 per liter (Moussa et al. 2013). Taking into account that quantification of low concentrations of N. fowleri in the environment is difficult (Pernin et al.1998), we have compared the filtration and the centrifugation methods using two counting procedures, the most probable number (MPN) and a more empirical method of counting the number of emerging amoeba clearing zones from filters cut into small pieces. Water samples containing 500, 50 and 5 amoebae per liter were filtered. The first results show that with filtration, a loss of 90 % and 85 % occurs for the first dilution with both methods (empirical method and MPN, respectively), a loss of 86% and 64 % for the second dilution, and for the third dilution a loss of 60% for the MPN method. This study is still ongoing to find the most precise and most simple method of counting low concentrations of N. fowleri

Poster

## Alkylphosphocholines – potential therapeutic agents of Acanthamoeba infections

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Diseases caused by Acanthamoeba spp. are requiring a high attention because of absence of a reliable therapy. High therapeutic potential show alkylphosphocholines (APCs) originally used as antineoplastic drugs which affect the membrane systems of various parasitic protists. We tested 13 APCs in three series on trophozoites of clinical isolates of Acanthamoeba spp. All the tested compounds demonstrated inhibitive effect which depended on the changes in the chemical structure in the series. The reference APC – miltefosine (HPC) showed weak cytotoxic effect on the isolates with MTC 250 – 500 μM after 24 h. In the group of four new synthesized heterocyclic analogues of HPC the increase of cytotoxic effect depended on the expansion of the heterocyclic ring. The compounds HPAzep and HPAzoc reached MTC values (50 μM) ten times lower than HPC. In further group of heterocyclic APCs, forming vesicles in the water solution, the distinct trophocidal effect of C10PC2N(Pyr)C12 (MTC 200 μM after 24 h) arose from the formation of giant vesicular aggregates (20 – 128 μm), which with their large surface contacted a higher number of trophozoites than the other compounds. Among the five APCs with different numbers of carbon atoms in branched alkyl chains (Isophol-PCs), the most effective was Isophol16PC with 16 carbon atoms (MTC 62.5 – 125 μM after 24 h). The high values of MTC ( 500 μM) of other Isophol-PCs with lower or higher number of carbon atoms in alkyl chains showed “cut-off” effect in the group. After the application of Isophol16PC, Isophol20PC and HPC, pseudocyst-like forms demonstrating their cytotoxicity were noted. A high amoebicidal effect of some tested APCs suggests their possible future use in the development of new drugs against Acanthamoeba infections.
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Poster

## Free-living amoebae in bat guano from karst caves

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Bats are migratory animals which form colonies in undisturbed spaces, such as church towers, roofs and caves, to hibernate over the winter, or to form nursery colonies in the summer period. Under a colony of bats, a heap of guano is formed, and for nutrient-poor environments, such as caves, guano represents an important source of nutrients and microorganisms. Bat guano is frequently characterized by elevated concentrations of heavy metals (e.g. Cd, Cu, Zn), and with aging it obtains a low pH (~3.0). Data on the presence and diversity of free-living amoebae in cave environments are scarce. In the current study, bat guanos of different ages from five caves in Slovenia (Huda luknja, Predjama, Spodnja klevevška jama, Škocjanske jame, Turjeva jama) were screened for the presence of free-living amoebae by culture and PCR. All guano samples were positive for amoebozoans by PCR and, except for two very old guano heaps (Turjeva jama, Škocjanske jame) where no fresh guano had been deposited for many years, all samples were also positive for free-living amoebae by culture. The isolated amoebae were identified as belonging to the genera Hartmannella, Vannella, Thecamoeba and/or to the mycetozoans. Interestingly, no representatives of the genus Acanthamoeba, one of the most ubiquitous amoebozoan genera, were found. Free-living amoebae were generally present in fresh guano samples that were minimum one season old and had high total counts of cultivable bacteria (~108 CFU/g) and fungi (~105 CFU/g), whereas old guano samples with low concentrations of or no cultivable bacteria and fungi ( 104 CFU/g) were negative for amoebae by culture. Altogether, this study indicates that the presence of cultivable free-living amoebae in guano is directly related to high concentrations of bacteria and fungi, and that old guano as a habitat with very low pH is less supportive for microbial life.

Poster

## Naegleria fowleri electrondense granules secretion (EDG): Ultrastructural study

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The free living amoeba Naegleria fowleri is potentially pathogenic for humans causing the primary amoebic meningoencephalitis, a low frequency but rapidly fatal infection in persons exposed to this amoeba in warm recreational waters. Infection occurs when trophozoites invade the nasal mucosa and migrates to the brain causing hemorrhagic necrosis such as destruction of tissues and extracellular matrix. Despite the gravity of this infection, to date the cytolytic mechanisms of Naegleria fowleri is scantily known. EDG are small structures present in many species of amoebae. Secretion of these granules was seen in Entamoeba histolytica in presence of target cells or collagen, the main component of extracellular matrix. X-ray microanalysis revealed that these granules contain a complex of cationic proteins with proteolytic activity, along with collagenase and it has been suggested that they participate in the pathogenesis produced by this invasive amoeba. Using electron transmission electron microscopy we analyzed the presence and secretion of EDG in N. fowleri trophozoites recovered from mice brain lesions. These trophozoites presented numerous granules in the cytoplasm or associated to the plasma membrane. When these trophozoites were incubated with collagen substrates or MDCK cell monolayers, EDG were identified protruding from the amoeba or secreted to the incubation medium. In addition, both collagen substrate and monolayer were phagocyted by the trophozoites which presented numerous endocytic invaginations medium. Our results suggest that these granules may have an important role in the cytophatic and invasive mechanisms of this amphizoic amoeba.

Poster

## Isolation of Acanthamoeba strains from fresh waters in Northeast and Lower Northern Thailand

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Acanthamoebae are free-living organisms that can be found in various types of environments, including fresh water. By morphology-based classification Acanthamoeba has been divided into three major morphological groups. Generally, non-pathogenic Acanthamoeba are in morphological group I, the majority of keratitis causing strains belongs to morphological group II and most GAE causing strains can be found in morphological group III. This study aimed to determine the presence of Acanthamoeba in fresh water habitats in Thailand. A total of 36 fresh water samples were collected from ponds, rivers, water reservoirs and waterfalls in recreation areas in Northeast and Lower Northern Thailand during the summer. Altogether, 31 Acanthamoeba strains were isolated from 17 water samples (47.2% of water samples positive for Acanthamoeba). The most frequently found Acanthamoeba morphological group was morphological group II with 24 isolates, followed by morphological group I having 6 isolates and group III with 1 isolate.

Poster

## Distribution of free-living amoebae in a textile wastewater treatment system

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It is well known that free-living amoebae can habitat a variety of environs, but few researches have been performance in industrial wastewater, specifically in wastewater from textile industry. For this reason the objectives of the research were to determine the presence and distribution of free-living amoebae in an activated sludge system that treats wastewater from a wool industry. Six samplings were carried out during a year; the samples were taken at the input, aeration tank, sedimentation tank and output of the system. Fifty milliliters of the samples were centrifuged and the sediment seeded on NNE, the plates were incubated at 30 and 37 °C. The isolated amoebae were identified morphologically. Free-living amoebae were detected in all the samples. A total of 13 genera were isolated at both incubation temperatures. Acanthamoeba, Dactylamoeba, Echinamoeba, Hartmannella, Mayorella, Naegleria, Platyamoeba, Saccamoeba, Thecamoeba, Vahlkampfia, Vannella, Vexillifera and Willaertia were isolated at 30°C and 37°C, but with the only difference that at 37°C was isolated Stachyamoeba instead of Willaertia. The amoebae most frequent were Acanthamoeba and Hartmannella at both temperatures. It was interesting the finding that free-living amoebae may be present in this kind of wastewater despite the presence of the chemical used in the stained and manufacture processes of wool. There were free-living amoebae in all the units of the system, but the sediment tank had the highest number of amoebae. The presence of free-living amoebae in the system could be due to great aeration that occurs to the wastewater in tank aeration, which favored high dissolved oxygen concentrations in the wastewater (2.8 to 5.1 mg per liter), and that the pH was not so acid (5.9 to 7.1).

Poster

## Presence of Free Living Amoebae in a Wastewater Treatment Plant used for Irrigation In Tenerife Island, Canary Islands, Spain

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Waste water samples from a local treatment plant in Tenerife island were collected and surveyed for the presence of Free-Living Amoebae (FLA), mostly Acanthamoeba (AC) spp. Samples were filtered and cultured in non-nutrient agar plates. The positive samples were cloned by dilution and checked for the presence of FLA and AC by microscopy and FLA universal and specific PCRs. Most samples were positive for Acanthamoeba. These strains were characterized to the genotype level by sequencing the diagnostic fragment 3 (DF3), belonging most of them to genotype T4. Therefore, the obtained results highlight the presence of potentially pathogenic FLA sources in waste water in Tenerife island.

Poster

## Genotyping and phylogenetic analysis of Acanthamoeba isolates associated with keratitis

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Corneal scraps and contact lenses from 261 patients were examined by cultivation from May 2003 to December 2012, in order to detect the presence of Acanthamoeba sp.. Sixteen cases were positive for Acanthamoeba and eleven could be maintained in culture. The genotypes of these eleven isolates with 9 additional ones were determined by sequencing a partial SSU-rDNA region that we named genotypic extended fragment (GEF), and compared to the strains examined in other studies. The phylogenetic tree inferred from this partial sequence allowed to assign isolates to genotypes. Among the 20 isolates examined, 16 were found to be the T4 genotype, 2 belonged to the T3, one isolate was a T5 and one was a T2, confirming the predominance of T4 in the infections. However, the study highlights other genotypes more rarely associated with infections, particularly the T2 genotype. Our study is the second one to detect this genotype associated with keratitis. Additionally, the phylogenetic analyses showed that five main clusters T4/T3/T11, T2/T6, T10/T12/T14, T13/T16, and T7/T8/T9/T17 emerged and were regularly obtained whatever the method used. A similar branching pattern was found when the full rDNA sequence was investigated.

Poster

## Preliminary survey of testate amoebae (Testacea) in Lithuania

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During 2010 the biodiversity of the testate amoebae in the Kaunas and Vilnius regions of Lithuania were studied. A total of 28 species of testate amoebae, belonging to 13 genera, were identified in moss Pleurozium schreberi. Twelve of them are announced for the first time in the Lithuanian fauna. It was found that the moss growing in a clean place, away from the road is characterized by the highest species diversity - a total of 16 species were found, whereas in moss growing beside the road the species diversity is smaller (8 species). In all of the studied biotopes the spread of the typical representatives of the genus was Centropyxis. Considering the relative significance of the genera, it was found out that the genera Trinema (22,15%), Euglypha (21,2%), Corythion (18,82%), , Centropyxis (9,53%), Assulina (7,8 %) have a dominant role. An attempt was made to explain the structure of the communities of testate amoebae in green and rhizoid parts of moss. In the green parts the numbers of species (25) were slightly higher than in rhizoids part (20 species), nevertheless abundance of testate amoebas was largest in rhizoids part of moss. In green and rhizoid parts of moss dominated species of genera Corythion, Euglypha, Trinema, Assulina.

Poster

## Free-living amoebae (FLA) as reservoir for Legionella pneumophila and other bacteria: Development of a screening system for water facilities in Austria

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Free-living amoebae (FLA) are well known as potential human pathogens. Acanthamoeba spp. and Balamuthia mandrillaris can cause granulomatous amoebic encephalitis (GAE) in immunocompromised humans. Additionally Acanthamoeba causes keratitis in contact lens wearers. Naegleria fowleri causes fatal primary amoebic meningoencephalitis (PAME) in completely healthy humans. Furthermore, FLA may serve as vehicles of dispersal and replicative niches for bacterial pathogens in natural and man-made habitats. In particular, Legionella pneumophila, the causative agent of Legionnaires´ disease, replicates in FLA. In addition, the amoebal cysts protect intracellular pathogens against disinfection measures. This can lead to colonization of air conditioning systems, cooling water devices and warm water preparation units, from where the bacteria spread via aerosols. Currently we are lacking suitable screening assays for fast and synchronous detection and identification of FLA. This is why, as part of an interdisciplinary project on the role of FLA as vehicles for bacteria in water systems, we aim to develop real-time PCR assays suitable for routine screening of water facilities and cooling towers in Austria.
The main aim of this study is to come up with a duplex screening system, detecting on one hand the amoebozoans, mainly focusing on Acanthamoeba and Hartmannella, and on the other hand Naegleria spp. and several other genera of the Vahlkampfiidae. For samples positive for amoebozoans a real-time PCR specifically detecting Acanthamoeba spp. will be duplexed with a newly designed real-time assay specific to Hartmannella vermiformis.
In parallel, sampling of water facilities and processing these water samples by filtration, cultivation and DNA isolation was started. The sampling will continue periodically and all collected isolates will be screened by real-time PCR and sequencing.

Poster

## Strains of free-living amoebae from Montego Bay, Jamaica: their morphology and fine structure

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Environmental samples were collected for isolation of free-living amoebae on the premises of the Iberostar hotel complex, Montego Bay, Jamaica, where the last Free-living Amoeba Meeting took place in 2011. In the limited area between the main hotel building and beach, various habitat types were present (e.g., palm garden, ornamental shrub garden near freshwater pool, and beach sand). Three types of samples taken from freshwater habitats included biofilm from palm tree trunk, soil crust, and sediment from koi carp tank. Seawater isolates originated from wet sand and washed-up algae Halimeda sp. on the beach. A total of 17 strains of free-living amoebae were isolated. Here, we introduce morphology and fine structure of some of those. Most of the isolated strains (10 marine and 2 freshwater strains) are members of Vannellidae of different trophozoite size and glycocalyx structure. From crust on soil that apparently had been chemically treated was isolated JAMF3C strain, a cyst forming undetermined amoeba of a most striking morphology, present in two morphotypes, one with smaller trophozoites of the flabellate type and the other one with larger trophozoites of branched type. All vannellid strains have been cryopreserved and supplement a dataset for a phylogeographic study of this widespread group of amoebae.

Poster

## Amoebicidal Efficacy of a Novel Multi-Purpose Disinfecting Solution: First Findings

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Contact lens disinfecting solutions are responsible for contact lens and lens case hygiene by killing pathogen microorganisms. Acanthamoeba is a free living amoebae causing a sight threatening corneal infection, Acanthamoeba keratitis, mostly in contact lens wearers. The use of ineffective contact lens disinfecting solutions is one of the most important risk factors for this infection. A novel multi-purpose contact lens disinfecting solution, OPTI-FREE® PureMoist®, was tested for its efficacy against Acanthamoeba trophozoites by using a most probable number technique for amoebic enumeration in this study. Trophozoites of Acanthamoeba castellanii ATCC 50373 and an environmental strain of Acanthamoeba genotype T4 isolated from tap water in İstanbul were used during experiments. After six hours of disinfection time, OPTI-FREE® PureMoist® achieved total kill against trophozoites of both strains. Since the cysts are more resistant than trophozoites to disinfectants, the efficacy of this disinfecting solution will be tested against Acanthamoeba cysts in next studies.

Poster

## Overexpression of nfa1 and nf-actin genes concerned with contact-dependent mechanism in Naegleria fowleri

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Naegleria fowleri, pathogenic free-living amoeba has been isolated from widespread areas in soil, water, swimming pools, exists as a virulent pathogen which causes fatal primary amoebic meningoencephalitis (PAM) in experimental animal and humans. N. fowleri destroys target cells though the contact-dependent mechanism such as a phagocytosis and the contact-independent mechanism such as a secretion of proteases. The nfa1 gene cloned from a cDNA library of N. fowleri consists of 360 bp, expresses a 13.1 kDa recombinant protein (rNfa1), and localizes on the pseudopodia. The Nf-actin gene consists of 1,124 bp, produces a 50.1 kDa recombinant protein (Nf-actin) and localizes on the cytoplasm, pseudopodia and food-cup structure. In this study, the nfa1 and nf-actin gene were amplified from gene-cloned vector, pEXQP5-T7/NT TOPO. The nfa1 and nf-acin genes were inserted into pEGFP-C2 vector, and then, the pEGFP-C2/nfa1 and pEGFP-C2/nf-actin were transfected to N. fowleri. The strong EGFP fluorescence was observed in N. fowleri transfected with pEGFP-C2/nfa1 and pEGFP-C2/nf-actin. And the expression of EGFP-Nfa1 protein was checked by Western blot. We investigated the activity of adherence, phagocytosis and cytotoxicity from the nfa1 or nf-actin overexpressed N. fowleri as comparison with wild type N. fowleri. The nfa1 or nf-actin overexpressed N. fowleri showed strongly increasing adherence in extracellular matrix components such as fibronectin, collagen I and fibrinogen compare to wild type N. fowleri. Moreover the nfa1 or nf-actin overexpressed N. fowleri showed increasing phagocytosis and cytotoxicity compare to the wild type N. fowleri. Finally, these results suggest that the nfa1 or nf-actin gene plays an important role in cell adhesion, phagocytosis and cytotoxicity of pathogenic N. fowleri.

Poster

## GROWTH ABILITY OF GRAM NEGATIVE BACTERIA IN FREE LIVING AMOEBA

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When bacteria and free living amoebas (FLAs) live both in natural waters and man-made water systems, they interact with each other constantly. Some bacteria can survive and grow within FLAs. So, it has been thought FLAs play an important role in spreading pathogen bacteria in water systems recently. In this study we investigated the growth ability of 7 different Gram negative bacteria (Pseudomonas fluorescens, Pseudomonas putida, Pasteurella pneumotropica, Aeromonas salmonicida, L. pneumophila serogroup 1, L. pneumophila serogroup 3, L. pneumophila serogroup 6) within four different FLA isolates (A1, A2, A3, A4). Of these, four bacteria isolates (Pseudomonas fluorescens, Pseudomonas putida, Pasteurella pneumotropica, Aeromonas salmonicida) and two free living amoeba isolates (A3, A4) were isolated from the tap water in our city (İstanbul). It was found that 4 different Gram negative bacteria could grow in A1, 2 different Gram negative bacteria could grow in A2, 4 different Gram negative bacteria could grow in A3, 1 Gram negative bacteria could grow in A4. In conclusion, we think that this growth ability could vary according to the characteristics of both bacteria and FLA isolates.

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