XVIII th International Meeting on the
Biology and Pathogenicity of Free-living Amoebae
Costa Rica 18- 22 november 2019

FLAM 2019

Puntarenas, Costa Rica

ABSTRACT BOOK

FLAM 2019

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Dr Lissette Retana-Moreira (University of Costa Rica)

Dr Mónica Prado-Porras (University of Costa Rica)

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Dr. Yann Hechard (University of Poitiers, France)

XVIII th International Meeting on the Biology and Pathogenicity of Free-living Amoebae
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FLAM 2019 PROGRAMME

TUESDAY 19TH NOVEMBER 2019

9.00 Opening Ceremony FLAM 2019

9.30-10.30 KEYNOTE LECTURE 1. Dr Paul A. Fuerst. Species, sequence types and alleles: dissecting genetic variation in *Acanthamoeba.*

COFFEE BREAK: 10.30-11.00

11.00-13.00 **ORAL SESSION 1: *ACANTHAMOEBA.***

Chairs: Dr Paul A. Fuerst and Dr Lissette Retana-Moreira

11.00-11.15. Or-1. Isabel Marcelino. Cartography of free-living amoebae in Guadeloupe using metabarcoding.

11.15-11.30: Or-2. Ascel Samba. Identification of the earliest pathways involved in the encystment of *Acanthamoeba castellanii*.

11.30-11.45: Or-3. Jesús Serrano Luna. *Acanthamoeba* cysteine proteases are able to degrade iron-binding proteins to acquire iron from the human host.

11.45-12.00. Or-4. A-Jeong Ham. Mouse model for an experimental *Acanthamoeba* keratitis.

12.00-12.15: Or-5. Maritza Omaña-Molina. Morphological description of the early events of the *Acanthamoeba castellanii* in a murine model of skin irradiated under UV-B light.

12.15- 12.30: Or-6. Dolores Hernández-Martínez. Stimulation of cytokines production in healthy and diabetic mice infected with *Acanthamoeba castellanii.*

12.30-12.45: Or-7. Martina Köshler. The thioredoxin reductase system in *Acanthamoeba* spp.

LUNCH BREAK: 13.00-15.00

15.30-17.00 **ORAL SESSION 2: *ACANTHAMOEBA* THERAPY-1.**

Chairs: Dr Julia Walochnik and Dr Donald Munson

15.30-15.45: Or-8. Julia Walochnik. *Acanthamoeba* disinfection.

15.45-16.00: Or-9. Christopher A. Rice. Screening the Calibr ReFRAME drug library yields new repurposing drug candidates for the treatment of diseases caused by pathogenic free-living amoebae.

16.00-16.15: Or-10. Christopher A. Rice. Discovery and development of trophocidal and cysticidal compounds for the treatment of *Acanthamoeba* infections.

16.15-16.30. Or-11. Sebastien Pomel. Evidence of a natural resilience phenomenon to amphotericin B in *Acanthamoeba* sp.

16.30-16.45. Or-12. Edyta B. Hendiger. *In vitro* effect of silver and gold nanoparticles conjugated with contact lens solutions against *Acanthamoeba.*

16.45-17.00. Or-13. Desirée San Nicolás-Hernández. *In vitro* activity evaluation of *Laurencia* derivatives against *Acanthamoeba castellanii* Neff.

COFFEE BREAK: 17.00-17.30

17.30-18.30 **POSTER SESSION.** *Flash Presentations of posters. Free-Living Discussion.*
Chairs: Dr Maritza Omaña-Molina and Dr María Reyes-Batlle

WEDNESDAY 20TH NOVEMBER 2019

9.30-10.30 KEYNOTE LECTURE 2. Prof. Dr. Patrick L. Scheid. *Vermamoeba vermiformis* - a free-living amoeba with public health and environmental health significance?

COFFEE BREAK: 10.30-11.00

11.00-13.00 **ORAL SESSION 3: *ACANTHAMOEBA* THERAPY-2.**

Chairs: Dr Fernando Lares Villa and Dr Yann Hechard

11.00-11.15. Or-14. Brian Shing. Identification of a novel Conazole that is both amebicidal and cysticidal against *Acanthamoeba castellanii.*

11.15- 11.30. Or-15. María Reyes Batlle. *In vitro* evaluation of combined commercialized ophthalmic solutions against *Acanthamoeba* strains.

11.30-11.45. Or-16. Atteneri López-Arencibia. Toxic effects of selected proprietary dry eye drops on *Acanthamoeba.*

11.45-12.00: Or-17. Ines Sifaoui. Programmed Cell Death in *Acanthamoeba* induced by Staurosporine isolated from *Streptomyces sanyensis*.

12.00-12.15: Or-18. Carlos J. Bethencourt Estrella. *In vitro* biological evaluation of phosphodiesterase inhibitors against *Acanthamoeba*: a preliminary study of cell death induction.

12.15-12.30: Or-19. Jennifer R. Cope. *Acanthamoeba* disease associated with the practice of nasal rinsing in immunocompromised patients.

12.30-12.45: Or-20. Maritza Omaña-Molina. Schwann cell's autophagy as mechanism of cell death by *Acanthamoeba*.

12.45-13.00: Or-21. Maritza Omaña-Molina. Effect of Taurine on the pathogenic mechanisms of *Acanthamoeba castellanii* in the *ex vivo* model of amoebic keratitis.

13.00-15.00 LUNCH BREAK

AN AFTERNOON WITH *Naegleria fowleri*: DEDICATED TO THE MEMORY OF JORDAN SMELSKI, HOSTED BY STEVE AND SHELLY SMELSKY.

Chair: Dr Lissette Retana Moreira, Dr Elizabeth Abrahams-Sandi and Dr Jacob Lorenzo-Morales

15.00-16.30 **JORDAN SMELSKI ORAL SESSION 4 PART 1. ADVANCES IN THE KNOWLEDGE OF VIRULENCE FACTORS AND INMMUNOBIOLOGY OF *Naegleria fowleri*.**

15.00-15.15: Or-22. Mineko Shibayama: A 23-kDa membrane protein involved in the virulence of *Naegleria fowleri.*

15.15-15.30: Or-23. Hae-Jin Sohn. Molecular cloning and characterization of *Naegleria fowleri* profiling.

15.30-15.45: Or-24. Maricela Carrasco Yepez. Immunogenicity and vaccine potential of a peptide from *Naegleria gruberi* Glyceraldehyde 3-phosphate dehydrogenase against *Naegleria fowleri* infection.

15.45-16.00- Or-25. Diego Alexander Rojas. Role of the FcγRI and FcγRIII receptors in the nasal mucosa of Balb/c mice in the MAP protection model.

16.00-16.15. Or-26. Frida Carrillo Morales. IgG, IgA and IgM antibodies response against *Naegleria fowleri* in healthy people from research laboratories.

16.15- 16.30. Or-27. Jennifer R. Cope. U.S. *Naegleria fowleri* updates.

COFFEE BREAK 16.30-17.00

17.00-18.30 **JORDAN SMELSKI ORAL SESSION 4 PART 2. NOVEL RESEARCH ON DIAGNOSTICS AND THERAPY AGAINST *Naegleria fowleri*.**

17.00-17.15. Or-28. Anjan Debnath. Identification of novel sterol biosynthesis inhibitors as new drug leads for *Naegleria fowleri* infection.

17.15-17.30. Or-29. Antoinette C. Russell. Discovery of microRNAs of *Naegleria fowleri* as potential biomarkers for early disease detection.

17.30-17.45. Or-30. Ikrame Zeouk. Evaluation of the in vitro activity of compounds isolated from bioguided fractionation of *Inula viscosa* crude extract against *Naegleria fowleri.*

17.45-18.00. Or-31. Olfa Chiboub. Bio-guided fractionation and isolation of bioactive molecules from *Streptomyces sanyensis* based on its *Naegleria fowleri* activity.

18.00-18.15. Or-32. Emma V. Troth. Development and applications of a novel screening assay using EdU.

18.15-18.30. Or-33. Aitor Rizo Liendo. *In vitro* activity of Statins against *Naegleria fowleri*.

THURSDAY 21ST NOVEMBER 2019

9.30-11.00 **ORAL SESSION 5. FLA AND THE ENVIRONMENT**

Chair: Dr María Reyes-Batlle and Dr Maritza Omaña-Molina

9.30-9.45. Or-34. Vincent Delafont. Free-living amoebae as hosts for Microbial Dark Matter: the case of the phylum Dependentiae.

9.45-10.00. Or-35. Yann Hechard. Warm and green with a pinch of salt, the regimen of ancient amoebae.

10.00-10.15: Or-36. Diego Arturo Castillo Ramírez. Morphologic and Molecular Identification of *Naegleria cardilemsis*, isolated from a Water Park in Hidalgo, Mexico.

10.15-10.30: Or-37. Natalia Karla Bellini. *Naegleria* spp. diversity on Monjolinho River basin at state of São Paulo – Brazil.

10.30-10.45: Or-38. Fernando Lares-Villa. Population dynamics of *Naegleria fowleri* in natural aquatic environments during the period from May 2017 to April 2018.

10.45- 11.00: Or-39. Yann Hechard. Amoebaiome, could we identify FLA in the environment without culture?

11.00-11.30: **FLAM 2021 CANDIDATES PRESENTATION and COFFEE BREAK: 11.00-11.30**

11.30-13.00 **ORAL SESSION 6. OTHER FLA AND INTERACTIONS WITH OTHER MICROORGANISMS**

Chair: Dr Patrick Scheid and Dr Mineko Shibayama

11.30-11.45: Or-40. Thelma Dunnebacke-Dixon. A double infection of an encephalitic survivor with the Ameba*, Balamuthia Mandrillaris,* and a fungus, *Aspergillus terreus.*

11.45-12.00: Or-41: Manuel Alejandro Borquez-Román. *Stenamoeba dejonckheerei* sp. nov., a free-living amoeba isolated from a thermal spring.

12.00-12.15: Or-42: Carolina Hurtado. The Influence of *Acanthamoeba-Legionella* interaction in the virulence of two different *Legionella* species.

12.15-12.30: Or-43. Ángela Magnet. Presence and interaction of free-living amoebae and amoeba-resisting bacteria in water from drinking water treatment plants**.**

12.30- 12.45: Or-44. Issam Hasni. Genome sequencing of *Willaertia magna* C2c Maky: insight into a potential pathogenicity by searching for horizontal gene transfers from pathogenic microorganisms.

12.45-13.00: Or-45. Jennifer R. Cope. U.S. *Balamuthia* case series.

13.00-18.00 SOCIAL AFTERNOON. The attendees of FLAM could choose one of the following activities: a coffee tour, a zip line tour or monkeys and crocodiles sightseeing tour (each tour will be offered only with a minimum of 10 people)

20:00-23:00: DINNER AT THE BEACH (CASUAL CLOTHING)

VOTING FOR NEXT FLAM2021 IF NEEDED WILL BE DONE DURING AND COUNTED DURING THE PARTY

FRIDAY 22ND NOVEMBER 2019

FREE DAY OF DISCUSSION BY THE BEACH/FAREWELL TO PARTICIPANTS

LIST OF POSTERS TO BE PRESENTED IN POSTER SESSION

P-1. Dolores Hernández-Martínez. Ultrastructural analysis of *Acanthamoeba polyphaga* invasion in deep stroma near Descemet's membrane: case report.

P-2. Dolores Hernández-Martínez. Adhesion of *A. castellanii* trophozoites to cosmetic contact lenses.

P-3. Issam Hasni. Differential behavior of *Legionella pneumophila* strains into different amoeba strains including *Willaertia magna* C2c Maky.

P-4. Roberto Mendoza García. Immunomodulatory effect of the 250 kDa glycoprotein of *Naegleria fowleri* on the dendritic cells of the NALT, nasal passage and cervical lymph node of Balb/c mice.

P-5. Mara Gutiérrez Sánchez. Identification of possible candidates for vaccines of immunogenic glycoproteins of *Naegleria fowleri* against primary amebic meningoencephalitis.

P-6. Itziel Berenice Rodríguez Mera. Role of Cathepsin B of *Naegleria fowleri* during Primary Amebic Meningoencephalitis.

P-7. Sául Rojas Hernández. Identification of immunogenic antigens of *Naegleria fowleri* adjuvanted by cholera toxin as vaccine candidates.

P-8. Maryam Niyyati. Molecular characterization of bacterial, viral and fungal endosymbionts of *Acanthamoeba* isolates in keratitis patients of Iran.

P-9. Moisés Martínez Del Castillo. Mucus degradation by *Naegleria fowleri* Glycosidase (Nf-GH).

P-10. Marcin Padzik. Tannic acid-modified silver nanoparticles conjugated with contact lens solutions enhanced their anti-amoebic activity.

P-11. Paul A. Fuerst. The DNA databases for the genus *Acanthamoeba*. An update to 2019.

P-12. Ascel Samba. The overexpression of two new *Acanthamoeba castellanii* proteins impaired the encystment process.

P-13. Patricia Bonilla Lemus. *Naegleria* spp. isolated from irrigation canals of Mexicali Valley, México.

P-14. Maria Wesolowska. *Acanthamoeba* infection as a cause of severe keratitis - the experience of Poland.

P-15. Elizabeth Ramírez Flores. Occurrence of *Acanthamoeba* genotype T10 in a geothermal power plant.

P-16. Rubén L. Rodríguez Expósito. Evaluation of the presence of Free-Living Amoeba in public indoor swimming pools in the North of Portugal.

P-17. Rubén L. Rodríguez Expósito. Isolation and molecular identification of different Free Living Amoeba (FLA) in water sources of Tenerife, Canary Islands, Spain.

P-18. Jacob Lorenzo-Morales. Evaluation of effects of chlorine and temperature in the development of *Naegleria fowleri* and other Free-Living Amoebae: applications in artificial lagoons of recreational use.

P-19. Christopher A. Rice. Discovery of anti-amoebic compounds from screening the MMV Pandemic Response Box on *Naegleria fowleri*, *Acanthamoeba castellanii* and *Balamuthia mandrillaris.*

P-20. Ángela Magnet. Search for natural extracts for the treatment of *Acanthamoeba* amebiasis. A comparative study of two viability methods.

P-21. Carolina Hurtado. Study of the presence of immunoglobulin A in tears from healthy people against *Acanthamoeba* sp.

P-22. Jennifer R. Cope. A Primary Amoebic Meningoencephalitis case associated with surfing in an inland surf park.

P-23. Luis Fernando Lares-Jiménez. Immunogenic surface proteins of *Balamuthia mandrillaris.*

P-24. Johan Alvarado-Ocampo. *In vitro* effects of environmental isolates of A*canthamoeba* T4 and T5 over human erythrocytes and platelets.

P-25. Elizabeth Abrahams-Sandí. *Acanthamoeba* genotype T5 with high patogenic potential isolated from a hospital.

P-26. Lissette Retana-Moreira. Exploring the extracellular vesicles´ world in *Acanthamoeba:* a preliminary study.

P-27. Rubén L. Rodríguez-Expósito. Isolation and molecular characterization of Free-Living Amoebae (FLA) in environmental sources of Santiago island, Cape Verde.

P-28. Katarina Trnkova. Genotyping of strain of free living amoebae isolated from indoor environment in Slovakia regarding to the quality of indoor environment.

KEYNOTE LECTURES

KN-1

Species, sequence types and alleles: dissecting genetic variation in *Acanthamoeba*

Dr. Paul A. Fuerst, University Professor Emeritus, Department of Evolution, Ecology and Organismal Biology, The Ohio State University, 318 W. 12th Avenue, Columbus, OH 43214, USA.

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Species designations within *Acanthamoeba* are problematic because of pleomorphic morphology. Molecular approaches, including DNA sequencing, offered a solution that has yet to be achieved. Alternative approaches were required. In 1996, the Byers-Fuerst lab introduced the concept of Sequence Types. Differences between isolates of *Acanthamoeba* could be quantitatively assessed by comparing sequences of the nuclear 18S rRNA gene, ultimately producing 22 Sequence Types designated T1 through T22. The concept of Sequence Types helps our understanding of *Acanthamoeba* evolution. Nevertheless, substantial variation in the sequence of the 18S rRNA gene differentiates many isolates within each Sequence Type. Genetic variation within the most variable portion of the 18S rRNA gene is of the greatest interest to us, since the majority of isolates in the international DNA databases are studied for only this ~400 bp segment, designated JDP1-JDP2. In 2002, we categorized variation in this region in a sample of isolates from Sequence Types T3 and T4 from Hong Kong. Ten “alleles” were observed within Type T4 and five “alleles” within T3. Subsequent studies by others expanded the number of alleles in Sequence Type T4 to over 40, but generated confusion when different labs used similar numerical labels to identify different alleles. A more unified approach was required. In our monitoring of sequence submissions to the DNA databases, we have tabulated alleles occurring in the data, and determined their frequency. Over 135 alleles have occurred more than once within 3400+ isolates of Sequence Type T4. The distribution of alleles within the universe of T4 isolates provides insight into major sub-types of T4. Allele frequencies of alleles existing in multiple isolates were tabulated, and the distribution of allele frequencies determined, providing insight into the evolutionary history of the T4 Sequence Type. Other Sequence Types also contain multi-isolate alleles. Within 229 isolates of Type T3, only 12 shared alleles occur, while 11 such alleles appear in 100 isolates of Type T11. For Type T5, 12 shared alleles appear within 328 isolates. Additional insights from the examination of alleles will be discussed. Information on allele sequences and frequencies can be found on our web site: http://u.osu.edu/acanthamoeba/alleles-within-sequence-types-2/.

KN- 2

Pathogenic free-living amoebae in humans

Prof. Dr. Patrick Scheid, Central Military Hospital Koblenz, Germany. Dep. XXI, Microbiology; Parasitology Lab; Rheinkaserne; Andernacherstr. 100; 56070 Koblenz, Germany

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Abstract:

The presence of several facultative **parasitic Free-living amoebae (FLA)** in habitats related to human activities supports their public health relevance. In several studies, *Vermamoeba vermiformis* has been found within the human environment, showing paradigmatically how easily humans may find themselves in close contact with these FLA. The medical significance of *V. vermiformis* (resp. *Hartmannella vermiformis*) has been reported. Two human cases involving *V. vermiformis* have been recently examined in Germany: *V. vermiformis* was detected within the contact lens cases of a bacterial keratitis patient. In this "case report" *V. vermiformis* seemed to be a contaminant without (significant) involvement in the pathogenesis. A second case included the only non-keratitis case report so far - with an exclusive isolation of *V. vermiformis* from a human.

V. vermiformis has been confirmed as potential etiological agent in a 27 years old female patient, who presented with a weeping wound developing as a painful ulcer on the upper eyelid at the medial angle of the right eye. The confirmed presence of *V. vermiformis* in the ulcer and the proven absence of the typical pathogenic bacterial microorganisms led to the strong assumption that *V. vermiformis* may be involved in the pathogenesis of this human case.

Additionally, a wide range of FLA is known as **vectors of pathogenic microorganisms** (**endocytobionts**), hereby emphasizing their environmental significance. Among those FLA serving as hosts for and vectors of (pathogenic) endocytobionts, there are also descriptions of *V. vermiformis* as a vehicle and a reservoir of those endocytobionts. These endocytobionts may also play a significant role in aggravating the infection or in enhancing inflammatory processes
The involvement in animal and human health, the role as vector of pathogenic microorganisms and the pathogenicity in cell cultures, led to the assumption that *V. vermiformis* should be considered relevant in terms of public health and environmental health.

ORAL SESSIONS

ORAL SESSION 1

Acanthamoeba

CHAIR:

Dr Paul A. Fuerst and Dr Lissette Retana-Moreira

Or-1

Cartography of free-living amoebae in Guadeloupe using metabarcoding

Yann Reynaud[1], Celia Ducat, Antoine Talarmin[1], **Isabel Marcelino**[1]

[1]TReD-Path Unit (Transmission, Réservoirs et Diversité des Pathogènes), LEMic (Laboratoire Interactions des Ecosystèmes Microbiens), Institut Pasteur de la Guadeloupe, Morne Jolivière 97183 Abymes, Guadeloupe, France.

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The free-living amoeba *Naegleria fowleri* is found in most geothermal baths of Guadeloupe and has been responsible for the death of a 9-year-old boy who swam in one of these baths in 2008. Our group determined that soil is the origin for the presence of *N.fowleri* and other thermophilic amoebae in these thermal recreational waters.

The work presented herein aimed to conduct a rRNA-targeted metagenomic analysis on free-living amoebae (FLA) present in soil in Guadeloupe, and hereby provide the first FLA environmental metabarcoding analysis in a Caribbean island.

A total of 107 soil samples were collected from 27 sites, nearby water ponds, hot springs and rivers. DNA was extracted directly from soil samples or from FLA cultivated at different temperatures (30, 37 and 44ºC). Metabarcoding studies were then conducted through FLA 18S amplicons sequencing; amplicon sequence variants (ASV) were extracted from each sample and taxonomy assigned against SILVA database using QIIME2 and SHAMAN pipelines.

Vermamoeba were detected in DNA extracted directly from the soil, but to detect other FLA an amoebal enrichment step is necessary. The cultures were mainly enriched with *Naegleria*, *Acanthamoeba* and *Vermamoeba*, at 37ºC and 44ºC. High differences in FLA diversity were observed between the 27 sites. *V. vermiformis* was by far the most represented species of FLA, being detected throughout the islands while the genus *Naegleria* was mainly found in Basse-Terre. Nevertheless, putative pathogenic *N. fowleri* was also detected in Grande Terre and in Les Saintes Islands. *Acanthamoeba* were mainly found in areas where temperature is approx. 30ºC.

Our results clearly show that FLA are widespread in Guadeloupe, posing a potential threat (direct or indirect) on human health. Similar studies could be performed in neighboring Caribbean islands.

Or-2

Identification of the earliest pathways involved in the encystment of *Acanthamoeba castellanii*

Cyril Noel[1], Clément Bernard[1], Yann Héchard[1] and **Ascel Samba-Louaka[1]**

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Most of free-living amoebae (FLA) have the ability to differentiate into cysts enabling them to survive under harsh environmental conditions. The process leading to the formation of a cyst, encystment, induces a decrease of the cell metabolism while allowing construction of a carbohydrates-containing cyst wall and energy storage. Even if several biological functions involved in the encystment have been characterized, the whole process remains unclear. To highlight the earliest pathways activated or repressed during the encystment, we performed a transcriptomics analysis of the FLA *Acanthamoeba castellanii* as soon as 1h, 4h and 8h after the addition of an encystment-inducing medium. We found thousands of transcripts up- or down-regulated during the encystment and at specific time-points. Transcripts differentially expressed during encystment seem involved in biological processes already reported such as autophagy. However, these data will allow exploration of new functions that could be involved in the encystment. For example, some transcripts related to the cell death were also differentially expressed and the analysis of the uncharacterized transcripts could reveal functions that are specific to *Acanthamoeba*. Interestingly, we have been able to detect an up-regulation of the cyst-specific protein 21 (csp21) as early as 1h after the addition of the encysting medium underlying the high sensitivity of the analysis. Further analysis of the earliest regulated transcripts will give us clues to uncover the initial pathways of the *Acanthamoeba* encystment.

Or-3

Acanthamoeba castellanii cysteine proteases are able to degrade iron- binding proteins to acquire iron from the human host

Jesus Serrano Luna[1], Gerardo Ramirez-Rico[1], Moises Martinez-Castillo[2,3], Mireya de la Garza[1], and Mineko Shibayama[2]

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Acanthamoeba castellanii, is a free-living amoeba, it is an amphizoic organism. It behaves as an opportunistic organism causing granulomatous amoebic encephalitis. In immunocompetent persons is able to cause corneal and cutaneous lesions. Acanthamoeba spp. are protists that are ubiquitously distributed throughout the environment. This protozoan can be in contact with different iron containing proteins such as; human holo-lactoferrin (Hholo-Lf), human holo-transferrin (Hholo-Tf), human hemoglobin (HHb), and horse spleen ferritin (HsF) in the human body. Since some years ago it is known that pathogenic microorganisms developed some mechanisms to acquire iron from the host. *A. castellanii* has different serine and cysteine proteinases that are able to degrade different host substrates. In this study we described that these proteases are able to degrade these human iron binding proteins.

Acanthamoeba castellanii trophozoites isolated from human cases of amoebic keratitis were kindly provided by Dr. Simon Kilvington (Public Health Laboratory, Bath, UK). We performed protease activities from the amoebic total crude extract (TCE) and from conditioned culture medium (CM). Proteases were analyzed using 10% SDS-PAGE gels copolymerized with 0.1% of the following substrates: Hholo-Lf, HholoTf, HHb or HsF at 37°C at different pHs (5.0-9.0) and with or without protease inhibitors.

In this study, we showed the presence of different *A. castellanii* proteolytic enzymes in both the TCE and CM that are able to degrade iron-binding proteins toward all of the tested substrates

within a wide range of pHs (5.0–9.0) at 37 °C. We also found that cysteine proteases participated importantly in the degradation of all tested iron binding proteins.

With all these data we conclude that *A. castellanii* trophozoites have several cysteine proteases that are able to degrade iron-binding proteins. These proteases could be involved in iron uptake and may represent a virulence factor for *A. castellanii*.

This study was supported by CONACyT grant numbers: 167431 and 237523.

Or-4

Mouse model for an experimental *Acanthamoeba* keratitis

A-Jeong Ham, Heekyoung Kang, A-Young Park, Jae-Ho Lee, Jeong-Heon Lee, Hae-Jin Sohn, Ho-Joon Shin*

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Acanthamoeba castellanii results in Acanthamoeba keratitis (AK), which occurs mainly in poor contact lens user. The findings of many in vitro studies of AK, especially the development of therapeutic drugs, need to be confirmed by in vivo experiments. For the establishment of an AK mouse model, in this study, *A. castellanii* cell suspensions (equal mixtures of trophozoites and cysts) were loaded onto 2-mm contact lens pieces to be inserted into mouse eyes that were scratched using an ophthalmic surgical blade under anesthesia; the eyelids of the mice were sutured. Keratitis symptoms were grossly observed, and PCR was performed using P-FLA primers for amplification of the Acanthamoeba 18S-rRNA gene from the mouse ocular tissue. The experimental AK mouse model, characterized by typical hazy blurring and melting of the mouse cornea, was established on day 1 post-inoculation. AK was induced with at least 0.3×10^5 *A. castellanii* cells (optimal number, 5×10^4), and the infection persisted for 2 months. In addition, PCR products amplified from the extracted mouse eye DNA confirmed the development of Acanthamoeba-induced keratitis during the infection periods. These results induced similar AK development even when inoculated with *A. castellanii* trophozoites only (5×10^4). In conclusion, it is suggested that the present AK mouse model may serve as an important in vivo model for various future studies.

Or-5

Morphological description of the early events of the *Acanthamoeba castellanii* in a murine model of skin irradiated under UV-B light

Mariana Hernández-Jasso[1], Dolores Hernández- Martínez[1], Guillermo Ávila- Acevedo[2], José del Carmen Benítez-Flores[3], Isis Gallegos-Hernández[4], **Maritza Omaña- Molina[1]**

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Acanthamoeba spp. are opportunistic protozoa having gained medical importance due to induce ulcers in people who suffer skin lesions or burns, as well as in immunosuppressed people, with HIV and cancer, constituting the first focus of infection, to later spread to other organs.

Early morphological events of *Acanthamoeba castellanii*-induced invasion are described in a murine model of skin infection in SKH-1 mice irradiated under UV-B light. Five male mice were chronically UV-B light irradiated (32 weeks). Two mice were selected showing obvious lesions on the skin (group 1), on which trophozoites (2.5 x 10_5) were placed on. One of them was sacrificed 48 h post-interaction. The other one remained under observation and sacrificed when signs of CNS infection were observed (18 days). The remaining three mice (group 2) although not shown skin lesions, were inoculated subcutaneously with 2.5 x 10_5 trophozoites in 5 pre-selected areas of the back of the irradiated mouse and were sacrificed at 24, 48 and 72h post-inoculation. The ventral area was considered as a control (non-irradiated zone). Skin cuts were processed by conventional immunohistochemistry technique using rabbit anti-*A. castellanii* hyperimmune serum at a 1:100.

In the group 1, trophozoites were observed in various dermal areas, penetrating epithelial tissue, and interacting with various dermal elements, such as the collagen (collagenolytic activity), hair cysts, sebaceous glands and blood vessels, which suggests their spread to the rest of the body. Similarly, as they do in human cornea and hamster cornea; trophozoites adhered, migrated and penetrated through cell junctions. Trophozoites were immunolocalized in the spleen and brain without the presence of apparent inflammatory infiltrate in samples from 18 days sacrificed mouse. In group 2, few trophozoites were observed in comparison to group 1, however, were located nearby collagenolytic activity. Control samples (non-irradiated/ventral zone) showed homogeneous cell layers, without collagenolytic activity.

The invasion of *A. castellanii* in skin with lesions caused by UV-B irradiation is significantly higher compared to the skin of non-irradiated control areas, suggesting that these are more susceptible

to infection, as well as the presence of amoebae in vessels demonstrates the spread to CNS through a skin lesion.

Or-6

Stimulation of cytokines production in healthy and diabetic mice infected with *Acanthamoeba castellanii*

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Acanthamoeba spp. are free-living organisms with the capacity to cause pathologies in humans such as granulomatous encephalitis, a chronic and necrotizing disease that is generally fatal, mainly associated with immunosuppressed persons or with chronic diseases such as diabetes mellitus. Until now considerable information remains to be clarified related with the factors of susceptibility and resistance established during the host-parasite interaction. In this study we evaluated the concentration the pro-inflammatory and anti-inflammatory cytokines during the early stages of *Acanthamoeba castellanii* infection.

Intranasal inoculation of 1 x 10^6 trophozoites of *A. castellanii* was performed in healthy and diabetic (previous induction with streptozotocin) BALB/c male mice of 14 weeks; accordingly to the following scheme: Healthy + *A. castellanii* (IH), Diabetics + *A. castellanii* (ID); Uninfected Healthy (UH) and Uninfected Diabetics (UD). Mice in study were sacrificed 24, 48 and 72h post-inoculation (PI), after taking a blood sample; nasal washes (NW) were also performed with sterile PBS. The concentration of GM-CSF, TNF-α, IFNγ, IL-1β, IL-2, IL-5, IL-4 and IL10 was determined using the Bio-PlexPro kit.

The cytokine concentration of all NW samples from the different groups was similar to the group of UH mice (≤ 20 pg/ml, generally), only in some of the times evaluated the GM-CSF, IL-1β, IL-2, IL-5 and IL-10 recorded concentrations in IH and ID mice slightly higher than controls. All cytokines evaluated in serum shown a higher concentration in IH and ID mice, being statistically significant only in GM-CSF (F=3.51 α 0.05). TNF-α and GM-CSF reached the highest serum concentrations, around 3000 and 300 pg/ml, respectively. Remarkably, in UD mice the concentration of cytokines was also higher than UH, which confirms the low initial proinflammatory status of diabetic individuals and is shown at slightly higher cytokine concentrations in ID mice.

Our results suggest that the early intranasal invasion of *A. castellanii* in IH and ID mice locally stimulates a weak inflammatory response, accompanied systemically by the antagonistic production of proinflammatory, anti-inflammatory and IL-10 cytokines, which could modulate the immune response and favor the invasion of these amoebae without stimulating an important inflammatory process as previously reported.

Or-7

The thioredoxin reductase system in *Acanthamoeba* spp.

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Acanthamoeba, like many organisms, have two essential redox networks for the defence against oxidative stress - the glutathione-mediated and the thioredoxin-mediated network. Of particular interest is the fact that the thioredoxin-mediated network of *Acanthamoeba* consists of two distinct thioredoxin reductase (TrxR) genes, one of the high molecular weight type (H-TrxR) and one of the low molecular weight type (L-TrxR). While H-TrxR has been identified in higher eukaryotes, including humans, L-TrxR has been found in archaea, bacteria and lower eukaryotes. To date *Acanthamoeba* is arguably the only organism known to have both types of TrxR. The uniqueness of this network in *Acanthamoeba* together with the fact that redox networks pose a highly promising drug target, clearly suggest to investigate the redox networks of *Acanthamoeba*.

In this study the expression of several key factors of both redox systems was investigated at the mRNA level. Clinical and environmental *Acanthamoeba* strains were grown in the presence of hydrogen peroxide and diamide, a thiol reducing agent. After RNA isolation and cDNA synthesis, qPCRs targeting two genes for thioredoxin (Trx1, Trx2), H-TrxR, L-TrxR, the glutathione reductase, three peroxiredoxins (Px2-4) and two glutaredoxins (GLR1, GLR2) were performed.

It was shown that, while both TrxR are expressed throughout the cell cycle of *Acanthamoeba*, treatment with H2O2 and diamide had only a pronounced effect on the expression of the L-TrxR, which was significantly increased, while the H-TrxR was unaffected. Also the expression of Trx1 and Px3 was significantly elevated. Enzymes involved in the glutathione-mediated network, however, were only affected in their expression by treatment with diamide.

Our results indicate that the L-TrxR is the first line of defence against different oxidative stressors in *Acanthamoeba*. The fact that proteins involved in the glutathione-mediated pathway only reacted to treatment with diamide, suggests this pathway is stimulated by different oxidative mechanisms.

ORAL SESSION 2

Acanthamoeba Therapy I

CHAIR:

Dr Julia Walochnick and Dr Donald Munson

Or-8

Acanthamoeba disinfection

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Objectives: Acanthamoebae are among the most abundant microorganisms, particularly in man-made habitats. This ubiquity of *Acanthamoeba* spp., even in treated waters, is mainly grounded by the exceptional resilience of their double-walled cysts. While in the past years quite some effort has been made to find sufficiently effective drugs to treat *Acanthamoeba* infections, the picture on anti-*Acanthamoeba* disinfection is incomplete and mostly focussed on contact lens disinfection. In the current study various established antimicrobial agents, including surface, hand and wound antiseptics were investigated for their *in vitro* efficacy against *Acanthamoeba* trophozoites and cysts.

Methods: All experiments were performed with an *Acanthamoeba* keratitis isolate, genotype T4. The compounds tested included various aliphatic alcohols, phenoxyethanol, octenidine dihydrochloride, polyhexamethylen biguanide (PHMB), potassium peroxymonosulfate, and peracetic acid. Efficacy against trophozoites and cysts was assessed after 1, 5 and 10 min of exposure and 5%, 50% and 95% effective concentrations (EC5/50/95) were determined.

Results: *Acanthamoeba* trophozoites were susceptible to all disinfectants tested, with alcohol-based ones being the least effective. *Acanthamoeba* cysts were inactivated reliably and within feasible time frames only with preparations containing PHMB, octenidine dihydrochloride or quaternary ammonium cations, the latter two being cysticidal even after 1 min of treatment, with EC50s of 0.196 and 15.25 mg/ ml, respectively.

Conclusions and significance: It was shown that *Acanthamoeba* trophozoites and also cysts are generally susceptible to various different disinfectants currently in use. The best efficacy was reached by antimicrobial agents containing octenidine dihydrochloide or quaternary ammonium. These disinfectants were able to kill trophozoites and even cysts reliably within 1 min and were more effective against *Acanthamoeba* than PHMB. PHMB and peracetic acid showed cysticidal effects after longer treatment times. Activated oxygen was effective only against trophozoites, while the alcohol-based disinfectants showed overall poor efficacy.

Or-9

Discovery and development of trophocidal and cysticidal compounds for the treatment of *Acanthamoeba* infections.

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The pathogenic free-living amoeba, *Acanthamoeba*, cause several diseases including a severe brain disease known as Granulomatous Amoebic Encephalitis (GAE), with a 90% mortality rate. *Acanthamoeba* also causes Amoebic Keratitis (AK), in association with poor contact lens hygiene, which may result in blindness. The current drug regimens were not originally discovered for *Acanthamoeba* infections but have shown some *in vitro* and *in vivo* potency against these organisms. The high mortality shows that these regimen(s) are not the best indicative treatment for the patients. New specifically developed drugs for these diseases need to be discovered, developed and implemented into the current drug regimen(s) to give patients a better chance of survival or prevent loss of eyes. Herein, using high-throughput screening methods we screened several large compound libraries from Calibr (11,968 compound ReFrame library), an FDA approved drug library (1,134 drug library - Dr. Kiplin Guy formerly at St. Jude Children's Research Hospital) and Medicines for Malaria Ventures (MMV Malaria Box (400 compounds) and Pathogen Box (400 compounds)) in search for new active chemical scaffolds. In our search we identified 67 compounds to possess sub micro to nanomolar potency against *Acanthamoeba* trophozoites, confirmed through dose response secondary screening. Fifty two of these compounds are newly described to have any activity against these amoebae, 15 compounds have been previously recorded within the literature and/or currently used therapeutics. Nanomolar potency compounds were tested for cysticidal activity in a newly developed 30 day high-throughput cysticidal screening

method developed in our lab. In addition via structure activity relationship (SAR) studies we discovered several compounds that possess anti-trophozoite activity. Only the nanomolar inhibitors were further selected and tested for cysticidal activity. Forty two compounds from the SAR studies were discovered to have cysticidal or cystistatic activity against three clinical isolates of *Acanthamoeba* tested at 6 hours of drug exposure. The compounds we discovered are more potent than other known cysticidal agents.

Or-10

Screening the Calibr ReFRAME drug library yields new repurposing drug candidates for the treatment of diseases caused by pathogenic free-living amoebae.

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Pathogenic free-living amoebae *Balamuthia mandrillaris*, *Acanthamoeba castellanii* and *Naegleria fowleri* were all previously identified as the etiological agents of several human diseases. *B. mandrillaris* and *A. castellanii* have both been described to cause cutaneous infections as well as diseases of the brain, *Balamuthia* Amoebic Encephalitis (BAE) or Granulomatous Amoebic Encephalitis (GAE), respectfully. *Acanthamoeba* can also cause ocular keratitis. *N. fowleri* exclusively causes a brain disease known as Primary Amoebic Meningoencephalitis (PAM). Due to the known difficulties in treating these diseases, the unmet clinical need is for highly potent and quickly acting newly drugs to reduce these high mortality rates. An additional need is for potent cysticidal action for diseases caused by *Acanthamoeba* and *Balamuthia*. Herein, we report our efforts for screening the world's largest drug repurposing library with the identification of potentially new anti-amoebic therapeutics. We used CellTiter-Glo 2.0 high-throughput screening methods to screen the Calibr at Scripps ReFRAME library in search for new active chemical scaffolds against pathogenic *B. mandrillaris, A. castellanii* and *N. fowleri*. The ReFRAME library now contains ~13,500 bioactive compounds comprising of roughly 38% FDA approved drugs, 30% of the library is in Phase II/III clinical trials, 19% is in Phase I/0, 3% are in preclinical development stage and ~10% of the library is in an undetermined clinical stage. Initially we screened the library as a single point assay at 5 µM. From our initial screen of ~12,000 compounds against logarithmic trophozoites we identified 220 hit compounds for *B. mandrillaris,* 184 compounds for *A. castellanii,* and 230 compounds against *N. fowleri*. Next we confirmed hits by conducting quantitative dose response assays and validated 63 hits against *B. mandrillaris*, 32 against *A. castellanii*, and 53 against *N. fowleri.* This is by far the largest screen of drugs for these neglected amoebae and these data identify new repurposing drug candidates for the treatment of amoebic diseases and putative drug targets for future studies.

Or-11

Evidence of a natural resilience phenomenon to amphotericin B in Acanthamoeba sp.

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Free-living amoebae (FLA) constitute a heterogeneous group of protozoa found in natural and artificial hydric environments. Several of them can be pathogenic for humans, such as FLA belonging to the *Acanthamoeba* genus which can cause either eye infections with keratitis (AK), or central nervous system disorders with Granulomatous Amoebic Encephalitis (GAE). No standard treatment is currently available for *Acanthamoeba* infections. Nevertheless, amphotericin B has been commonly used for the treatment of GAE, in association with different other drugs. In this work, we analyzed the *in vitro* antiacanthamoebal activity of amphotericin B after different times of treatment. Interestingly, we obtained an IC50 at the micromolar range after 3 days of treatment, which increased markedly at 4 and 5 days of treatment. The amoebae susceptibility to amphotericin B cultured in the presence of 250 µM of the drug was similar to the one of a naive control, revealing that no resistant strain could be selected. However, the amoeba susceptibility always returned to an initial level at each passage. This natural and non-acquired adaptation to amphotericin B, qualified as resilience, was observed in several species of *Acanthamoeba*. Using a pharmacological approach with effectors of different cellular mechanisms, and an ultrastructural analysis of amphotericin B-treated amoebae, the involvement of mitochondria-dependent pathways was determined in the amphotericin B resilience phenomenon.

Or-12

In vitro effect of silver and gold nanoparticles conjugated with contact lens solutions against *Acanthamoeba*

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Acanthamoeba trophozoites are able to attach to the surface of contact lenses. Our previous studies have shown that multipurpose contact lens disinfection systems are not fully effective against *Acanthamoeba*. There is an urgent need to expand research in order to develop contact lens solutions with better anti-amoebic activity. Rapid and successful development of nanotechnology might be a promising approach to achieve this goal. In our previous studies, we showed that nanoparticles can be effective against *Acanthamoeba* clinical strains. The aim of this study was to evaluate the activity of nanoparticles conjugated with selected multipurpose contact lens solutions as well as to test the induced cytotoxicity of these conjugates. To check and compare the activity of three commonly used in Poland contact lenses solutions and their conjugates with gold and silver nanoparticles the Alamar Blue oxido-reduction cytotoxicity assay was used. All tested compounds were used *in vitro* against the trophozoite stage of axenically cultured ATCC 30010 type *Acanthamoeba castellanii* strain. The cytotoxicity assay was performed using a fibroblast HS-5 cell line, based on the measurement of lactate dehydrogenase activity release. Pierce lactate dehydrogenase cytotoxicity assay kit (88953, 88954) was used as per protocol. Results were statistically analyzed by ANOVA and Student-Newman-Keuls tests using the

p>0.05 level of statistical significance. Obtained results showed enhancement of the anti-amoebic activity of selected nanoparticles and contact lens solutions conjugations with a low cytotoxicity profile. Silver nanoparticles conjugation with Solo Care Aqua and Renu contact lenses solutions as well as gold nanoparticles conjugation with Solo Care Aqua enhanced the anti-amoebic effect within the minimal disinfection time recommended by the manufacturers. However, the enhanced dose-dependent anti-amoebic effect with favourable relation to the cytotoxicity was achieved only for silver nanoparticles conjugation with Solo Care Aqua.

In conclusion, silver nanoparticles may be considered as a component of contact lens solutions that increase their anti-amoebic activity without increasing cytotoxicity, which is promising in the field of *Acanthamoeba* keratitits prevention actions.

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Or-13

In vitro activity evaluation of *Laurencia* derivatives against *Acanthamoeba castellanii* Neff

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The amoebas from genus of *Acanthamoeba* spp. are, ubiquitous, free-living amoebae that inhabit a variety of air, soil, and water environments. The amoeba's life cycle includes an active feeding trophozoite stage and a resistant dormant cyst stage. *Acanthamoeba* is an opportunistic pathogen involved in keratitis, in granulomatous amebic encephalitis and cutaneous amebiasis in immuno-compromised patients. Moreover, *Acanthamoeba* can also serve as hosts for endosymbionts, representing a significant reservoir for environmental pathogen/opportunistic microorganisms and food-borne pathogens, with important implications for human health. Furthermore, the antiprotozoal agents, used for the treatment of *Acanthamoeba* are often endowed with unpleasant side effects, irritating and toxic for the host, and the trophozoites and cyst have gained resistance to many disinfectants and chemicals. Recently, the study of bioactive principles from seaweeds shows that they are effective to the treatment of several diseases, including antiprotozoal.

In this sense, the genus *Laurencia* is one of the richest sources of novel secondary metabolites among red algae. Additionally, the chemistry of *Laurencia* species has been exhaustively investigated, and the biological activity of the isolated secondary metabolites has been revealed to

possess antiparasitic properties against several parasites and their vectors. Beside this, the amoebicidal activity of *Laurencia* metabolites has been already reported. The aim of the present study was to evaluate the activity of various compounds derivate from *Laurencia* genus against *Acanthamoeba castellanii* Neff trophozoites and cyst. As well as the cytotoxicity was studied against murine macrophages. The IC50 and LC50 were calculated for all the derivatives. The molecules with the higher amoebicidal activity and the lowest cytotoxicity were selected to study their action mode on the parasite.

Finally, considering all results from the amoebicidal and cytoxicity assays, the derivatives of *Laurencia* genus have a remarkable potential for development of new amoebicidal compounds.

Projects PI18/01380, RD16/0027/0001, CTQ2014-55888-C03-01 and FEDER.

ORAL SESSION 3

Acanthamoeba Therapy II

CHAIR:

Dr Fernando Lares-Villa and Dr Yann Hechard

Or-14

Identification of a Novel Conazole that is both amebicidal and cysticidal against *Acanthamoeba castellanii*

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Objectives: Current treatments for *Acanthamoeba* keratitis rely on disinfectants chlorhexidine, propamidine isethionate, and polyhexamethylene biguanide. The treatment is painful and 10% of cases result in recurrent infection due to the difficulty in killing both *Acanthamoeba* trophozoites and cysts. Therefore, the development of efficient and safe drugs for treating *Acanthamoeba* keratitis is a critically unmet need for averting blindness from *Acanthamoeba* keratitis. The objectives of this study are to identify specific and effective therapeutics capable of curing *Acanthamoeba* keratitis and ultimately preserving vision.

Methods: Since *A. castellanii* CYP51 shares high identity with fungal CYP51 and both trophozoites and cysts contain sterols, we hypothesize treatment with CYP51 inhibitors (azole drugs known as conazoles) would have a deleterious effect on *A. castellanii* trophozoites and cysts. We performed a systematic screen of azole drugs against *A. castellanii* trophozoites and the most potent conazoles were further evaluated against cysts by a newly developed automated cyst counting method.

Results and Conclusions: Two conazoles were identified as having inhibitory activity against trophozoites that exceeded those of standard-of-care drugs. Both drugs demonstrated low

nanomolar potency against three clinical strains of *A. castellanii*. One drug killed trophozoites within 24 hours and suppressed excystment of pre-formed *Acanthamoeba* cysts into trophozoites. This conazole was never reported to show activity against *Acanthamoeba* and is well tolerated in humans when administered systemically.

Significance of Work: This study identified an azole drug that is the most potent amebicidal agent among all the azoles tested thus far against *Acanthamoeba*. In contrast to other CYP51 inhibitors, pre-formed cysts treated with this azole did not excyst when placed into excystation medium. For the first time, we also developed a machine learning-based algorithm for automated image analysis to identify and score the number of *Acanthamoeba* cysts in a drug treatment study. The identification of a target-specific FDA-approved azole that inhibits *Acanthamoeba* cyst viability opens an opportunity for a cost-effective, repurposed treatment for *Acanthamoeba* keratitis.

Or-15

In vitro evaluation of combined commercialized ophthalmic solutions against *Acanthamoeba* strain

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Free-living amoebae of the genus *Acanthamoeba* are present worldwide in natural and artificial environments. Furthermore, they are also clinically important as causative agents of diseases in humans and other animals and are responsible for causing infections such as fatal encephalitis or sight threatening *Acanthamoeba* keratitis (AK) in humans. Lately, studies have been focused on the search of novel therapeutic options in order to treat AK but also to prevent these infections. The evaluation of commercialised products seems to be an option for this case since not clinical assays would be required. Therefore, the activity of the commercial products: Systane® Ultra eye drops (previously reported to present anti-amoebic activity) and Naviblef® Daily Care eye foam was evaluated in this study. In addition, mixtures of both ophthalmic products against *Acanthamoeba* spp. and their cytotoxic effect in murine macrophages was also evaluated using a colorimetric assay. Overall, both compounds were active against *Acanthamoeba* although Naviblef® Daily Care showed to be the most active product. The combination of Systane® Ultra and Naviblef® Daily Care enhanced anti-amoebic activity with low toxic effects.

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Or-16

Toxic effects of selected proprietary dry eye drops on *Acanthamoeba*.

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Amoebae of the genus Acanthamoeba are ubiquitous protists that have been isolated from many sources such as soils, water and the air. They are responsible for infections including fatal encephalitis and a severe keratitis in humans[1]. The keratitis caused by *Acanthamoeba* is increasingly being recognized as a serious infection of the cornea that can lead to a permanent visual impairment or even blindness. To date, there is no satisfactorily effective therapeutic agent against this pathogen and the infections it causes are exacerbated by the existence of a resistant cyst stage produced by this amoeba.

Dry eyes disease (DED) is a more common ocular surface disease that has a severe impact both on quality of live and on cost but it is also a predisposing risk factor for the development of Acanthamoeba keratitis (AK).

In this work we have evaluated the anti-Acanthamoeba activity of a variety of proprietary eye drops intended to treat dry eye syndrome. The ingredients of this eye drops range from compounds as hyaluronic derivatives to sugars as threhalose or carmellose.

From the nine eye drop formulations tested, the one named as “Systane Ultra”, based on propylene and polyethylene glycol, was determined to be the most active against all tested *Acanthamoeba* strains, on trophozoite as well as on cyst stage. During our investigations into the mode of action of Systane Ultra, we discovered that it decreases mitochondrial membrane potential and ATP levels, induces chromatin condensation, and increases the permeability of the plasma-membrane in the trophozoite stage.

All these results lead us to conclude that the Systane Ultra can promote a programmed cell death mechanism on the *Acanthamoeba* strain tested.

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Or-17

Programmed Cell Death in *Acanthamoeba* induced by Staurosporine isolated from *Streptomyces sanyensis*

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Microorganisms namely bacteria and fungi are widely used for various area such as food, pharmaceutical industries among others. Actually, they produce several metabolites namely organic acids, phenyllactic acid, fatty acids, bacteriocins, exopolysaccharides, antibiotics, statins. Furthermore, marine actinomycetes have recently been reported as highly rich in bioactive agents including salinosporamides, xiamycines, indolocarbazoles, naphtyridines, phenols, dilactones such as antimycines, macrolides among others. Those metabolites exhibited interesting activities such as anti-diarrhoea, antimicrobial, anti-tumoral, anti-inflammatory and antioxidant (Demain & Adrio, 2008; Fontana et al, 2013).

In this study, the fractionation of *Streptomyces sanyensis* extract, led to the identification of two amoebicidal compounds the staurosporine and 7 oxo-staurosporine. The action mode of both molecules was studies by the detection of changes in the choramtin condensation, the mitochondrial membrane potential and the ATP levels, the permeability of the plasma membrane and the production of ROS (Reactive oxygen species) in the treated cells. Staurosporine was

shown to induce plasma membrane damage, chromatin condensation, decrease of mitochondrial membrane potential and ATP level as well as increased levels of ROS. Therefore, we could suggest that Staurosporine inhibit the parasite by activation of Programmed Cell Death via the mitochondrial pathway.

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Or-18

In vitro biological evaluation of phosphodiesterase inhibitors against Acanthamoeba: a preliminary study of cell death induction

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Free Living Amoebae (FLA) importance have been *increased* worldwide in the last decades. As an emerging pathogen, widely distributed in different environments, it can produce serious pathologies like *Acanthamoeba* Keratitis (AK) and Granulomatous Amoebic Encephalitis (GAE). Therefore, it is important to develop new treatments against this protozoan due to the nonefficiency of the actual treatments or due to their side effects. Actually, the phosphodiesterase inhibitors (IPDE) have been used in several clinical pathologies such as erectile dysfunction, pulmonary arterial hypertension or others skin diseases, like alopecia or psoriasis.

In the present study, different IPDE have been tested *in vitro* against trophozoite stage of *Acanthamoeba spp*. The first group belongs to different chemical families of human IPDE, and the second one was identified as *in silico* IPDE by a computational study. The activity assays were carried out using the colorimetric assay based on alamarBlue® reagent. To evaluate the cytotoxicity effects in murine macrophages (J774A.1) the colorimetric assay was as well used. In order to determine the type of cell death, assays to study the chromatin condensation and the production of Reactive Oxygen Species (ROS) among others, were performed. Some of the compounds tested in this study were active against *Acanthamoeba spp*. Considering the fact that some products could induce a collapse in the mitochondrial membrane potential as well as chromatin condensation, they could inhibit the parasite growth by Programed Cell Death (PCD). In conclusion, and even though further studies are needed to confirm this action mode, those

products could be considered as a source of potential novel therapeutic agents.

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Or-19

Acanthamoeba disease associated with the practice of nasal rinsing in immunocompromised Patients

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Background: The genus *Acanthamoeba* are free-living amebae found worldwide in water, including tap water, and soil that can cause rare but severe infections of the eye, skin, and central nervous system. *Acanthamoeba* spp. generally cause disease in immunocompromised persons including those with HIV, hematologic malignancies, and solid organ transplants. The route of transmission and incubation period are not well known in humans but animal studies have shown that disease can be produced via the intranasal, intrathecal, and intravenous routes. We describe five cases of *Acanthamoeba* disease among immunocompromised patients who practiced nasal rinsing prior to becoming ill.

Methods: The Centers for Disease Control and Prevention (CDC) offers a clinical consultation service for free-living ameba infections and maintains a Free-living Ameba laboratory with confirmatory diagnostic testing capabilities. When an *Acanthamoeba* case is confirmed in the United States, details about the case are collected on a standardized case report form which includes questions about the case-patient's water and soil exposure prior to becoming ill. Questions about nasal rinsing were added to the form in 2011.

Results: Five *Acanthamoeba* case-patients in CDC's free-living ameba database were reported to have performed nasal rinsing prior to becoming ill. The median age was 60 years (range 36–73 years) and 3/5 patients were female. Two were solid organ transplant patients (heart and kidney), 2 had chronic lymphocytic leukemia, and 1 had HIV. Three patients presented only with encephalitis and died. The two organ transplant patients had a combination of rhinosinusitis, osteomyelitis, and skin lesions. One survived and the other died, the cause of which was unrelated to *Acanthamoeba*. All reported using tap water to perform nasal rinsing, most for sinus congestion using a neti pot or similar device and one for religious purposes.

Conclusions: *Acanthamoeba* is an inhabitant of water, including treated tap water. Immunocompromised patients, like those presented here, might be at risk for infections caused by *Acanthamoeba* transmitted via tap water used for nasal rinsing. Clinicians caring for immunocompromised patients should advise their patients not to use tap water for nasal or sinus rinsing.

Or-20

Schwann cell's autophagy as mechanism of cell death by *Acanthamoeba*

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Several free-living amoebae of the genus *Acanthamoeba* are etiological agents of Granulomatous Amebic Encephalitis (GAE) a necrotizing and hemorrhagic infection of the Central Nervous System (CNS). Although the tropism of the amoebae is mostly to CNS cells, recently, through an *in vivo* GAE model, *Acanthamoeba* trophozoites were observed in contact with Schwann Cells (SC) apparently without cytological damage. As known SC surround nerve sheaths in the peripheral nervous system, produce the myelin that protects these structures and facilitates nerve impulse, the alteration of this cells induce atrophy and destruction of the nerve losing motor function. In this study, we expose the ultrastructural *in vitro* description of the cytopathic effect during the interaction of trophozoites of *A. culbertsoni* (ATCC 30171) and SC (ATCC CRL2941) at early times (1, 2, 3 h). The cytopathic effect was evaluated on SC cultures of *Rattus norvegicus* in a 2:1 ratio. Samples were fixed with glutaraldehyde, then processed for scanning electron microscopy (SEM) and transmission (TEM). Through SEM; at first hour post interaction amoebae were observed adhered to SC cultures, emitting cytoplasmic prolongations or sucker structures in contact with the SC, this was corroborated by TEM, with the observation of microphagocytic channels acquiring plasma content of the SC in digestive vacuoles. In addition, edematous organelles characteristic of

necrosis and multivesicular and multilaminate bodies showing micro and macro-autophagy were observed in SC. At 2 h amoebae migrated and penetrated culture cells, emitting a greater number of sucker structures; necrosis and autophagy persisting in the SC. By 3 h post interaction extensive lytic areas deprived of SC were evidence. It has been reported that amoebae of the genus *Acanthamoeba* are able to cause cell death by apoptosis and necrosis in the cells that invade, we showed that they can also cause cell death by autophagy.

Or-21

Effect of Taurine on the pathogenic mechanisms of *Acanthamoeba castellanii* in the ex *vivo* model of amoebic keratitis

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Amoebae of the genus *Acanthamoeba* spp. are etiological agent of amoebic keratitis (AK); a sight threatening corneal infection.Iit is well known that tear film is the first line of defense during eye infection, nonetheless until now the role played by most of its components on *Acanthamoeba* trophozoites is unknown, specifically if are able to prevent their adhesion and invasion of the corneal tissue. In this study we evaluate the effect of taurine present in high concentrations in tear (195.1 +/- 26.9 µM), on trophozoites of *Acanthamoeba castellanii*; one of the most frequently isolated species of cases of AK.

$2.5x10^5$ trophozoites of *A. castellanii* (Ac) were interacted with hamster cornea (*Mesocricetus auratus*), for 3 and 6 h:

Group a) Corneas + Medium in which the amoeba grew (MA) + Taurine (200 µM).

Group b) Corneas + MA + Ac.

Group c) Corneas + taurine (200 µM) interacting 15 minutes before adding trophozoites of Ac.

Group d) Ac trophozoites interacted 15 min with Taurine (200 µM) before placing them on the cornea.

Group e) Corneas + Ac interacted for 15 minutes and subsequently taurine (200 µM) was added.

Samples were processed according to the conventional technique for scanning electron microscopy.

The results obtained are similar in both times evaluated, for that reason they are shown indistinctly: in **Group a**; no alterations were observed in the corneal surface. In corneas of **Group b**, few adhered trophozoites were observed on corneal surface, some of these initiated migration through cell junctions, however, in corneas of **Groups c**, **d** and **e**, abundant trophozoites were observed, penetrating through different corneal cell areas, emitting phagocytic mouths and destabilizing the corneal architecture in areas far from cell junctions.

Taurine does not prevent the adhesion of the amoebae, neither favors detachment once the amoebae have adhered to the cornea. Besides, promotes penetration into different areas of the epithelium and exacerbate phagocytosis.

According to these results, taurine in physiological concentrations and without the presence of other components of the tear, stimulate pathogenic mechanisms of *A. castellanii* on the corneal surface, instead of preventing its adhesion and invasion of the corneal tissue.

JORDAN SMELSKI ORAL SESSION 4 PART 1

Advances in the knowledge of virulence factors and inmmunobiology of N*aegleria fowleri*

CHAIR: Dr Lissette Retana-Moreira, Dr Elizabeth Abrahams-Sandí and Dr Jacob Lorenzo-Morales

Or-22

A 23-kDa membrane protein involved in the virulence of *Naegleria fowleri*

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The plasma membrane is crucial in the recognition and adhesion of several pathogen microorganisms.
The protozoan *Naegleria fowleri* produces an acute and fatal disease called Primary Amoebic Meningoencephalitis (PAM). Diverse virulence factors have been describe, however, there are few studies related to the membrane proteins of *N. fowleri* involved in the pathogenic mechanisms of this amoeba. The objective of this work was to analyze and compared the expression of a 23-kDa membrane protein (Nf23) in three *Naegleria* species. We realized the *in silico* analysis of the Nf23, and determine the presence of the membrane protein by WB and DB assays. Moreover, we analyze their localization by confocal microscopy studies and the expression by qRT-PCR. These methodologies were realized compared *N. fowleri*, *N. lovaniensis* and *N. gruberi*. The results showed by *in silico* studies that the Nf23 membrane protein is present in the three *Naegleria* species, showing 85.6% of identity. The WB showed that *N. fowleri* and *N. lovaniensis* as well the amoebae recovered from mice brains (Nf23-r) were present in total crude extracts (TCE) and the membrane proteins, the band was more intense in Nf23-r and was absent in *N. gruberi*. The analysis of the mRNA level of *nf23* showed a higher level of expression in *N. fowleri* than in the non-pathogenic amoebae (4-folds and 40,000-fold in *N. fowleri* compared with *N. lovaniensis* and *N. gruberi* respectively). The overexpression of the *nf23* in amoebae recovered from the brain was 5-fold compared from trophozoites axenically cultivated. The established interactions of this protein with others, whether cytosolic or membrane, can influence its role in the molecular mechanisms of pathogenicity in which it is involved, also, could participate in the degree of virulence of *N. fowleri*. We conclude that the Nf23 membrane protein could be participating as another virulence factor in this amoeba. This work contributes to the knowledge of a membrane protein involved in the molecular pathogenesis of *N. fowleri*.

Or-23

Molecular cloning and characterization of *Naegleria fowleri* profilin

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Ubiquitous free-living *Naegleria fowleri* causes a fatal primary amoebic meningoencephalitis (PAM). *N. fowleri* trophozoites are encyst in adverse condition such as lack of food, desiccation and cold temperature. In our previously RNA-Seq (RNA-sequencing) assay, the differentially expressed transcriptome profiles of *N. fowleri* cyst or trophozoites were identified. The upregulated 143 genes in cysts showed 2-fold expression in comparison with trophozoite and 163 genes were downregulated in comparison with trophozoite. In the *N. fowleri* cyst, protein kinase or lipid metabolisms related protein was upregulated, as which serine/threonine protein kinase or sphingosine plays a role in the regulation of cell proliferation, differentiation and death. Especially, *N. fowleri* profilin known as an actin-binding protein involved in the dynamic turnover and restructuring of the actin cytoskeleton was cloned in this study. We constructed the cloning vector for *nf-profilin* gene in *N. fowleri* cyst. The *nf-proflin* gene is composed of 450 bp (encodes 135 amino acid) and produced 16 kDa recombinant protein (rNf-profilin). And the sequence identity was 83 and 38% with nonpathogenic *N. gruberi* and *Acanthamoeba castellanii* respectively. The *nf-profilin* gene was amplified with an *nf-profilin* cloned vector (pET30a vector) and transformed into BL21(DE3) *Escherichia coli*. The recombinant nf-profilin protein (rNf-profilin) was purified with His-conjugated Ni-NTA column. Anti-nf-profilin monoclonal antibody was produced using a cell fusion technique form BALB/c mice immunized with rNf-profilin and observed immunochemical characters. In addition, the rNf-profilin reacted with *N. fowleri* lysates but did not bind to lysates of other amoeba in western blot analysis. The Nf-protein was mainly localized on the cell membrane in *N. fowleri* cyst using immunofluorescence assay. In the further study, we will observe the intracellular localization shifting of the Nf-profilin and explore the correlation with Nf-actin.

Or-24

Immunogenicity and vaccine potential of a peptide from Naegleria gruberi Glyceraldehyde 3-phosphate dehydrogenase against *Naegleria fowleri* infection

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We previously found that intranasal administration of 37 kDa protein in association with cholera toxin increased protection against *Naegleria fowleri* meningoencephalitis in mice. The protein of 37 kDa was immunoreactive by Western blot to monoclonal antibodies for human glyceraldehyde 3-phosphate dehydrogenase (G3PDH).
In the present work, a synthetic peptide, called M1 derived from the primary sequence of *Naegleria gruberi* G3PDH was selected based on lowest homology to human G3PDH. The peptide was designed through theoretical methods where the protein sequence was submitted for multiple alignment analysis, and their three-dimensional models (3-D) were then built by using homology modelling. The sequence was submitted epitope predictors, which included the major histocompatibility complex type II (MHC II) and B-cell peptides.
Balb/c mice were intranasally immunized with M1 peptide in association with cholera toxin. The peptide induced significant increase in the survival of mice vaccinated after the challenge with trophozoites of *N. fowleri*, and it was associated with the production of mucosal and systemic IgA and IgG antibodies that reacted with *N. foweleri* lysates.
The data together indicated the effect of immune responses of G3PDH-derived peptide might be considered as a candidate component for a vaccine against *Naegleria fowleri* infection. These types of tools such as theoretical predictions of immunogenic peptides, nasal immunization and mucosal adjuvants represent promising strategies to be used in experimental vaccine against microorganisms that colonize through the nasal cavity.

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Or-25

Role of the FcγRI and FcγRIII receptors in the nasal mucosa of Balb/c mice in the MAP protection model.

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Naegleria fowleri provoke an acute meningitis that cause death in few days, little is known about the mechanisms involved in the activation of the inflammatory cells of innate immunity during the infection. Therefore, understanding the role of PMN and natural antibodies on infection and pathogenesis of *N. fowleri,* are necessary to generate actions for future treatments and preventions of this infection.
The objective was to determinate the presence and role of the FcγR in the nasal mucosa of mice in the protection model, through of the interaction between neutrophils FcγR and the antigen-antibody complexes.
Immunizations were performed by intranasal route with 100 µg of *N. fowleri* extract, four times in seven days intervals. Then, mice were challenged with lethal doses of live trophozoites of *N. fowleri* by intranasal route.
Neutrophils were purified from peripheral blood of mice and interacted with opsonized *N. fowleri* trophozoites with anti-*N. fowleri* IgG from immunized mice for 60 min. Then, samples were processed by immunofluorescence.
For *in vivo* test, histological sections of 7 µm were obtained from the immunized and challenge mice and stained with fluorescent antibodies then observed by confocal microscopy.
In the interaction experiments, we detected that the PMN from control mice barely approach to the opsonized trophozoites; while, the opsonized trophozoites where interacted with PMN from immunized mice; the trophozoites where surrounded by many PMN. The FcγR was observed on the surface of PMN from the control and immunized group, however, the stain was higher in immunized mice.
We first observed the presence of the FcγR in the nasal cavity of the control and the infected mice, these with increased expression of the receptors compared with control. In the nasal cavity of immunized mice, we found the presence of inflammatory exudate, with positive stain to FcγR, IgG and histone, this stain was observed around some trophozoites.
The presence of the FcγR in the mice nasal mucosa was demonstrated for the first time, and it´s suggested that the receptors induce the possible elimination of *N. fowleri* in immunized animals.

Or-26

IgG, IgA and IgM antibodies response against *Naegleria fowleri* in healthy people from research laboratories.

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Introduction: *Naegleria fowleri* is a free-living amoeba causes Primary Amoebic Meningoencephalitis (PAM) in humans, its mortality rate is 95%. In Mexico have been reported in scientific rigor only 10 cases of PAM from 1978 to 2007, there are many cases underdiagnosed due to ignorance of the disease or similarity with other CNS diseases. The immune factors that protect most people against *N. fowleri* infections are unknown. It has been proposed that IgA and IgG antibodies contribute to protection against *N. fowleri* in murine models. Given the etiology and lack of a vaccine in humans, it is important to analyze the response of specific IgA, IgG and IgM antibodies against *N. fowleri* to observe how the specific immune system react in people exposed to this pathogen and develop tools that facilitate the prevention or timely treatment, thus preventing it from becoming a public health problem.

Objective: To determine the specific serum and saliva IgG, IgA and IgM antibodies response against *Naegleria fowleri* in exposed people to the amoeba.

Materials and methods: Saliva and serum samples were obtained from healthy subjects from research laboratories that manipulate *Naegleria fowleri*, and the specific antibodies response was analyzed by immunoassay (Western blot and ELISA).

Results: We showed the existence of specific IgG, IgA and IgM antibodies response against *Naegleria fowleri* antigens in people that work in research laboratories. Some of these proteins match those already have been reported as immunogenic in our mice protection model against PAM.

Conclusions: The specific antibodies response found to certain *N. fowleri* antigens could be considered in the selection of proteins candidates for the design of a vaccine against MAP.

Perspectives: Currently working on the development of a vaccine based on the band that presented the greatest recognition in subjects with different isotypes (50 kDa).

This work was supported by PAPPIT-UNAM IN224519.

Or-27

U.S. *Naegleria fowleri* Updates

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Background: Primary amebic meningoencephalitis (PAM) is a devastating infection of the brain caused by the thermophilic free-living ameba, *Naegleria fowleri*. Infection can occur when water containing the ameba enters the body through the nose, usually during recreational water activities such as swimming or diving. Historically, in the United States, cases were mostly reported from the warmer southern-tier states. In the last decade, several notable changes have been documented in PAM epidemiology including a northward expansion of infections and new types of water exposures.

Methods: The Centers for Disease Control and Prevention (CDC) offers a clinical consultation service for free-living ameba infections and maintains a Free-living Ameba laboratory with confirmatory diagnostic testing capabilities. When a PAM case is confirmed in the United States, details about the case are collected on a standardized case report form which includes questions about the case-patient's water exposures prior to becoming ill.

Results: From 1962–September 2019, 147 PAM cases have been reported to CDC with a range of 0–8 cases annually. The median age of patients is 12 years (range 8 months–66 years) with over 75% of cases occurring in males. Most cases occur in the southern tier of the United States with over half of all cases being reported from the U.S. states of Florida and Texas. Four cases have occurred in the northern states of Minnesota, Indiana, and Maryland. Most PAM cases occur in the summer months of July and August. Six PAM cases have been associated with nasal exposure to tap water. Two recent cases have been associated with exposure to novel recreational water venues (artificial whitewater river and inland surf park).

Conclusions: Surveillance for PAM cases in the United States demonstrates recent changes in the epidemiology of PAM including northward expansion and new types of water exposures. Efforts to track this high consequence infection should continue in order to inform prevention and control measures.

JORDAN SMELSKI ORAL SESSION 4 PART 2

Novel research on diagnostics and therapy against *Naegleria fowleri*

CHAIR: Dr Lissette Retana-Moreira, Dr Elizabeth Abrahams-Sandí and Dr Jacob Lorenzo-Morales

Or-28

Identification of Novel Sterol Biosynthesis Inhibitors as New Drug Leads for *Naegleria fowleri* Infection

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Objectives: Primary Amebic Meningoencephalitis (PAM), caused by a free-living ameba *Naegleria fowleri*, has a fatality rate of over 97%. PAM has a rapid clinical course and this brain infection lacks effective therapy. Despite the fact that no more than a dozen patients out of about 350 PAM cases reported worldwide have survived, economic interests to invest in development of anti-PAM drugs by the pharmaceutical industry are lacking. Therefore, development of safe and rapidly acting drugs remains a critical unmet need to avert future deaths.

Methods: Since ergosterol is one of the major sterols in the membrane of *N. fowleri*, disruption of isoprenoid and sterol biosynthesis by small-molecule inhibitors may be an effective, novel intervention strategy against PAM. HMG-CoA reductase (HMGR) is the rate-limiting enzyme in the pathway which catalyzes the conversion of HMG-CoA into mevalonate. HMGR inhibitors prevent the conversion of HMG-CoA to Lmevalonate resulting in the inhibition of the downstream sterol biosynthesis and numerous isoprenoid metabolites such as geranylgeranyl pyrophosphate and farnesyl pyrophosphate. Farnesyl pyrophosphate is the natural substrate of farnesyl transferase (FT), which catalyzes the transfer of a farnesyl moiety from farnesyl pyrophosphate to proteins. In this study, we tested well-tolerated and widely used HMGR inhibitors and a novel FT inhibitor against *N. fowleri*. We also undertook a biochemical study to confirm the on-target activity of HMGR inhibitors. Since a successful treatment of PAM requires combination therapy, we determined the growth inhibitory effect of the combination of a novel HMGR inhibitor and the FT inhibitor.

Results: A fast-acting, blood-brain barrier permeable novel HMGR inhibitor was identified as a potent amoebicidal against different clinical strains of *N. fowleri*. Our biochemical studies validated HMGR as a potentially 'druggable' target in *N. fowleri*. A combination of the HMGR inhibitor and the FT inhibitor showed synergy at different drug ratios.

Conclusions and significance of work: This study identified new anti-*Naegleria* compounds via newly validated molecular target in *N. fowleri*. The use of drugs already FDA-approved or in advanced clinical development opens the possibility to cost-effectively repurpose these drugs for the treatment of PAM.

Or-29

Discovery of microRNAs of *Naegleria fowleri* as potential biomarkers for early disease detection.

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The pathogenic free-living amoeba, *Naegleria fowleri* is the causative agent for primary amoebic meningoencephalitis (PAM), an acute brain disease with a case mortality rate of >97%. Several factors contribute to this considerable degree of mortality including delayed diagnosis, ineffective therapeutics and a lack of understanding of the amoebic pathogenesis. Recently, there have been notable advances in the study of the parasitic biology, but the genome still remains poorly annotated and the understanding of the molecular basis for parasite-host interactions is lacking. MicroRNAs (miRNAs) are small secretory molecules that can be found in many bodily fluids. These have been implicated as regulators of gene expression and are currently used as biomarkers for the diagnosis and prognosis of various diseases such as cancer. These miRNAs are also secreted by viruses and parasites to modulate the host immune response and mediate infection. As such, we reasoned that *N. fowleri* might also produce miRNAs for similar purposes. By utilizing deep-sequencing of small RNA libraries, we have computationally predicted 12 novel miRNAs and are validating their presence with qPCR in several *N. fowleri* clinical isolates both *in vitro* and *in vivo*. We aim to uncover conserved miRNAs excreted by clinical isolates of *N. fowleri* to identify biomarkers that can be easily detected and used to further understand the mechanistic basis of PAM.

Or- 30

Evaluation of the in vitro activity of compounds isolated from bioguided fractionation of *Inula viscosa* crude extract against *Naegleria fowleri*

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According to the World Health Organization, about 80% of individuals from developed countries use traditional medicine. Natural compounds could constitute a significant source of new antimicrobial agents taking into consideration that lots of drugs used against infectious diseases are natural or natural derived products. The current study is a bioguided fractionation of the crude ethanolic extract of *Inula viscosa* leaves in order to isolate pure effective compounds against the free-living ameba *Naegleria fowleri*, popularly known as the “brain-eating ameba” which has been classified in category B priority pathogen by the National Institute of Allergy and Infectious Diseases (NIAID). The fractionation led to the isolation of two known and active compounds: a sesquiterpene the Inuloxin A and a flavonoid, the Sakuranetin. Data from this work suggests that *Inula viscosa* might be an interesting natural source of antiamoebic compounds.

This work was funded by PI18/01380, RD16/0027/0001and SAF2015-65113-C2-1-R Spanish MINECO co-funded by FEDER.

Or-31

Bio-guided fractionation and isolation of bioactive molecules from *Streptomyces sanyensis* based on its *Naegleria fowleri* activity

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Introduction: *Naegleria fowleri* is a free-living protist also known as brain eating amoeba. The disease caused by this pathogen is a central nervous system infection known as primary amoebic meningoencephalitis. The high mortality of this disease associated to the limits of treatments available and the increasing incidence of cases urge the need of effective agents against this parasite. Mangrove ecosystem can be a good starting point considering their high biodiversity and richness on organisms such as fungi and actinomycetes that can be an interesting source of bio-active compounds and have been associated to several biological activities such as anti-proliferative, antimicrobial, antiviral, among others. The objective of this current research is to isolate the active compounds from *Streptomyces sanyensis* extract associated to the activity against *Naegleria fowleri.*

Material and methods: *Streptomyces sanyensis* isolated from mangrove was cultured in seawater-based medium. Crude extract was tested for its *in vitro* activity against *Naegleria fowleri* using Alamar Blue assay ® measuring fluorescence which allow us to calculate the concentration inhibition 50% of cells population by nonlinear regression. Bio-guided fractionation of this extract

was performed using several separation methods including column chromatography using silica and sephadex® matrix.

Results: The fractionation of the crude extract of *Streptomyces sanyensis* bioguided by the in vitro activity against *Naegleria fowleri* led to the isolation of four known and active indolocarbazole. Among them Staurosporine and 7-oxo-Staurosporine showed the highest amoebicidal activity. These bioactive molecules will be further experimented in order to evaluate their cytotoxicity and their potential action mode against this life-threating protist.

Keywords: *Naegleria fowleri*, drug discovery, secondary metabolites, bio guided fractionation

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Or-32

Development and applications of a novel screening assay using EdU incorporation for *Naegleria fowleri*.

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Naegleria fowleri is a pathogenic free-living amoeba that is commonly found in warm, freshwater and can cause a rapidly fulminant disease known as primary amoebic meningoencephalitis (PAM). Given that the fatality rate of PAM is >97%, new drugs are urgently needed to treat the disease and until recently, few advances have been made in the discovery of new drugs for *N. fowleri* as well as other pathogenic free-living amoebae. One drawback in advancing the study of the biology of these amoebae is the lack of validated tools and methods to enhance drug discovery and diagnostics. In this study we aimed to validate alternative methods to assess cell proliferation that are commonly used for other cell types and develop a novel drug screening assay to evaluate drug efficacy on *N. fowleri* replication. EdU (5-ethynyl-2´-deoxyuridine) can be used as a quantitative endpoint for cell proliferation; the pyrimidine analog is taken up by the cell and incorporated in place of thymidine in actively replicating cells. EdU is detected via a copper catalyzed click reaction with an Alexa Fluor linked azide. EdU incorporation in replicating *N. fowleri* was validated using fluorescence microscopy and flow cytometry. Quantitative methods for assessing EdU incorporation in *N. fowleri* was developed using the Amnis Imagestream flow cytometer. The Amnis Imagestream offers the tremendous advantage of the ability to phenotypically assess drug treatment on individual *N. fowleri* cells. Herein, we assed currently used PAM therapeutics for their ability to inhibit *N. fowleri* replication *in vitro* using this newly developed EdU methodology. Treated *N. fowleri* were also phenotypically assessed for their response to drug treatments. EdA (5'ethynyl-2'-deoxyadenosine), an adenine analog, functions on the same premise as EdU. EdA was evaluated by a similar methodology for the ability of *N. fowleri* cells to incorporate this analog while replicating. Further applications for the EdU assay to assess investigative new drugs and their efficacy against *N. fowleri* are currently being investigated. We propose the EdU assay could be used as a complimentary method for drug discovery for these neglected pathogens.

Or-33

In vitro activity of Statins against *Naegleria fowleri*

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Currently there isn´t established specific medical protocol for treatment against *Naegleria fowleri* infections. The anti-protozoan treatments available to treat these infections include an experimental combination of drugs including amphotericin B, miltefosine, fluconazole and rifampicine, among others. These treatments usually present secondary effects for the patient. Furthermore, due to the problems presented by some treatments to pass the blood-brain barrier or with intracranial pressure, there is a great need to develop new anti-protozoal agents with an acceptable efficacy and safety profile for the patients.

Statins are a group of molecules commonly used in medical practice to lower cholesterol and triglycerides in patients. In fact, they are inhibitors of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), an enzyme that regulates the conversion of HMG-CoA into mevalonate, being one of the precursors of cholesterol in humans and ergosterol in protozoa, fungi and plants. Previous studies against *Acanthamoeba* spp., reveal good result as a possible target for future treatments of infection by these protozoa.

In this study six types of statins were tested. The in vitro activity against *Naegleria fowleri* and the cytotoxicity towards murine macrophages were based on alamarBlue® reagent. Assays to measure the mitochondrial membrane potential using JC-1, or the permeability of the plasma membranes using SYTOX Green, among others, were performed with the most active compounds in order to study the type of cell death. Most of compounds showed a significant dose-dependent inhibition effect on the proliferation of the parasites, with an IC_{50} values lower than 0,3 µM. Moreover, the actives compounds have shown low cytotoxic effects.

In conclusion, the statins tested in this study showed to be active against *N. fowleri*, at low doses, being able to consider them as good molecules for further studies.

Projects PI18/01380, RD16/0027/0001 and FEDER.

ORAL SESSION 5

Free Living Amoeba and the environment

Chairs: Dr María Reyes-Batlle and Dr Maritza Omaña-Molina

Or-34

Free-living amoebae as hosts for Microbial Dark Matter: the case of the phylum Dependentiae

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Dependentiae (formerly TM6) designates a major bacterial phylum, widespread and frequently detected in natural and built environments. Accumulating evidence suggests that members of this phylum along with other phyla of the so-called "microbial dark matter" are living in association with (eukaryotic) hosts. This study aimed at shedding light on the ecology and lifestyle of this elusive phylum. Screening for 16S rRNA signatures affiliated to the Dependentiae in public repositories, we recovered more than 400,000 sequences, which clustered in 1390 OTUs97%, reflecting a moderately diversified bacterial phylum. Based on environmental metadata, Dependentiae were frequently found in aquatic and soil environments, with highest prevalence in freshwater sediments, where protists are both abundant and diverse. As current knowledge about Dependentiae is mainly based on metagenomic data, we performed an in-depth analysis of one of the rare isolate available, *Vermiphilus pyriformis,* which was found recently in the free-living amoeba *Vermamoeba vermiformis*. This association was further characterized using fluorescence *in situ* hybridization, transmission electron microscopy, cryo-electron tomography, and genome analysis. *V. pyriformis* shows a Gram-negative type cell envelope; pili-like structures densely cover its outer membrane, and conspicuous invaginations of the cytoplasmic membrane were seen. Tracking *V. pyriformis* during infection of its original host, major morphological changes were observed including the formation of filamentous structures preceding cell division. The *V. pyriformis* genome shows hallmarks of a hostdependent intracellular lifestyle, a feature we noted in our comprehensive analysis of the 22 Dependentiae genomes available. Remarkably, (eukaryotic) effector proteins carrying a sec-signal sequence were abundant; in the absence of other known secretion systems, Dependentiae bacteria thus seem to rely solely on type 2 secretion for host interaction, representing an unprecedented strategy for intravacuolar microbes. Studying these bacteria within their protists' hosts highlighted unique developmental features and uncovered original adaptations to intracellular life of a widespread, yet largely unexplored bacterial phylum.

Keywords: Free-living amoeba, TM6, Dependentiae, microbial dark matter, endosymbiosis

Or-35

Warm and green with a pinch of salt, the regimen of ancient amoebae.

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Free-living amoebae could be found in various environment, such as water and soil. More extreme environments have been poorly studied; however they might be highly interesting as a potential source of new findings. Thalassohaline ecosystems are hypersaline lacustrine environments whose water is of marine origin. The lake Dziani Dzaha, located in Mayotte island presents high salinity (around 52 psu), high temperature (29-30°C) and alkaline pH (between 9 and 9.5).
The aim of our study was to assess the presence of amoebae in lake Dziani Dzaha by a culture-based approach. Their presence was researched in samples collected at different seasons and at several heights along the water column. Also, grazing experiments were performed to know if the most abundant natural preys, filamentous cyanobacteria, were potentially grazed by the isolated amoebae.
Our study ended with the isolation of two amoebae from the lake. Partial sequencing of the 18S rRNA gene sequence allowed us to identify a new isolate of the *Pharyngomonas* genus. The second isolate displayed rather moderate sequence similarity (85%) to *Euplaesiobystra hypersalinica,* suggesting that this amoeba might represent a new genus. Both isolate belong to the Heterolobosea, a group gathering ancient amoebae. Light and electronic microscopy allowed us to describe their morphology. Regarding their salt tolerance, both isolates displayed an optimum growth around 15-40 psu. Because the cyanobacteria *Arthrospira fusiformis* is the most abundant bacteria in the lake (ca. 95 % of the total photosynthetic biomass), grazing experiments were performed by mixing these cyanobacteria and the amoebae. Both FLA isolates appeared to ingest Arthrospira filaments and to digest it partly within few hours.
In conclusion, our study described two new amoebae from the hypersaline lake Dziani Dzaha that were able to phagocyte the cyanobacteria predominantly found in the lake. It would be interesting to better characterize the trophic interactions between these organisms and their preys in this environment.

Or-36

Morphologic and molecular identification of *Naegleria cardilemsis*, isolated from a water park in Hidalgo, Mexico

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Naegleria genus is an anfizoic protozoon from the soil, natural and artificial tempered waters, they belong to the *Vahlkampfiidae* family; *Heterolobosea* class and *Excavata* super group (Adl et al., 2012). To these days there are many species of *Naegleria* genus, many have just identified for the first time and others have been re-classified on basis its genetic sequences. (De Jonckheere, 2006). Some of the most important species of this genus are *Naegleria gruberi, Naegleria lovaniensis,* and *Naegleria fowleri*, this last one causal microorganism of Primary Amoeba Meningoencephalitis.

The aim of this work was to isolate and identify this microorganism. *Naegleria cardilemsis* is an amoeba isolated from a water sample taken from a swimming pool of a water park in Hidalgo, Mexico.

Morphological and physiological features were examined showing that this amoeba grew at 25°C on Ringer medium. This amoeba was unable to kill young mice that were intranasal inoculated, suggesting that this amoeba is not a pathogenic specie. The trophozoite morphology is highly characteristic of *Naegleria* genus where the cyst present pores in the wall; while, flagellated form showed the presence of one or two flagella. The amoeba molecular identification was performed by amplifying the ITS by PCR, whose products were sequenced and analyzed using MEGA7 software. A total of 58 sequences of *Naegleria* genus were downloaded from GenBank to build a similarity matrix using ClustalW for alignment. Phylogenetic analyses was conducted to asses phylogenetic relationships within *Naegleria* species using Neighbor-Joining method.

Based on the morphological and molecular evidences we demonstrate that *Naegleria cardilemsis* is a species that had not been previously described. It is important to highlight the use of accurate molecular tools to the identification of these microorganisms, which are in direct contact with humans representing a risk as they could have a pathogenic potential.

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Or- 37

Naegleria spp. diversity on Monjolinho River basin at state of São Paulo - Brazil

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Protozoan organisms of the genus *Naegleria* are worldwide distributed mainly in environmental sources. It has been associated with vertebrates as a facultative parasite capable of causing a highly lethal brain infection named Primary Amoebic Meningoencephalitis (PAM). The knowledge of *Naegleria* geographical distribution has been an effective way to recognize its ecological niches, to map its presence as a strategy to both evoking new contaminations and accessing evolutionary patterns that reveal relationships among the species. To date, Brazil has a lack of *Naegleria*'s occurrence reports, although relying on having about twenty percent of the global freshwater which indicates its potential to harbor a diversity of FLA. The purpose of this research is contribute to narrow the Brazilian gap through the environmental evaluation of the *Naegleria* diversity in the Monjolinho River Basin at São Carlos-SP. Water collection of five sampling sites, limnological water analysis to obtain its physical and chemical characterization, together morphological and molecular biology approaches to isolate and identify the trophozoites comprise the central methodology. Regarding the morphological assays, after filtering the water through a 0.45µm membrane, pieces of the membrane were transferred onto non-nutrient agar plates cultivated under a range of temperature (26, 37 and 44º C). For the molecular biology technics, after purifying the genomic DNA, there were performed PCRs against internal transcribed spacers (ITS) regions and 5.8S rDNA, which products were verified by electrophoresis and Sanger sequencing. The results revealed trophozoites growing in all sampling sites and the identification of *N. philippinensis, N. canariensisi, N. australiensis, N.gruberi, N. dobsoni* besides a *Valhkampfia* genus. The temperature 44 ºC, assessed to inspect thermotolerance capability, revealed that solely *N. australiensis* could withstand. These findings configure this research as the first Brazilian *Naegleria spp.* report on a freshwater system that could isolate the aforementioned species. Our results imply the urgency of enlarging similar investigations including other FLA genera with potential pathogenic species as observed in *Acanthamoeba spp., Balamuthia spp., Sappinia spp.* and *Vermamoeba spp.*

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Or- 38

Population dynamics of *Naegleria fowleri* in natural aquatic environments during the period from May 2017 to April 2018

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Free-living pathogenic amoebae are protozoa capable of existing in the environment, as well as within a host organism, and have a wide distribution in nature. Within this classification there is the genus *Naegleria*, which has a thermophilic pathogenic species called *N. fowleri* which causes a rapid and fatal infection in the central nervous system known as primary amoebic meningoencephalitis. The present study was developed to determine the concentration of N. fowleri in recreational water bodies of the Yaqui Valley during May 2018 to April 2019. The samples were taken monthly at La Isleta, Las Palmas, Agua Caliente and Laguna de Náinari. The amoebae count was performed by the most likely number method and the identification of *N. fowleri* was performed by endpoint PCR. The maximum concentration of thermophilic amoebae and thermophilic *Naegleria* spp. was obtained at Agua Caliente with 2,398 and 788 NMP L-1, respectively. The maximum presence of *N. fowleri* was detected during September with 70 NMP L-1 at Laguna de Náinari. La Isleta and Agua Caliente showed a concentration of 4 and 18 NMP L-1, respectively during October. For Las Palmas, two concentrations were obtained, being 22 and 16 NMP L-1 during the months of August and September, respectively. We took some representatives of *N. fowleri* from each sampling site and month to genotype and all were genotype 2. Due to the variations presented in the population dynamics and the ecological diversity of the sites, it can be deferred that different factors besides temperature influence the presence or absence of this species. However, given the presence of *N. fowleri* in this region, it is recommended to implement preventive measures, as well as to carry out further studies since it is unknown whether the climate change and / or the increasing environmental pollution could increase the concentration of this pathogen.

Or- 39

Amoebaiome, could we identify FLA in the environment without culture?

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Free-living amoebae (FLA) identification in the environment is a central question in studying these microorganisms. Culture-based identification has been used for a long time, but it presents many drawbacks: it is fastidious, selective and some FLA might be non-cultivable. Besides, high-throughput sequencing is currently used as a standard method for identification of many microorganisms without isolation nor culture. Our objective was to improve the identification of FLA in the environment via high-throughput sequencing.

In this aim, we performed a four-step study. First, a review of the literature was made to collect almost all primer pairs, around twenty, that have been described. Second, these primers and newly designed primers were tested *in silico* against the Silva database to compare their coverage and specificity on various FLA genera. This step underlined huge differences between the primers and suggested that some pairs might be more efficient to amplify the 18S rRNA gene. Third, a selection of these primers was tested in vivo to amplify the 18S rRNA gene of 25 FLA isolates, belonging to various genera (i.e. Acanthamoeba, Vermamoeba, Naegleria, Echinamoeba, Tetramitus and Vahlkampfia). Fourth, four primer pairs, two targeting Amoebozoa and two targeting Heterolobosea, were used to perform high-throughput sequencing of 18s amplicons. Our aim was to identify all FLA from a river water sample. The Amoebozoa primers were not specific enough and most sequences did not belong to FLA genera. In contrary, the Heterolobosea primers pairs gave rather interesting results since they display a high specificity (up to 66% of amplicons belong to Heterolobosea) and they covered several of FLA genera, even some that have been poorly recovered by culture.

In conclusion, our work allowed to compare many primer pairs dedicated to FLA amplification and to design a new primer pairs that has a high potency to identify Heterolobosea in the environment to get a better knowledge on their diversity.

Or-40

A double infection of an encephalitic survivor with the ameba*, Balamuthia mandrillaris,* and a fungus, *Aspergillus terreus*

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The cultivation of biopsy tissues on Vero monkey kidney cells from an encephalitic survivor has led to the recovery of the ameba*, Balamuthia mandrillaris,* plus an unexpected (unex) companion, a thin shelled cell identified by sequence analysis as the fungus *Aspergillus terreus.* Cultivation and maintenance of the amebae was consistent with that of the Balamuthia originating from soil, animals, or humans and was maintained by feeding on cultured mammalian cells. The unex fungal cells underwent closed mitosis, had a chitin shell and have been cultivated for more that 5 years in axenic mammalian cell media, by feeding on the amebae, or in ambient temperature storage (7+ years). Inter-activities of the co-cultured ameba and unex cells included the approach of an ameba to an inactive unex, the extension of its pseudopodia to touch, retreat, touch again several times before wrapping the unex within its pseudopodia followed by the release of the unex's crushed shell. Another interaction was the approach and attachment of the unex to the body of an ameba where they stayed for hours as the ameba was digested.

After years of cultivation in axenic media, the unex cells became darkened and appeared to be an encasement of multiple smaller cells. Upon a period of slow drying, the blackened unex gave rise to numerous smaller (2µm) cells, each seen to produce a single filament that joined with others to form hyphal structures typical of the *Aspergillus terreus*. Sequence analysis of the ameba-active unex cells as well as the blackened-unex, performed at two different facilities confirmed the unex cells to be *Aspergillus terreus*. Treatment of thin sections of brain biopsy samples from the patient confirmed the presence of the *Balamuthia mandrillaris* as well as the unex-*Aspergillus terreus* cells within the brain parenchyma and in the lumen of the vasculature, thus indicative of a dual brain invasion.

Or- 41

Stenamoeba dejonckheerei sp. nov., a free living amoeba isolated from a thermal spring

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Two isolates of free-living amoebae were obtained from water samples taken from a thermal spring, "Agua Caliente", in Northwestern Mexico. The spring had a water temperature of 45.5 °C at the time of collection. The isolates were obtained when samples were cultivated at 37 °C on non-nutrient agar coated with *Escherichia coli*. The initial identification of the isolates was performed morphologically using light microscopy. Samples were found to have trophozoite morphology consistent with members of the genus *Stenamoeba*, a genus derived in 2007 from within the abolished polyphyletic genus *Platyamoeba*. Further analysis was performed by sequencing PCR products obtained using universal eukaryotic primers for the SSU rRNA gene. Sequencing primers were designed to allow the comparison of the 18S rRNA gene sequences of the new isolates with previous sequences reported for *Stenamoeba*. Sequences in the DNA databases were available from *Stenamoeba stenopodia*, *S. limacina*, *S. berchidia*, *S. amazonica*, *S. sardiniensis* and *S. polymorpha*, as well as several sequences classified as being from *Stenamoeba* sp. DNA analysis of the two isolates produced sequences of length 2029 and 1976 nucleotides, differing by a single nucleotide in the 1976 base overlap. The sequences were confirmed to be most closely related to sequences attributed to members of *Stenamoeba*. Phylogenetic relationships among sequences from *Stenamoeba* were determined using Maximum Likelihood analysis. The results showed the two "Agua Caliente" sequences to be closely related, while clearly separating them from those of other *Stenamoeba* taxa. The phylogenetic analysis suggests that *Stenamoeba berchidia* is the

closest known relative of the isolates, with the highest sequence similarity (94.5%). Sequence similarities with the other *Stenamoeba* taxa having near-full length 18S rRNA gene sequences ranged between 87.8% and 92.0%. The degrees of sequence differentiation from other taxa were considered sufficient to allow us to propose that the Mexican isolates represent a new species, designated *Stenamoeba dejonckheerei* sp. nov. to honor Dr. Johan F. De Jonckheere for his valuable contributions in the field of free-living amoebae. Electron microscopy and pathogenicity tests are in process, which will be used to fully analyze the isolates and provide a detailed species description.

Or-42

The Influence of *Acanthamoeba-Legionella* interaction in the virulence of two different *Legionella* species

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The genus *Legionella* comprises more than 60 species, and about half are associated with infection. *Legionella pneumophila* is the most commonly associated with these infections and by far the most studied, but *L.* non-*pneumophila* species, such as *L. feeleii*, *L. anisa*, etc., may also present clinical importance. Free-living amoebae are their preferred environmental host, where these bacteria not only survive but also succeed in multiplying, and this relationship can lead to an increase in bacterial virulence. The goal of this study was to evaluate the alterations of *Legionella* pathogenicity due to its interaction with *Acanthamoeba*. For this, the expression of protein effectors SdhA, LegK2, and SidK were evaluated by Retrotranscripción-PCR, in *L. pneumophila* and *L. feeleii*, before and after infecting *Acanthamoeba*. Additionally, the host response was evaluated by measuring the production of IL-6, IL-8, and IFN-γ in infected macrophages, by Enzyme-Linked ImmunoSorbent Assay. Regarding the virulence factors, an increase in SdhA expression was observed after these bacteria infected *Acanthamoeba*, with a higher increase in the macrophage cultures infected with *L. feeleii*. Also, an increase in the expression of LegK2 was observed after infecting *Acanthamoeba*, but it was more intense in the cultures infected with *L. pneumophila*. With regard to SidK, it was increased in *L. feeleii* after infecting *Acanthamoeba*, however the same effect was not observed for *L. pneumophila*. In cytokine production, the effect on IL-6 and IL-8 was similar for both cytokines, increasing their concentration, but higher production was observed in the cultures infected with *L. feeleii*, even though it demonstrated slightly lower production with the inoculum obtained from *Acanthamoeba*. Concerning IFN-γ, induction was observed in both species but higher in the infection by *L. pneumophila*. Nevertheless, it is not known if this induction is enough to promote an efficient immune response against either *L. pneumophila* or *L. feeleii*. Altogether, these alterations seem to increase *L. feeleii* virulence after infecting *Acanthamoeba*. However, this increase does not seem to turn *L. feeleii* as virulent as *L. pneumophila*. More studies are necessary to understand the aspects influenced in these bacteria by their interaction with *Acanthamoeba* and, thus, identify targets to be used in future therapeutic approaches.

Or- 43

Presence and interaction of free-living amoebae and amoeba-resisting bacteria in water from drinking water treatment plants

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Free-living amoebae (FLA) are ubiquitously distributed in nature and many isolates have shown to be infected with amoeba-resisting bacteria (ARB). *Acanthamoeba-Legionella* interaction is an example in which these bacteria not only survive inside amoebae but also replicate. Due to the high environmental prevalence of *Acanthamoeba* spp. in Central Spain, the aims of this work were to investigate the occurrence of *Acanthamoeba* and other FLA in water from several sampling points from four Drinking Water Treatment Plants (DWTP) and to investigate the presence of *Legionella* spp. and other ARB in biofilms and in both raw and finished water, comparing the effectiveness of the treatments employed in these DWTPs. *Acanthamoeba* was detected at different sampling points, and sand filters seemed to contribute to amoebic enrichment. After ozonation, however, a temporary decrease of viable amoebae was clearly observed. The genotypes detected were T3, T4/1, T4/7, T4/8, T4/12, T4/13, T4/16, T4/22, T4/27, T4/30, T4/34, T4/36, T4/37 and T5, revealing the first report of genotype T5 in waters from this region. Regarding other amoebae, *Naegleria fowleri* was not detected and *Balamuthia mandrillaris* was detected once, while *Vermamoeba vermiformis* and *Paravahlkampfia* sp. were detected mostly associated with biofilm and mud. Regarding *Legionella*, PCR detection in raw and finished water was higher than by agar culture, but even higher after *Acanthamoeba* co-culture. Also, *Legionella* presence was higher in raw water than in finished water. However, the decrease of free *Legionella* observed from raw (27.5%, by PCR) to finished water (3.4% by PCR) contrasted with the increase of *Legionella*-infected FLA from raw (30.7%) to finished water (52%). At biofilm, however, it was not detected free *Legionella*, and the percentage of infected FLA was low (3.8%). *Legionella* species identified in these samples were *L. drozanskii*, *L. donaldsonii* and *L. feeleii*. Additionally, *Acanthamoeba* co-culture led to the isolation of other bacteria, such as *Pseudomonas aeruginosa*, *P. stutzeri*, *P. fluorecens*, *Achromobacter xylosoxidans* y *Stenotrophomonas maltophilia*. Therefore, the highly disseminated presence of *Acanthamoeba* and the detection of ARB inside amoebae highlight the importance of developing methods for controlling FLA in order to limit human pathogenic ARB survival to the water purification processes.

Or- 44

Genome sequencing of *Willaertia magna* C2c Maky: insight into a potential pathogenicity by searching for horizontal gene transfers from pathogenic microorganisms

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Willaertia magna C2c Maky is a thermophilic amoeba closely related to the genus *Naegleria*. This free-living amoeba has the ability to eliminate *Legionella pneumophila*, which is an amoeba-resisting bacterium living in an aquatic environment. To prevent the proliferation of *L. pneumophila* in cooling towers, the use of *W. magna* C2c Maky as natural biocide was proposed. To improve a better understanding of the *W. magna* C2c Maky characteristics, whole-genome sequencing was performed. These data were computed for the study of virulence factors and horizontal gene transfers. This amoeba harbors a genome of 36.5 megabases with 18,519 predicted genes, including 73,3 % of the annotated genes and 26,7% of ORFans. Best hit analysis reported a diverse origin of the *W. magna* genes. The core genome of *Vahlkampfiidae* includes 1,795 genes, and the ratio core genome/pangenome is less than 0,09. BLASTp analyses reported protein homology between 136 *W. magna* C2c Maky sequences and amoeba-resistant microorganisms. Horizontal gene transfers were demonstrated based on phylogenetic reconstruction hypothesis. We detected 14 homologs of *N. fowleri* genes related to virulence, although these latter were also found in the genome of *N. gruberi*, which is a non-pathogenic amoeba. Furthermore, the cytotoxicity test performed on human cells supports the hypothesis that the strain C2c Maky is a non-pathogenic amoeba. This work explores the genomic repertory for the first genome of genus *Willaertia* and provides genomic data for further comparative studies on virulence of related pathogenic amoeba, *N. fowleri*.

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U.S. Balamuthia case series

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Background: *Balamuthia mandrillaris* is a free-living ameba that causes rare, nearly always fatal disease in humans and animals worldwide. *B. mandrillaris* has been isolated from soil, dust, and water. Initial entry of *Balamuthia* into the body is likely via the skin or lungs. To date, only individual case reports and small case series have been published.

Methods: The Centers for Disease Control and Prevention (CDC) maintains a free-living ameba (FLA) registry and laboratory. To be entered into the registry, a *Balamuthia* case must be laboratory-confirmed. Several sources were used to complete entries in the registry, including case report forms, CDC laboratory results, published case reports, and media information. SAS© version 9.3 software was used to calculate descriptive statistics and frequencies.

Results: We identified 109 case reports of *Balamuthia* disease between 1974 and 2016. Most (99%) had encephalitis. The median age was 36 years (range 4 months to 91 years). Males accounted for 68% of the case patients. California had the highest number of case reports followed by Texas and Arizona. Hispanics constituted 55% for those with documented ethnicity. Exposure to soil was commonly reported. Among those with a known outcome, 90% of patients died.

Conclusions: *Balamuthia* disease in the United States is characterized by a highly fatal encephalitis that affects patients of all ages. Hispanics were disproportionately affected. The southwest region of the U.S. reported the most cases. Clinician awareness of *Balamuthia* as a cause of encephalitis might lead to earlier diagnosis and initiation of treatment, resulting in better outcomes.

POSTER SESSION

P-1

Ultrastructural analysis of *Acanthamoeba polyphaga* invasion in deep stroma near descemet's membrane: case report

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Amoebic keratitis (AK) is a sight threatening corneal infection. Recently, increasing number of AK cases have been reported in Mexico, specifically in the hospital to avoid blindness in Mexico. The present study describes by histology and transmission electron microscopy (TEM) the deep stromal invasion of *A. polyphaga* in a severe case of AK, in which patient was multitreated with antibiotics and corticosteroids, even so, required keroplasty. In the initial diagnosis, a corneal scraping was performed to determine bacterial, fungal or amoebic origin. Growth was obtained only for *Acanthamoeba*. The strain was axenized, in PYG medium, with an optimal temperature of growth of 30 °C. Amoebic virulence was evaluated through intranasal inoculation of trophozoites (1 x10^6) in 5 mice groups (BALB / c), accordingly to granulomatous amoebic encephalitis (GAE) model. Inducing death of two of five mice (40% virulence), recovering trophozoites from brain and lung.

Corneal segments obtained from surgery were processed according to conventional histology and transmission electron microscopy techniques. Histological analysis of samples showed stromal lamellar disarrangement and necrotic tissue intermingled with neutrophils, some cysts and few trophozoites. The Descemet's membrane was preserved.

By TEM it could be possible to observe that *A. polyphaga* was able to migrate and invade the deep stroma where few trophozoites, pre-cysts and viable cysts were detected, as well as abundant empty cystic forms recognized by the double characteristic wall of the genus. It is important to emphasize that numerous apparently viable and empty cysts, were observed surrounded by cells of the immune system, which possibly deposited on its surface electrondense material. Which

suggests an intensified inflammatory response, associated with extensive destruction of corneal tissue. Besides, the presence of some electrodense excrescences on the cyst membrane were observed, resembling deposits of ab-ag immune complexes observed in the basement membrane of the glomeruli in post-estreptococcal glomerulonephritis.

Although numerous trophozoites invaded the deep stroma, many of them began the process of encystment either due to lack of oxygen, combined with the effect of drugs and direct action of the immune cells in response to the amoebic invasion, exacerbating the inflammatory process damaging the corneal tissue.

P-2

Adhesion of *A. castellanii* trophozoites to cosmetic contact lenses

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Amoebae of the genus *Acanthamoeba* are opportunistic pathogens able to cause amoebic keratitis (AK); a sigh threatening corneal infection. The use of contact lenses (CL) is the main risk factor for the AK.

Recently, it has reported an increasing number of cases AK related to the use of cosmetic contact lenses (CCL). In Mexico, there is no regulation of the CCL in quality and marketing. Teenagers are the main users. Importantly these lenses can be acquired in stores, markets and Internet, without receiving any adaptation, handling and cleaning recommendations, besides poor hygiene.

It has been suggested that numerous factors converge to promote adhesion of amoebae to the lens, highlighting; polymers and materials from which the lenses are manufactured, its surface, water content and ionicity.

The purpose of this study was to evaluate quantitatively and qualitatively the adherence of *A. castellanii* (isolated from an AK case), to segments (25 mm^2) of two different CCL, one of them approved by FDA (Acuvue), and Magic eye CL non approved by FDA, as well as the effect of two lens multipurpose solutions (MS). The interactions were conducted using 250 trophozoites in 300 µl of MS, culture medium (PYG) and saline solution, during 4 h (minimum time recommended for disinfection). Samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1h. Adhering trophozoites were quantified by light microscope.

Although the solutions induce the death of more 80% of amoebae, viable trophozoites were observed adhered to both lens surface; observing a greater number of trophozoites adhered to Acuvue CL. Besides, Magic eye CL shown irregular edges, rough zones and discontinuous surface, which can cause abrasions to the corneal epithelium. Several amoebae were observed adhered inside fissures of the lenses.

It is well known that the use of contact lenses is a risk factor for AK, however several polymers, ionicity and hydrophobicity, facilitates or not amoebic adhesion. In the case of lenses not approved by the FDA, the risk of manufacturing defects exacerbates the risk of infection. Moreover, the solutions must guarantee the elimination of 100% of trophozoites, otherwise few amoebae can invade the cornea.

P-3

Differential behavior of *Legionella pneumophila* strains into different amoeba strains including *Willaertia magna* C2c Maky

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Free-living amoebae are the main site of *Legionella pneumophila* development in aquatic environment. The pathogen bypasses the elimination mechanism to replicate within amoebae.

However, not all amoeba species support the growth of *L. pneumophila*. *Willaertia magna* C2c maky, a non-pathogenic amoeba had previously demonstrated ability to eliminate *L. pneumophila* strain Paris. For this reason, the Amoéba company developed it as a natural biocide to control *L. pneumophila* proliferation in cooling towers.

In this work, we studied intracellular behavior of three *L. pneumophila* strains, Paris, Philadelphia, and Lens, with *W. magna* C2c Maky and compared it to *Acanthamoeba castellanii* and *Willaertia magna* Z503 used as controls. Co-culture in adhesion are performed according two temperature conditions (22°C and 37°C). The assay lasts five day with a daily count of intracellular *L. pneumophila* and amoebas by culture and Trypan blue staining.

At the end of the experiment at 22°C, remaining intracellular *L. pneumophila* are harvested from *W. magna* C2c Maky assays and used as inoculum in a new flask containing fresh strain C2c Maky during three days at 22°C to verify that these bacteria are not resistant to amoeba digestion. For this set of experiment, a multiplicity of infection of 1 (MOI 1) was studied.

We observed intracellular growth of strain Lens within *W. magna* Z503 and *A. castellanii* at 22°C and 37°C. Strain Paris multiplicated within *A. castellanii* at any temperatures and only at 22°C within *W. magna* Z503. Strains Philadelphia proliferated only within *A. castellanii* at 37°C. As for the *W. magna* C2c Maky, we did not report any intracellular growth for all legionella strains used, regardless of temperature. Furthermore, freshly *W. magna* C2c Maky have the ability to eliminate the *L. pneumophila* cells remaining from first co-incubation of 96 hours.

The study demonstrated that *W. magna* C2c Maky has a different behaviour compared to *A. castellanii and W. magna* Z50*3* with legionella strains at 22°C and 37°C. Therefore, the behavior towards amoeba-resistant bacteria is multifactorial and depends especially of amoeba-microorganism strain association, and temperature conditions.

P-4

Immunomodulatory effect of the 250 kDa glycoprotein of *Naegleria fowleri* on the dendritic cells of the NALT, nasal passage and cervical lymph node of Balb/c mice.

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The nasal cavity is the entry route for many pathogens, among them, *Naegleria fowleri*, a free-living amoeba, which causes Primary Amebic Meningoencephalitis (PAM), a disease with a high mortality rate. There are not studies that describe the mechanisms that may protect individuals against this microorganism, therefore, an option it's the induction of a protective immunity on mice models using *N. fowleri* antigens.

The objective was to determine the immunomodulatory role of the 250 kDa glycoprotein of *N. fowleri* on dendritic cell populations in the nasal cavity of immunized mice.

The 250 kDa band was purified by electroelution from *N. fowleri* extract and used to immunize mice. To determine the activation of dendritic cells, groups of mice were immunized in two and four times at seven-day intervals and from theme, different sections of the nasal cavity (NALT, nasal passage) and cervical lymph node (CN) were obtained of which the leukocyte fraction were purified and processed by flow cytometry, also, sera and nasal washes to determine the presence of antibodies anti-*N. fowleri* through immunoblots. Subsequently, mice were immunized intranasally four occasions with the purified glycoprotein alone or coadjuvanted with Cholera Toxin (CT). After, the mice were challenged with lethal dose of trophozoites and observed for 60 days to determine the survival percent.

The activated DC number were higher in NALT and NP than CN, when mice were immunized in two times. When mice were immunized in four times, the activated DC number were higher in CN than NALT and NP. In immunoblot test, the 250 kDa glycoprotein was recognized by IgA and IgG antibodies from nasal washes and serum. The protection rate in immunized mice with 250 kDa glycoprotein was 80%, whereas, when immunizing with the 250 kDa glycoprotein coadministered with CT a 100% protection was obtained.

We suggested that the 250 kDa glycoprotein of *N. fowleri* is a very strong immunogen that can modified the activated DC population, the specific antibodies production so then this immune

factor may participate a high levels of protection obtained. Therefore, these glycoprotein it is a good candidate to be use on vaccine design against PAM.

P-5

Identification of possible candidates for vaccines of immunogenic glycoproteins of *Naegleria fowleri* against primary amoebic meningoencephalitis

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Naegleria fowleri, a pathogenic free-living amoeba causes primary amoebic meningoencephalitis (PAM) a fatal disease in humans and animals. The trophozoites of *N. fowleri* enter the central nervous system through the olfactory bulb when penetrating the nasal epithelium. The adherence of the amoeba is a critical step in the infection process. Recently, 108, 50 and 19 KDa molecules have been found to be highly immunogenic, in addition; these molecules have carbohydrate residues therefore, they might be participating in amoeba adhesion to the nasal epithelium and consequently in the infection process.

Due to the above and considering that there is no completely satisfactory prophylactic or therapeutic treatment against PAM, it is important to develop new strategies for the search of candidates for vaccines administered intranasally.

The objective of this work was determine if the immunogenic proteins of 108, 50 and 19 KDa were exclusive of *N. fowleri*, compared with total extracts of *N. lovaniensis* a non-pathogenic free-living amoeba.

For this, the proteins were purified from total extracts of *N. fowleri* and *N. lovaniensis* by electroelution technique and the immunodetection was performed by using specific antibodies and lectins. The identification of the proteins of interest was carried out using the 2D technique and mass spectrometry.

The results obtained show that despite the electroelution process, the structure of the proteins and the immunogenicity were not modified. Notable differences were found on the spots of 108, 50 and 19 KDa proteins in terms of the intensity between *N. fowleri* and *N. lovaniensis* by 2D technique. Particularly, the 19 kDa spots showed to be different between the species.

Regarding to mass spectrometry, a great number of proteins were identified, which are included only for 19 KDa spots. The main identified antigen is a membrane protein which could play an important role as a virulence factor.

Therefore, in this work we found differences in protein expression between *N. fowleri* and *N. lovaniensis* that could be used to design a vaccine against PAM.

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P-6

Role of Cathepsin B of *Naegleria fowleri* during Primary Amoebic Meningoencephalitis.

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Naegleria fowleri causes primary amoebic meningoencephalitis (PAM) in humans and experimental animals, which leads to death within 7–14 days. In some studies, it has been suggested that cysteine proteases of parasites play key roles in nutrient uptake, host tissue invasion, and immune evasion.

The objective of this work was to determine the role of Cathepsin B of *N. fowleri* during the early stages of PAM in nasal cavity of mice.

In this study, we produced anti-Cathepsin B polyclonal antibodies and designed a probe from mRNA of this *N. fowleri* enzyme. First, we determine the presence of mRNAs as well as the enzyme in fixed trophozoites by in situ hybridization and immunocytochemical techniques. After that, the initial stages of *N. fowleri* meningoencephalitis in mice were characterized immunohistochemically after the first 12 and 24 h post-nasal inoculation.

After experiments, we were able to observe positive markers both mRNAs and enzyme in fixed trophozoites and histological sections of infected mice. As for the mRNAs, the marks were observed with greater intensity in the nucleus and it was decreasing along the cytoplasm of fixed trophozoites. Unlike the *in vitro* part, the mark was observed homogeneous throughout the cytoplasm in histological sections. On the other hand, when we incubated the trophozoites with our purified antibody we found rounded marks along the cytoplasm and in histological sections, the marks were found along the membrane of the trophozoites.

These results suggest that *N. fowleri* could be using this enzyme during its migration through the olfactory epithelium.

Based on these results, we could suggest that Cathepsin B of *N. fowleri* could be used as a candidate for the design of prophylactic or preventive vaccines.

P-7

Identification of immunogenic antigens of *Naegleria fowleri* adjuvanted by cholera toxin as vaccine candidates

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We previously reported that intranasal administration of *Naegleria fowleri* lysates plus Cholera toxin (CT) increases protection against *Naegleria fowleri* meningoencephalitis in mice and we suggested that STAT6-dependent humoral immune response is crucial to induce protection against the infection.

In this study, we applied protein analysis for the detection and identification of immunogenic antigens from *N. fowleri*. Serum and nasal washes from protected immunized mice with total extracts of *N. fowleri* plus cholera toxin (CT) were used to identify vaccine candidates as responsible for such protection. A Western blot analysis was performed using the serum and nasal washes from different groups of immunized mice: a) one immunization, 2) two immunizations, 3) three immunizations and 4) four immunizations. All groups were followed by challenge with trophozoites of *N. fowleri*.

All mice mounted either nasal or sera IgA, IgG and IgM antibody response, which was progressively stronger as the number of immunizations was increased, and that response was mainly directed to 250, 108, 50 and 37 kDa proteins bands especially in the third and fourth immunization. To identify proteins corresponding to that immunogenic bands, the total extract of *N. fowleri* was separated by 1D gel electrophoresis, and reactive sera from animals immunized four times was used. Finally, nano-LC–ESI-MS/MS mass spectrometric analysis was employed to identify the corresponding proteins. This analysis identified 32 proteins, 18 peptide masses were matched with the theoretical peptide of *N. fowleri* and 15 peptides were matched with *N. gruberi*. Our results support the notion that the proteins identified in the present study could serve as antigen candidates for the development of vaccines against *N. fowleri infection*.

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P-8

Molecular characterization of bacterial, viral and fungal endosymbionts of *Acanthamoeba* isolates in keratitis patients of Iran

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Objectives: Free-living amoebae belong to the genus *Acanthamoeba;* can feed on microbial population by phagocytosis, and with the capability to act as a reservoir and a vehicle of microorganisms to susceptible host. Therefore, the role of endosymbiosis in the pathogenesis of *Acanthamoeba* is complex and not fully understood. The aim of the present study was to identify bacterial, fungal, and human *adenovirus* (HADV) endosymbionts as well as evaluating the endosymbionts role of such organisms in the pathogenesis of *Acanthamoeba* in keratitis patients living in Iran.

Methods: Fifteen *Acanthamoeba* (T4 genotype) isolates were recovered from corneal scrapes and contact lenses of patients with keratitis. Cloning and purification was performed for all isolate. Gram staining was performed to identify bacterial endosymbionts. DNA extraction, PCR, and nested PCR was set up to identify endosymbiont of amoeba. Evaluation of pathogenicity was conducted by osmo-tolerance and thermo-tolerance assays and cell culture, and then CPE (cytopathic effect) was survey. Statistical analysis was used between *Acanthamoeba* associated endosymbionts and *Acanthamoeba* without endosymbiont at 24, 48, 72, and 96 hours.

Results: A total of 9 (60%) *Acanthamoeba* (T4 genotypes) isolates were successfully cloned for detecting microorganism endosymbionts. The only isolate negative for the presence of endosymbiont was ICS9. ICS7 (*Pseudomonas aeruginosa*, *Aspergillus* sp., and human *adenovirus* endosymbionts) and ICS2 (*Escherichia coli* endosymbiont) isolates were considered as

Acanthamoeba associated endosymbionts. ICS7 and ICS2 isolates were highly pathogen whereas ICS9 isolate showed low pathogenicity in pathogenicity evaluated. Positive CPE for ICS7 and ICS2 isolates and negative CPE for ICS9 isolate were observed in cell culture. The average number of cells, trophozoites, and cysts among ICS7, ICS2, and ICS9 isolates at 24, 48, 72, and 96 h was significant.

Conclusions: Our study demonstrated that microbial endosymbionts can affect the pathogenicity of *Acanthamoeba*; however, further research is required to clarify the exact pattern of symbiosis, in order to modify treatment protocol.

Keywords Endosymbiont · *Acanthamoeba* · Pathogenicity · keratitis

P-9

Mucus degradation by *Naegleria fowleri* Glycosidase (Nf-GH)

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Background: *Naegleria fowleri* is the etiological agent of primary amoebic meningoencephalitis (PAM). The infection starts with the entrance of the trophozoites by the nasal route, then penetrates the epithelium of the olfactory mucosa, and migrates through the olfactory nerves until reach the olfactory bulbs. In our laboratory, it has been observed in the mouse model that mucus production is insuficient to eliminate *N. fowleri* trophozoites. Likewise, we previuosly reported that proteases present in total crude extracts can be related with mucus degradation. However, the identification and the role of this proteases in the secretion products (SP) and their role as pathogenic mechanism during PAM has not been evaluated.

Objective: In the present work, we identify, characterize and evaluate the pathogenic role of a mucinolytic activity released by *N. fowleri* trophozoites.

Methods: Previously, we reported the detection of mucinolitic activities at pH 7.0 and 37°C in the SP after ethanol precipitation (3:1). Zimography assays were used for biochemical caracterization using cysteine, serine, and metalloproteases inhibitors. To evaluate the production of specific proteases, we incubated SP with bovine submaxilary mucin at different times of co-incubation. Inducible mucinase was partial purified by anion-exchange chromatography; the identification was determined by mass spectrometry (MALDITOF-MS). To evaluate human MUC5AC mucin degradation, cytopathic effect and survival curves of animals we produce specify antibodies against mucinase (Nf-GH). The antibodies were co-incubated with mucoepithelial cell line NCI-H292 in presence or not of *N. fowleri* trophozoites; MUC5AC degradation and cytopathic effect was compared without the antibody. The survival curves of animals instilled with trophozoites (1.2 x104) pre-treated with antibodies against Nf-GH was compared with animals infected with trophozoites without treatment.

Results: A 94-kDa protein with mucinolytic activity was inducible and abolished by pHMB. MALDI-TOF-MS identified a glycoside hydrolase (GH). Specific antibodies against Nf-GH inhibit cellular damage, human MUC5AC mucin degradation, and delay mouse mortality.

Conclusion and significance of the work**:** Secretory products from *N. fowleri* participates in the degradation of mucus allowing the invasion process. The GH secreted can be used for diagnosis or even as drug target against *N. fowleri***.**

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P-10

Tannic acid-modified silver nanoparticles conjugated with contact lens solutions enhanced their anti-amoebic activity

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Proper contact lens use is crucial to avoid *Acanthamoeba* keratitis infection. In our previous studies we showed, that multipurpose contact lens disinfection systems are not fully effective against *Acanthamoeba* and need improvement on this matter. Synthesized nanoparticles are currently proposed as a new generation of anti-microbial agents. It is also known that some plant metabolites present anti-parasitic activity. Tannic acid (penta-m-digalloyl glucose) is the simplest, hydrolysable tannin with confirmed anti-microbial, anti-cancer and anti-oxidant activity. In our previous studies, we showed that tannic acid-modified silver nanoparticles are effective against *Acanthamoeba* clinical strains. The aim of this study was to evaluate the anti-amoebic activity and cytotoxicity of the tannic acid-modified silver nanoparticles (AgTANPs) conjugated with selected multipurpose contact lens solutions.

Three commonly used in Poland contact lens solutions conjugated with AgTANPs in concentration of 0.25–2.5 ppm were used *in vitro* against the axenically cultured ATCC 30010 type *Acanthamoeba castellanii* strain. The activity assays were performed after 6-96h of incubation using Alamar Blue oxido-reduction reaction. The cytotoxicity assays were performed using a fibroblast HS-5 cell line, based on the measurement of lactate dehydrogenase activity release. Results were statistically analyzed by ANOVA and Student-Newman-Keuls tests using the $p>0.05$ level of statistical significance.

Obtained results confirmed lack of amoebicidal effect of the tested contact lens solutions. AgTANPs conjugated with Solo Care Aqua and Renu Multiplus solutions enhanced significantly their anti-amoebic activity within the minimal disinfection time recommended by the manufacturers (6h) on the dose-dependent manner. This result was achieved without increased

toxicity to the human cells. The synergistic anti-amoebic effect for Opti-Free solution conjugated with the nanoparticles was revealed just after 48h of incubation.

Summarizing, conjugation of selected contact lens solutions with AgTANPs might be a promising approach to prevent *Acanthamoeba* keratitis among contact lens users. Nevertheless, further studies should be conducted to elucidate stability of the conjugation and activity against *Acanthamoeba* cysts.

P-11

The DNA databases for the genus *Acanthamoeba*; an update to 2019

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Amoebae in the genus *Acanthamoeba* were discovered by Castellani and described by Volkonsky in 1930. Morphological characters (primarily cyst structure), and cytological characteristics of nuclear division (Pussard and Pons, 1977) were used to describe different species. Questions regarding morphological approaches led to the hope that molecular methods would provide more definitive subgeneric classifications. Comparison of isolates focused on the nuclear small subunit ribosomal RNA gene (*Rns*), with sequences deposited in the International DNA databases (GenBank, DDBJ and EMBL). Accumulation of information for the *Rns* gene led to the development by our lab of the Sequence Type classification, now routinely used for classifying isolates of *Acanthameoba*. Since 1996, when 4 sequence types were identified, additional reports elevated the number of types to 22. In 2019, data on full or partial *Rns* sequences were available for almost 5100 isolates of *Acanthamoeba*, an increase of ~1200 sequences since FLAM2017. Information from "almost complete" *Rns* sequences (sequences > 2000 bases) exist for 435 isolates, with 282 sequences identified as T4 isolates (65%), a percentage similar to that seen for isolates with only partial *Rns* sequences. T4 is easily the most common Sequence Type in either environmental or clinical samples. The only other Sequence Type exceeding 5% of *Rns* sequences is Type T5. Additionally, no Sequence Type identified post-1998 represents more than 0.65% of isolates, except Type T15, associated with *A. jacobsi*. Several Sequence Types also contain significant phylogenetic subgroups (sequence divergence between 1-5%). At least six significant sub-types exist within T4, and five sub-types exist within the boundaries of the super-Type T2-T6. Beyond a doubt, current species nomenclature within *Acanthamoeba* is only loosely related to phylogenetic classification. Major additional expansions of the DNA databases included information on mitochondrial loci (199 mitochondrial *rns* sequences, 127 sequences for cytochrome oxidase subunit I, and complete mitochondrial genome sequences for 21 isolates). Multi-isolate information exists for a small number of nuclear proteins, including 50 sequence of beta-tubulin, 65 sequence of elongation factor-1, 55 sequences of glyceraldehyde-3-phosphate dehydrogenase, and over 30 sequences of three other genes. Information on all of these sequences, including much additional analysis is summarized at our website: http://http://u.osu.edu/acanthamoeba/.

P-12

The overexpression of two new *Acanthamoeba castellanii* proteins impaired the encystment process.

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Background: In response to environmental stresses, amoebas can differentiate into a resistant form called cyst, protecting them from adverse conditions. The encystment process involves morphological, biochemical and genetic modifications. It has been described that some proteins involved in sugar metabolism, autophagy or proteolytic enzyme are important to encystment process. However, the understanding of this mechanism is still poorly understood.

Objectives: The aim of this study is to identify new proteins involved in encystment of the free-living amoeba *Acanthamoeba castellanii*.

Methods and results: From a proteomic analysis using *A. castellanii*, we have selected the hypothetical protein ACA1_384820 and the G-protein coupled receptor putative ACA1_383450. Using RT-qPCR approaches, we show that the expression of the *ACA1_384820* and ACA1_383450 genes were down-regulated as early as 2 hours after induction of the *Acanthamoeba* encystment process. For ACA1_384820 gene, the downregulation was more important and was observed until 24 hours after induction of the encystment. Expressing plasmid vectors were constructed to overexpress the gene, based on an effective and stable transfection approach in *A. castellanii*. The overexpression of both genes did not affect the growth of *Acanthamoeba castellanii* but impaired the formation of cysts. For *ACA1_383450* gene, the BLAST analysis showed a homology with diverse G-protein coupled receptors including the human GPR107 and murine GPR108. For *ACA1_384820* gene, the bioinformatics analysis showed that the protein possesses a N-Acetyltransferase domain and the best score were obtained with bacterial proteins, suggesting that its gene could have a prokaryotic origin.

Conclusion: This study allowed us to describe new *Acanthamoeba* genes involved in the encystment process.

P-13

Naegleria spp. isolated from irrigation canals of Mexicali Valley, México

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Among the free-living amoebae of the genus Naegleria, is the pathogenic species Naegleria fowleri which causes Primary Amebic Meningoencephalitis (PAM). The PAM is mainly associated with the recreational use of water contaminated with these amoebae. Because clinical cases of PAM have been previously registered in Mexicali Valley, the objective is to isolate and identify amoebae of the *Naegleria* genus in irrigation canals of the Mexicali Valley. Water and sediment samples were taken in ten canals. Temperature, pH, conductivity and dissolved oxygen were determined in situ. Water samples were filtered in duplicate through membrane filters which were placed in non-nutritive agar plates (NNE) and incubated at 30 ° C and 37 ° C. The sediment samples were also inoculated in duplicate, dispersing approximately 0.1 g on the surface of the NNE and incubated at the same temperatures. The plates were observed to detect amoebae growth and were subcultured in obtain axenic cultures. The pathogenicity test was made. Morphological identification of isolates was performed using taxonomic keys and molecular identification was carried out by amplification and sequencing of the ITS regions. Isolates of genus *Naegleria* belonging to 4 species were identified in 9 of the 10 canals; *N. fowleri, N. gruberi, N. pagei* and *N. australiensis*. *N. fowleri* isolates were pathogenic and *N. australiensis* isolates showed low virulence. The results show that the genus *Naegleria* is widely represented in the irrigation canals of the Mexicali Valley, and that pathogenic species are also present, which represents a risk for the population that has contact with the water of these canals.

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P-14

Acanthamoeba infection as a cause of severe keratitis the experience of Poland

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Objectives: *Acanthamoeba* is a human pathogen widely distributed in the environment and recognized as etiological factor of *Acanthamoeba* keratitis (AK). AK occurred most frequently in contact lens wearers. The aim of the study was to examine the frequency of occurrence of *Acanthamoeba* keratitis in patients treated in the Clinic of Ophthalmology, Wrocław Medical University and in the group of volunteer students.

Methods: A total of 69 people (51 women and 18 men) aged from 16 to 64 were examined. Patients with clinical symptoms have undergone thorough ophthalmologic examinations with a particularly in-depth assessment of the local corneal state with a slit lamp.

Contact lenses and preservative fluid were collected from all subjects, and corneal scrapings from patients with clinical symptoms have been examined. The obtained material was used for the *in vitro* culture. *Acanthamoeba* parasites were isolated from clinical specimens by standard techniques. *Acanthamoeba* were cultured on no-nutrient agar plates spread with *Escherichia coli* or *Enterobacter aerogenes* (PCM 532 strain). All the plates were incubated at 37ºC and 22ºC for up to 14 days and examined every 24 h for amoeba growth under a microscope (200x and 400x magnification). Positive results of the culture were confirmed by confocal microscopy and diagnosed using molecular techniques PCR.

Results: *Acanthamoeba* was detected in five patients, including four people with full clinical symptoms and one person with slight redness and discomfort in the eye. The prevalence of AK was estimated as 7.3%.

Based on the *Acanthamoeba* sequences available in the GenBank we confirmed that the PCR products are fragments of *Acanthamoeba* 18S rRNA gene and that isolates represent T4 genotype. T4 is known as the most common strain characteristic for AK cases.

Conclusions and Significance of the work: *Acanthamoeba* keratitis should be taken into consideration in patients presenting clinical features suggesting HSV keratitis with contact lens history and severe pain compliance. Correct diagnosis of AK is a serious diagnostic problem, due to the clinical similarity to inflammatory states caused by viruses, bacteria and fungi. The lack of availability of anti- *Acanthamoeba* - keratits drugs in Poland delays the treatment process.

P-15

Occurrence of *Acanthamoeba* genotype T10 in a geothermal power plant.

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Free-living amoebae can withstand adverse environmental conditions in their cyst phase, mainly those belonging to *Acanthamoeba* genus, due to the presence of cellulose in the cyst wall. This feature allows to find *Acanthamoeba* in extreme environments, such as textile wastewater, salt water and air. A geothermal power plant is a facility where electricity is generated by geothermal energy; geothermal energy is obtained by harnessing heat from inside the earth. The objective of the study was to determine the occurrence of free-living amoebae in cooling channels and lagoon of a geothermal power plant. Water samples of 250 mL were collected from cooling channels and lagoon. Temperature, pH, dissolved oxygen and conductivity were measured on site. Water samples were filtered trought cellulose nitrate filter (1.2 µm pore size); filters were placed inverted on non-nutritive agar plates seeded with *Enterobacter aerogenes* (NNA), the plates were incubated at 30 and 37 °C. A preliminary morphological identification were done to genus level. For the classification of the isolates at the genotype level, the diagnostic fragment 3 of the 18S rDNA gene was amplified. The obtained products were purified and sequenced and the DNA sequences were compared to the ones available in the Genbank database. The isolated strains belonging to *Acanthamoeba* genus. Phylogenetic analysis revealed that all strains belonging to genotype T10, *Acanthamoeba culbertsoni*. Amoebae growth at 37 and 42 °C. The strains were pathogenic when they were inoculated in mice. Lagoon parameter ranges were: temperature of 19 to 22 °C, pH of 6.6 to 6.7, dissolved oxygen of 5.0 to 5.8 and conductivity of $4.1x10^4$ to $5.2x10^4$ μScm^{-1}; channels parameter ranges were: temperature of 34 to 54 °C, pH of 7.3 to 8.1, dissolved oxygen of 1.2 to 2.2 and conductivity of $4x10^4$ to $4.4x10^4$ μScm^{-1}. *Naegleria* spp. were not present in cooling water, due to high salt concentration of water. It is the first time genotype T10 is reported in Mexico.

P-16

Evaluation of the presence of Free Living Amoeba in public indoor swimming pools in the North of Portugal

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Free Living Amoeba (FLA) are widely distributed in the environment and have been isolated from different sources such as water, sand, soils, dust and air. To date, six groups of FLA, namely *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, *Vahlkampfia* spp. *Sappinia pedata* and *Vermamoeba vermiformis*, are reported to be harmful to humans and other animals' health. The FLA-related health risk can result from their known ability to induce infection-associated pathologies and to act as vehicles of other pathogens such as bacteria and viruses. *Acanthamoeba* spp*., N. fowleri* and *V. vermiformis* are among the most common species found in water bodies. In this work, the presence of FLA was evaluated in water collected from the pools and shower rooms of 20 public indoor swimming pools located in the North Region of Portugal. No positive detection was obtained for the pools' water samples, however positive results were found for the water collected from the shower rooms of two of the studied facilities (SP01 and SP16). For SP01 and SP16, a second more comprehensive sampling campaign, in which the evaluation of FLA was extended to the rest of the facilities' water systems, was conducted. In this second sampling, *Acanthamoeba griffini* genotype T3 and *V. vermiformis* were isolated in SP01 whereas *Vannella planctonica* and *Naegleria canariensis* were the species identified in SP16 water samples. Because some of these amoebae can cause fatal infections and act as environmental carriers of other pathogens of high medical relevance, in order to safeguard the health of swimming pool's users, our results should raise awareness about the need for controlling these pathogens in the heated water reservoirs that serve the public swimming pool's facilities.

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P-17

Isolation and molecular identification of different Free Living Amoeba (FLA) in water sources of Tenerife, Canary Islands, Spain.

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Free-Living Amoebae (FLA) are widely distributed microorganisms worldwide and have been isolated from many sources such as dust, soil and water. Furthermore, some genera/species of FLA such as *Acanthamoeba* spp., *Naegleria fowleri* and *Balamuthia mandrillaris* among others, are able to cause opportunistic infections in humans and other animals. More recently, FLA have been reported to be environmental carriers of pathogenic bacteria, fungi and viruses, and thus have gained further importance from the public health point of view. Regarding the prevalence in water bodies, *Acanthamoeba* spp. and *Vermamoeba vermiformis* are among the most common species described in the literature. In this study, 59 water samples from Tenerife Island (Canary Islands, Spain), including tap water, irrigation water, fresh water, recreational fountain water and bottled water, were analysing in order to evaluate the presence of FLA 13,6 % (8/59) of the samples were positive for FLA, including *Acanthamoeba* spp., *Naegleria gruberi*, *Vermamoeba vermiformis* and *Vannella* spp. To the best of our knowledge, this is the first report of *N. gruberi* and *Vannella* spp. in water samples of Canary Islands.

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P-18

Evaluation of effects of chlorine and temperature in the development of *Naegleria fowleri* and other Free-Living Amoebae: applications in artificial lagoons of recreational use

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Free-Living Amoebae (FLA) are causative agents of lethal encephalitis and amoebic keratitis among other infections. *Naegleria fowleri* also known as the *Brain Eating Amoeba* has gathered relevance recently due to increase awareness and also widely covered infection cases in the US and worldwide.

Among these cases, the death of a surfer who was using an artificial lagoon in the USA was widely covered in the media. Moreover, risk activities such as swimming in pools and lakes allowing amoebae-contaminated water enter through the nose have also been highlighted recently and in the past.

Therefore, in this study which has gathered the collaboration of a company and the academia, key questions were risen and checked for an answer such as the effect of different parameters against the development of FLA and specifically *Naegleria fowleri.* Among the tested factors, temperature of the water and chlorine levels were evaluated. The obtained results are presented in this communication, with the idea of opening a path for the future development of amoebae free recreational water related areas.

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P-19

Discovery of anti-amoebic compounds from screening the MMV Pandemic Response Box on *Naegleria fowleri*, *Acanthamoeba castellanii* and *Balamuthia mandrillaris*.

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Pathogenic free-living amoebae*, Naegleria fowleri, Acanthamoeba castellanii* and *Balamuthia mandrillaris* are the etiological agents of severe brain diseases known as Primary Amoebic Meningoencephalitis (PAM), Granulomatous Amoebic Encephalitis (GAE), and *Balamuthia* Amoebic Encephalitis (BAE), respectively. The case mortality rates are greater than 90%, with PAM being the most acute and lethal due in part to delayed diagnosis and partially effective therapeutics. Given the difficulties with diagnosis and the fact that most cases are diagnosed very late, the unmet medical need is for rapidly acting, highly potent new drugs to reduce mortality rates. Herein, we report on efforts to discover new drugs as anti-amoebic therapeutics. We used CellTiter-Glo 2.0 high-throughput screening methods to screen the Medicines for Malaria Ventures (MMV) Pandemic Response Box in a search for new active chemical scaffolds against pathogenic *N. fowleri*, *A. castellanii* and *B. mandrillaris.* The Pandemic Response Box contains 400 bioactive compounds with known efficacy against viral, bacterial, and fungal diseases. Initially we screened the full library as a single point assay at 10 µM; from these data we identified 107 compounds that were active against *N. fowleri*, 24 compounds for *A. castellanii,* and 43 compounds for *B. mandrillaris*. Next we confirmed hits by conducting quantitative dose response assays and we validated 29 hits against *N. fowleri*, 14 against *A. castellanii,* and 12 against *B. mandrillaris.* The activity of the validated hits against the trophozoite stages of the 3 amoebae ranged from nanomolar to low micromolar potency. We counter screened all of the reconfirmed hits with a cytotoxicity screen against A549 mammalian cells and identified only 10 compounds that displayed cytotoxicity from 310 nM to 8 µM, all others tested were > 10 µM. We prioritised the anti-amoeba compounds by determining the selectivity index (Cytotoxic cell line IC_{50}/Amoebae IC_{50}), with an index of $\geq$ 10 being the standard for the consideration of further evaluation as a potentially useful drug for treatment of these parasitic diseases. These data identify new starting points for discovery of drugs to treat diseases caused by pathogenic free-living amoeba and demonstrate the utility of phenotypic screening for drug discovery for *Naegleria*, *Acanthamoeba*, and *Balamuthia*.

P-20

Search for natural extracts for the treatment of *Acanthamoeba* amebiasis. A comparative study of two viability methods

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The treatment of *Acanthamoeba* is a problem due to the toxicity of the different compounds used in humans, and by the high resistance of their cysts. Many studies are focused on the search for medicinal plants as a source of molecules with high anti-*Acanthamoeba* activity but with lower toxicity. It had been described, that different extracts and essential oils are effective in controlling the growth of a wide variety of microorganisms, including bacteria, parasites, yeast, and filamentous fungi. In the present study, an evaluation of anti-*Acanthamoeba* activity in petals' ethanolic extract of *Rosa gallica L.* and in flower's ethanolic extract of *Santolina chamaecyparissus* was carried out in comparison with *Matricaria recutita* ethanolic extract that has a known amoebicidic effect. *Acanthamoeba* USP-CR5-A45 strain from a keratitis patient and incubated with different concentrations of the plant extracts which were further evaluated for their amoebicidal activity. Viability was assessed using two staining methods, Trypan Blue stain and CTC stain at different time intervals (1, 24 and 48 h). Trypan Blue (TB) viability was obtained by manual count with light microscopy while the CTC stain was determined using a fluorimetry.

The IC50 at 1h for *M. recutita* (control) by TB was 2.12 mg/ml and 3.91 mg/ml by CTC. The other extracts also showed low IC50. In the case of *S. chamaecyparissus* the IC50 was 5,94 mg/ml with both techniques. However, in the case of *R. gallica* viability studied with CTC was inviable as no variation in fluorescence could be observed; by TB the IC50 was 9.29 mg/dl.

The obtained results revealed that ethanolic extracts of *R. gallica L.* and *S. chamaecyparissus* could be considered as new natural agents against *Acanthamoeba* spp. It's important to highlight that the use of CTC together with fluorimetry revealed similar results to TB. Nevertheless, in the case of *R. gallica* this technique was useless probably due to the natural pigments presented in the extract that could be interfering with the measurement.

At the light of these results, the viability test should be designed considering that natural pigments could lead to false results like the ones observed for *R. gallica*.

P-21

Study of the presence of Immunoglobulin A in tears from healthy people against *Acanthamoeba* sp

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Acanthamoeba spp. is a free-living amoeba with a wide environmental distribution, found in hot springs, lakes, swimming pools, air conditioning ventilation ducts, in tap water, air, soil, and in contact lenses or their cases.

In humans it can produce different pathologies, such as granulomatous amoebic encephalitis or cutaneous amebiasis associated mainly with immunocompromised people, while *Acanthamoeba* keratitis is associated with contact lens wearers who do not comply with adequate hygienic measures or with previous eye trauma.

In the current work, the presence of specific immunoglobulin A (IgA) against *Acanthamoeba* has been studied by an indirect immunofluorescence method. Volunteers were recruited from a Spanish University and High School. Volunteers collaborate with two samples one from each eye. The samples were used undiluted against trophozoites of *Acanthamoeba* USP-CR-A45 (T4 genotype).

The results obtained show that a percentage higher than 50% of the people studied present a specific response to *Acanthamoeba* in tears. To our knowledge this is the first study of the presence of IgA in tears in Spanish population. This high prevalence is in accordance to the high levels of *Acanthamoeba* in environmental samples from Spain. The presence of IgA in tears could be a protective factor for possible new contacts with these amoebas.

P-22

A primary amebic meningoencephalitis case associated with surfing in an inland surf park

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Background: *Naegleria fowleri* is a thermophilic ameba that is found in freshwater and causes primary amebic meningoencephalitis (PAM; 0–8 infections per year in the United States) when it enters the nose and migrates to the brain. Patient exposure to water containing the ameba typically occurs in warm freshwater lakes and ponds during recreational water activities. In September 2018, a 29-year-old man died of PAM after visiting a Texas inland surf park.

Methods: To determine water exposures, we reviewed medical records and conducted interviews with family and individuals who had traveled with the patient. To further investigate the inland surf park as a possible exposure source, we visited the facility and collected water, biofilm, and sediment samples from the surf park and other venues (water slides, lazy river, and cable park) within the facility. We assessed water sources and treatment practices, performed water quality tests, and tested for the presence of *N. fowleri* by culture and real-time PCR.

Results: Interviews revealed that the case-patient's most probable water exposure in the 10 days before becoming ill occurred while surfing in an inland freshwater surf park where he fell off the surfboard into the water multiple times. The onsite investigation of the facility revealed a practice of manual chlorine treatment with monitoring, but no water filtering or record keeping to document water quality. Surf park water temperature was warm (25 °C) and chlorine residual was negligible. *N. fowleri* was detected in 1 water and 1 sediment sample collected at the cable park venue, and viable thermophilic amebae were detected in all samples collected from the surf park, water slide, and cable park venues, as well from the sediment in the open-air ground water reservoir feeding the venues.

Conclusions: This investigation documents a novel exposure in an inland surf park as the likely exposure causing PAM. Conditions in the surf park were conducive to amebic growth. Novel types of recreational water venues that do not meet traditional definitions of swimming pools, such as this surf park, might not meet the water quality standards for pools or similar treated venues. Clinicians and public health officials should remain vigilant for non-traditional exposures to water.

P-23

Immunogenic surface proteins of *Balamuthia mandrillaris*

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Balamuthia mandrillaris is a free living amoeba that can be isolated from soil and water. It is an emerging pathogen causing skin lesions as well as CNS involvement with a fatal outcome if untreated. The infection has been described more commonly in immunocompetent individuals, mostly males, many children, and with a predilection for population with Hispanic background. Rapid identification of balamuthiasis is critical for effective therapeutic intervention and case management. The objective of the present work was to analyze the membrane protein profile of *B. mandrillaris* searching for immunogenic surface specific proteins which could be used as targets for identification and/or their participation in adhesion processes as a possible pathogenic mechanism. For this purpose, whole cell protein extract of the pathogenic *N. fowleri*, *Acanthamoeba* T4, and of *B. mandrillaris*, as well as surface membrane proteins of the latest were compared by Western Blot with rabbit hyperimmune serum against surface membrane protein extract. Preliminary results showed a strong detection of a band around 50kDa with no cross reaction with the other two genera. Further studies will be carried out using human sera to see if the recognition of these proteins remains the same.

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P-24

In vitro effects of envionmental isolates of *Acanthamoeba* T4 and T5 over human erythrocytes and platelets

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Free-living amoebae of the genus *Acanthamoeba* are widely distributed microorganisms considered emerging amphizoic parasites[1] and have been associated with keratitis and encephalitis. Through molecular techniques, it has been determined that most of the isolates from human and environmental infections belong to the T4 genotype[2]. Some factors related to their pathogenic potential have been described, including the release of hydrolytic enzymes, and the adhesion and phagocytosis processes that leads to cytopathic effects. These damage mechanisms may involve cells such as erythrocytes and platelets[3]. Despite there are few studies regarding the effects of Acanthamoeba on hemodynamics and microcirculation; thrombi, necrotizing vasculitis, and hemorrhages[3, 4] have been described as part of the clinical manifestations.
The aim of this study was to evaluate the capacity of damage of some environmental isolates of *Acanthamoeba* and their products over blood elements. These amoebae were already tested for other characteristics of pathogenic potential by our research group[5, 6].

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P -25

Acanthamoeba genotype T5 with high patogenic potential isolated from a hospital

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Several isolates of *Acanthamoeba,* obtained from samples of different sources in Costa Rica have been axenically grown and characterized using molecular techniques. Besides, the pathogenic potential of these amoebae has been evaluated. Osmo- and thermotolerance, the secretion of proteases and hemolytic activity of some of these isolates have been proven using in *in vitro* models.

An *Acanthamoeba* genotype T5 was isolated from a water sample in the Internal Medicine Unit of a hospital in Nicaragua and sent to our laboratory. This isolate resulted thermotolerant and secrete serine and cystein proteases. Moreover, the cythopatic effect of this isolate over Vero and MDCK cell monolayers was evaluated using confocal microscopy and the cell imaging multi-mode reader Cytation 5. An important decrease in the percentage of live cells after 8 h and 12 h post infection was observed for using the Hoechst fluorescent stain. Intracellular morphological were also observed.

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P-26

Exploring the extracellular vesicles´world in *Acanthamoeba*: a preliminary study

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Extracellular vesicles (EVs) have become subject of intensive research due to their proven participation in intercellular communication, immunomodulation, inflammation, pathogenesis, diagnosis, metastasis and drug delivery, among others. First described as a mechanism for the elimination of membrane cellular waste, they are now considered as possible active elements of communication inside an organism or among different ones. Due to their size, composition and site of origin, EVs can be classified in apoptotic bodies, ectosomes and exosomes. The secretion of EVs in protozoan parasites has been widely studied, especially in *Trypanosoma cruzi*, *Toxoplasma gondii*, *Leishmania* sp., *Trichomonas vaginalis* and *Giardia intestinalis*.

In this study, we performed the first characterization of the EVs of *Acanthamoeba* using AFM. *Acanthamoeba* T5 were incubated at two different temperatures and the resultant EVs were quantified and measured using different techniques. AFM was employed for both topography and force spectroscopy measurements, as for the determination of biophysical properties. Finally, analyses of the protease content of the EVs using zymography were also included. Our results suggest that EVs of *Acanthamoeba* T5 could be important effectors in the pathogenicity of this organism.

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Isolation and molecular characterization of Free Living Amoeba (FLA) in environmental sources of Santiago Island, Cape Verde

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Free-Living Amoebae (FLA) are protozoa widely distributed worldwide and have been isolated from many environmental sources such as dust, soil and water. They have been described as causal agents of lethal encephalitis or sight-threatening keratitis. On the other hand, there are many studies which have demonstrated their important role in the environment, as well as vehicles of other pathogenic bacteria, fungi and viruses. Thus, lately they have gained further importance from the public health point of view. Related to other pathogens internalization, *Acanthamoeba* spp. and *Vermamoeba vermiformis* are among the most common species described in the literature. In the present study, the presence of FLA was evaluated in 39 samples from Santiago Island (Cape Verde), including tap water, sea water, fresh water, depurated water and soil. 67% of the samples were positive for FLA (26/39), including *Acanthamoeba* spp. T4, *Vermamoeba vermiformis* and *Vannella croatica*. To the best of our knowledge, this is the first report of FLA in Cape Verde.

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Genotyping of Free Living Amoebae strains isolated from indoor environment in Slovakia regarding to the quality of indoor environment

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It is generally known that free living amoebas (FLA) are widespread in nature, but no less serious, due to their pathogenic potential, is their occurrence in an artificial environment used by the public. Monitoring of FLA in various facilities (indoor and outdoor swimming pools, drinking water, air conditioning, etc.) in Slovakia over the past decade confirmed their frequent occurrence in the environment. The aim of our work was to confirm the possible pathogenicity of selected strains of environmental isolates by molecular analyzes.

Methods: A selected set of 36 FLA strains isolated from different artificial sites were tested by PCR and genotyping. DNA extraction from NNA plates + PAS (Page´s Amoeba Solution), Centrifuge, DNA purification kit Maxwell 16®, Quantification DeNovix®, PCR of rDNA 18S universal primers for FLA and *Acanthamoeba* primers JDP1, JDP2.

Results: After the molecular characterization, 30 samples were positive for FLA (83%). The presence of *Acanthamoeba* sp. T4 was recorded in 8 samples (26,7%, 8/30), *A. hatchetti* T4 in one of these samples. However, the most numerous was *V. vermiforiis*, confirmed in 21 samples (70%). Interestingly, we have isolate and characterize *Paravahkampfia ustiana* in one of the swimming pool waters (3%, 1/30).

Our findings suggest the potential risk of acquisition amoebic infections, and the need to demonstrate their interactions with pathogenic bacteria in drinking water network as an important public health concern. This fact should help rising awareness of the problem and allow to prepare a complex support of diagnostic procedures, epidemiological investigation, disinfectants system practice setup to prevent the mutual occurrence of FLA and amoeba-resisting bacteria with aim to underline the importance of considering amoebae for water control measures.

THANK YOU FOR ATTENDING

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