FLAM 2025

XXTH INTERNATIONAL MEETING ON THE BIOLOGY AND PATHOGENICITY OF FREE LIVING AMOEBAE

Puerto Morelos, Quintana Roo, Mexico

November 3rd – 7th , 2025

ABSTRACT

BOOK

Dear FLAM family,

It is with great joy and enthusiasm that we welcome you to the 2025 edition of the XXth International Meeting on the Biology and Pathogenicity of Free-Living Amoebae (FLAM).

As many of you know, FLAM is a biennial meeting that brings together researchers, clinicians, and experts from around the world, united by a common passion: the fascinating world of Free-Living Amoebae. These gatherings showcase groundbreaking work — from the discovery of new species and endocytobionts to major advances in pathogenesis, immunology, diagnostics, and therapeutics.

But FLAM is not only science, FLAM is also a celebration of friendship, collaboration, and the strong international community we have built over the years.

This year, Mexico has the honor of hosting the meeting, and we are delighted to share not only our love for science but also the natural beauty, cultural richness, and warm hospitality of our country.

We hope that your experience in our Mexico is as inspiring professionally as it is memorable personally.

Organizing committee

Dr. Maritza Omaña Molina
Universidad Nacional Autónoma de México

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Universidad Nacional Autónoma de México

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Instituto Tecnológico de Sonora

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Isabel Marcelino Institut Pasteur de la Guadeloupe, French West Indies.	**Jacob Lorenzo Morales** Instituto Universitario de Enfermedades Tropicales y Salud Pública, Universidad de La Laguna, Tenerife, Spain.
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KEYNOTE LECTURES

DR. MARTIZA OMAÑA-MOLINA

Dr. Maritza Omaña Molina is a biologist, microbiologist, and Doctor of Science with a specialization in Experimental Pathology from CINVESTAV-IPN. With more than 35 years of academic experience at UNAM, she is a Full Professor ("Titular C") at FES Iztacala, where she has trained generations of medical and scientific professionals. Since 2016, she has also served as Head of the Laboratory of Amphizoic Amoebae.

Her research focuses on the biology and pathogenic mechanisms of free-living amoebae, being a pioneer in Mexico in the study of amoebic keratitis. She has published over 60 scientific articles, several book chapters, and has delivered more than 170 presentations at national and international conferences. She has also supervised and advised undergraduate, master's, doctoral, and postdoctoral theses, consolidating her role as a key mentor in biomedical sciences.

She is a Level II National Researcher (SNI) and a member of UNAM's PRIDE program (Level C). Her work has been recognized with several awards, including the Miguel Alemán Valdés Medical Research Award (2017) and the State of Mexico Award for Science and Technology (COMECYT, 2020).

Dr. Omaña is recognized not only for her solid scientific and academic contributions, but also for her commitment to teaching, professional ethics, and knowledge dissemination, making her a national and international reference in the study of free-living amoebae and their impact on human health.

KL1. Mexico's contributions to understanding the pathogenic mechanisms of amphizoic amoebae

Maritza Omaña-Molina[1,*]

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The amphizoic amoebae of the *Acanthamoeba* genus, and *Naegleria fowleri* are opportunistic pathogens of medical importance due to the pathologies they cause, for which, up to now, there has not been a treatment of choice. The study of the pathogenic mechanisms of these amoebae can open new perspectives for the design of alternative treatment therapies.

The early morphological and electrophysiological events occurring during *the in vitro* interaction of *Acanthamoeba* spp. an *N. fowleri* with different blank tissues has been analyzed.

Initially, the AK *ex vivo* model was implemented to describe the interaction with intact or physically damaged hamster corneas (Mesocricetus auratus) using light microscopy and scanning electron microscopy. The invasion and disruption of corneal tissue is performed by the adhesion, migration, and amoebae penetration through cell junctions, either by the action of proteases, only promoting cellular separation but not their destruction, and/or a mechanical effect exerted by amoebae. Phagocytosis of recently detached cells leads to the modification of their architecture, facilitating the migration and destruction of deeper layers of the corneal epithelium, suggesting that the contact-dependent activity is an important pathogenic mechanism of *Acanthamoeba* spp. The most important remark in this study is that the presence of proteases in both total extracts and conditioned medium is not determinative in tissue destruction, and previous physical damage to the corneas was not a prerequisite for the development of amebic corneal ulcerations.

This was further confirmed by using *in vivo* and *in vitro* experimental models, which described the invasion processes of EAG in murine models of healthy and diabetic mice, as well as skin lesions, also suggesting hematogenous dissemination.

Independent contact mechanisms, such as the presence of electron-dense granules (EDGs) in the cytoplasm of trophozoites, have been reported in highly virulent *Acanthamoeba* species.

N. fowleri trophozoites are capable of migrating through the neuroepithelium without producing any apparent damage, suggesting that this migration could occur via a paracellular route, disrupting tight junctions by degrading ZO-1 and claudin-1 using amoebic cysteine proteases.

The interactions with target tissues in the studied species can reduce transepithelial resistance. Besides, extracellular vesicles induced by strains in study actively participate in intercellular communication; regarding pathogens, facilitating paracellular invasion, migration, and damage caused by trophozoites playing a significant role in pathogenic processes as part of a contact-independent mechanism, which, in conjunction with a contact-dependent mechanism, enhances our

understanding of the pathogenicity exhibited by this amphizoic amoeba during its invasion of target tissues.

DR. ASCEL SAMBA-LOUAKA

Dr. Ascel SAMBA-LOUAKA is an Associate Professor of Microbiology in the Department of Biochemistry at the University of Poitiers, France, a position he has held since 2013. He holds a Master's and PhD in Cancerology (focused on the role of bacteria in cancer) from the University Paul Sabatier in Toulouse, completed in 2006 and 2009, respectively. He later pursued postdoctoral research at the Institut Pasteur, investigating interactions between *Listeria monocytogenes* and human cells. In 2018, he earned his Habilitation (HDR), qualifying him to supervise doctoral research.

His research focuses on the interactions between pathogenic bacteria such as *Legionella pneumophila* and *Mycobacterium avium*, and free-living amoebae (*Acanthamoeba* spp.), particularly in understanding the molecular mechanisms of amoebal encystment. He led the "AMOCYST" project (2017–2022), aimed at unraveling the signaling pathways involved in this process. His scientific contributions have been recognized through several awards, including distinctions for research and doctoral supervision in 2017 and 2021.

Dr. Samba has an active publication record with 27 original research articles (5 as first author, 8 as last author), 5 review articles (4 as first author), 3 opinion pieces, and 1 editorial. His work has an H-index of 13, with an average impact factor of 4.35 per publication. He has supervised one postdoctoral fellow, one research engineer, three PhD candidates, and twelve Master's students.

In teaching, he contributes to international academic programs, serving since 2021 as a lecturer in the Master's course "Water Quality and Human Health" at the University of Science and Technology of Hanoi (USTH), Vietnam. Since 2019, he has been Head of the Master's Program in Microbiology and Immunology at the University of Poitiers.

Dr. Samba also holds various institutional and scientific responsibilities. He is a member of the Research Committee and the Faculty Council at the University of Poitiers and currently serves on a scientific evaluation panel for the French National Research Agency (ANR). Nationally, he is a member of the "Biodiversity, Evolution and Biological Adaptation" section of the National Committee for Scientific Research (CoNRS) and previously served on the board of the "Microbial Pathogenesis" section of the French Society for Microbiology (SFM).

KL2. Exploring the molecular mechanisms of encystment in free-living amoebae

Ascel Samba-Louaka[1,*].

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Free-living amoebae are able to survive in stressful and rapidly changing environments by undergoing encystment—a differentiation process that transforms them into cysts and ensures their long-term survival. While gene expression profiles and protein modifications associated with encystment have been identified in certain amoebae, such as *Acanthamoeba* spp., the initial signals that trigger this differentiation remain poorly understood. Gaining insight into these early events could make it possible to inhibit encystment in pathogenic amoebae, rendering them more vulnerable to biocides. If broad omics-based approaches fall short in identifying these early triggers, should we consider alternative strategies? Could microorganisms capable of influencing or manipulating encystment hold the key?

DR. ISABEL MARCELINO

Dr. Isabel MARCELINO is a biochemist with a PhD in Chemical Engineering and a Habilitation in Biology, Medicine, Health. She has over 20 years expertise in vaccine R&D and microbial pathogenesis. She first worked at ITQB/IBET (Portugal), then at CIRAD (Duclos, Guadeloupe, FWI) and in December 2016, she joined the Institut Pasteur de la Guadeloupe to work on free-living amoebae (FLA). Since 2022, she leads the Amoebae Team and her research topics include (1) the biology and pathogenesis of *N. fowleri*, (2) the diversity and distribution of FLA and their associated microbiome within water ecosystems and (3) public health services (for the detection and identification of amoebae involved in meningitis, keratitis, or others).

KL3. Free-living amoebae in Guadeloupe: a 15-year journey through water, soil, and microbial ecology

Isabel Marcelino[1,*], Youri Vingataramin[1], Aurélie Delumeau[1], Nina Allouch[1], Isaure Quétel[1], Virginie Nerriere[1], Vincent Guerlais[1], Alexis Dereeper[1], Yann Reynaud[1], Mirna Moussa[1] and Antoine Talarmin[1].

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The Guadeloupe archipelago, located in the Caribbean, is known for its diverse water sources, warm climate, stunning landscapes, and biodiversity-rich soils. While its natural beauty attracts many tourists, microbial hazards often go unnoticed.

One of them came to light tragically in 2008, when a young child died from *Naegleria fowleri* infection after bathing in a natural hot spring. This incident marked the beginning of the scientific investigations into the presence and risks of free-living amoebae (FLA) across the island's waters and soils.

Since 2010, our team has collaborated with the Regional Health Agency to screen for *Naegleria* and other FLA in recreational waters, soils, and, more recently, in drinking water distribution systems. The unique ecosystems of these small islands offer ideal conditions for such investigations and have supported the development of a long-term research program at the Institut Pasteur de la Guadeloupe (IPG).

In this presentation, I will provide an overview of the key research conducted at IPG over the past 15 years. This includes recent findings on the geographic distribution of FLA communities in untreated and treated waters, as well as the bacterial taxa they can harbour. This descriptive work lays essential groundwork for understanding the functional role of FLA in water quality. I will also highlight genomics and transcriptomics studies on *Naegleria*, aimed at advancing our understanding of *N.fowleri* virulence.

My objective is to present a comprehensive synthesis of our findings, and to encourage future collaboration among scientists studying amoebae and those working in related fields. This interdisciplinary approach is essential to deepen our understanding of these fascinating organisms, and to raise awareness, particularly as climate change may affect their presence, with potential consequences for water quality and human health.

ORAL PRESENTATIONS

SESSION I
CELL BIOLOGY

Chair: Dr. Sutherland Maciver, Dr. Ismael Castelan-Ramírez

Or1. SARS-CoV-2 reservoir: *Acanthamoeba* and its link to long COVID

Ana Bernárdez[1], Dolores Ollero[1], Angela Magnet[1], Lucianna Vaccaro[1], Carolina Hurtado-Marcos[1], Elizabeth Valdivieso[1], Alejandra Álvarez[1], Rubén Agudo[1], Soledad Fenoy[1], Maritza Omaña[2], Fernando Izquierdo[1] and Carmen del Aguila[1,*].

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Acanthamoeba, is a cosmopolitan free-living amoeba known for harboring a high variety of microbial endosymbionts, including pathogens that pose considerable risks to human health. The goal of this study was to evaluate the *Acanthamoeba* potential as a reservoir of SARS-CoV-2. Eighty water samples from two drinking water treatment plants (DWTPs) and three wastewater treatment plants (WWTPs) were analyzed. *Acanthamoeba* was identified in 82.5% water samples, predominantly in WWTPs (93.75%) compared to DWTPs (65.63%). The T4 genotype, which is associated with human infections, was identified in 92% of the isolates examined.

RT-qPCR analysis revealed the presence of SARS-CoV-2 RNA in 34% of water concentrates and 41% of *Acanthamoeba* isolates, with a higher prevalence observed in WWTPs, though it was also detected in DWTP outlets. Microscopy techniques (immunofluorescence, confocal microscopy, and transmission electron microscopy) were employed to confirm the intracellular localization of the virus, the expression of viral proteins, and the highly vacuolated cytoplasm and damaged mitochondria suggestive of apoptosis.

These results lend support to the hypothesis that *Acanthamoeba* may be a niche and reservoir for SARS-CoV-2 in aquatic environments, thereby contributing to viral environmental stability, transmission, and the long-term health effects, such as long COVID. Ultimately, this study highlights the importance of enhancing monitoring and refining disinfection protocols for *Acanthamoeba* eradication in water treatment systems, given its significant impact on public health.

Or2. Microsporidia-like parasites of amoebae

Elena Nassonova[1,*], Oksana Kamyshatskaya[1], Arseniy Shklyar[2], Yelisei Mesentsev[2], Mike Rayko[1], Natalya Bondarenko[1], Anna Glotova[1], Rolf Michel[3], Julia Walochnik[4], Daniel Corsaro[5] and Alexey Smirnov[2].

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Amoebae serve as hosts for various types of endobionts, including viruses, bacteria, and eukaryotic organisms. Among them, the microsporidia-like parasites from the phylum Rozellomycota deserve special attention. While several groups of these parasites have been identified in amoebae, they were considered to be rather rare and remained poorly studied. However, in recent years, significant new morphological and molecular data were obtained, revealing at least some of them as widely distributed groups comprising multiple sequence-rich lineages in the phylogenetic tree. Despite this, only a few representatives have been isolated and studied at the organismal level. Three genera were described in Europe: *Paramicrosporidium* (2 species), *Nucleophaga* (ca 7 species), and *Morellospora* (1 species). The latter parasitize the cytoplasm of amoebae, while *Paramicrosporidium* and *Nucleophaga* develop within the nucleus. They form multinucleate plasmodia, with sequential plasmotomy resulting in spore formation within the sporophorous vesicles. Some aspects of their life cycle remain unclear, such as the mode of entry into the amoeba nucleus and the mechanism of function of the invasion apparatus. The recent discovery of a new *Nucleophaga* species in the Far East of Russia demonstrates the wide geographic distribution of these organisms. Phylogenetically, they are considered to represent early lineages in the tree of Rozellomycota. They retain their own mitochondria and mitochondrial genomes. Phylogenomic studies and investigations into their genome organization and gene content suggest they exhibit intermediate features between typical fungi and canonical microsporidia. This study was supported by RSF project № 23-74-00071.

Or3. Inhibition of cyst formation in *Acanthamoeba castellanii* by endosymbiosis with *Legionella pneumophila*

Hye-Jeong Jo[1,*], Hae-Ahm Lee[2], Fu-Shi Quan[2], Eun-Kyung Moon[2] and Hyun-Hee Kong[3]

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Acanthamoeba is a genus of free-living amoebae commonly found in soil, water, and other habitats. It has two stages in its life cycle: trophozoites and cysts. Under unfavorable conditions, trophozoites transform into cysts, making them resistant to harsh physical and chemical conditions. Infection by *Legionella pneumophila* can reduce the number of cysts formed by *Acanthamoeba* trophozites, however, the mechanisms underlying this process are not well understood. In this study, differentially expressed genes (DEGs) in *A. castellanii* infected by *Legionella pneumophila* or *Escherichia coli* were identified based on mRNA sequencing. We investigated whether the encystment of *A. castellanii* infected with *L. pneumophila* was inhibited and explored the genes involved in this process. After inducing encystment for 72 h, it was found that *Acanthamoeba* infected with *Legionella* showed a 45.8% decrease in encystment compared to the control, while *Acanthamoeba* that had phagocytosed bacteria exhibited a 21.7% decrease. To explore the genes involved in this process, real-time PCR was performed on 20 genes known to have increased expression during encystment to confirm their expression patterns at 24, 48, and 72 h. Among them, thirteen genes, including cyst-specific protein 21, RSNARE, encystation-mediating cysteine proteinase, encystation-mediating serine proteinase, and cellulose synthase, showed increased expression in both the control and bacteria-phagocytosed *Acanthamoeba,* but not in *Legionella*-infected *Acanthamoeba.* This suggests that various cellular processes, including autophagy and cell wall formation, are inhibited in *Legionella*-infected *Acanthamoeba,* leading to reduced encystment. These results are expected to provide important insights for future studies on the encystment mechanism of *Acanthamoeba* and the effects of *Legionella* infection.

Or4. Multidisciplinary approach to study interactions between host cells and free-living pathogenic amoebae

Jan Mach[1,*], Ronald Malych[1], Taťána Baudišová[1], Jana Pilátová[2,3,4], Eliška Drncová[1] and Róbert Šuťák[1].

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The free-living pathogenic amoebae *Naegleria fowleri* and *Acanthamoeba castellanii* remain very dangerous human pathogens despite many studies and new potential drugs. Our research focuses on the interaction between these amoebae and host cells. In particular, we are interested in how quickly they can adapt to their environment and what proteomic and physiological changes occur to make them capable of a parasitic lifestyle. In our study, we searched for virulence factors that might be responsible for the ability of amoebae to invade the host. We monitored by label-free proteomic analysis which proteins and how fast they change their expression during the transition from axenic to host cell culture and vice versa. We identified several proteins with strongly altered expression rates, which we further characterized. We suggest that these proteins may be potent virulence factors used in host invasion and thus promising targets for therapy. Using flow cytometry, advanced fluorescence microscopy and expansion microscopy, we observed how amoebae ingest host cells. We have employed mass spectrometry to identify the proteins secreted by amoebae after contact with the host cell. Finally, we used Raman microscopy to analyze the substances they can utilize from host cells.

Or5. Molecular and cellular approach to understanding pathogenesis of *Balamuthia mandrillaris*

Kasem Kulkeaw[1,2,3,*], Cherie Tsz-Yiu Law[4], Sukanya Chumchuen[5], Nongnat Tongkrajang[1], Pratima Dubey[1], Porntida Kobpornchai[1,2], Urai Chaisri[6], Patsharaporn Sarasombath[1], Stephen Kwok-Wing Tsui[4], Lu Chenyang[7] and Christopher Rice[7].

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Amoebic encephalitis is a rare fatal disease in Thailand. Only three BAE cases are reported without survivors. Given its rarity and difficult-to-culture, genetic variation, pathogenesis, and drug susceptibility of a clinical isolate of *B. mandrillaris* remain unexplored. To analyze phylogeny and identify pathogenic determinants of a clinical isolate of the *B. mandrillaris* KM-20 strain. *B. mandrillaris* trophozoites were isolated from a biopsied brain tissue using co-culture with human lung carcinoma A549 cells. Following adaptation to feeder-free growth (axenic culture), the mitochondrial genome was subjected to phylogenetic study. Transcriptome of the *B. mandrillaris* was analyzed among: 1) the axenic culture, 2) co-culture with human feeder cells, and 3) the biopsied brains. For drug susceptibility, the trophozoites were exposed to drugs commonly used for BAE treatment. The effect of drugs on the inhibition of brain damage was assessed using cerebral organoids. Phylogenetic and comparative analyses revealed a range of diversification in the mitochondrial genome of KM-20 and nine other *B. mandrillaris* strains. The most variable regions were in the ribosomal protein S3 (*rps3*), which was caused by an array of novel protein tandem repeats. The repeating units in the *rps3* protein tandem region present significant copy number variations among *B. mandrillaris* strains and suggest KM20 as the most divergent strain for its highly variable sequence and highest copy number in *rps3*. Among all drugs tested, nitroxoline is the most potent amoebicidal drug without recrudescence. Histological examination showed amoebic invasion and neuron damage following coculture with the trophozoites. The transcript profile suggested an alteration in neuron growth and a proinflammatory response. The release of intracellular proteins specific to neuronal bodies and astrocytes was detected at higher levels postinfection. Nitroxoline could reduce the number of invading trophozoites in human cerebral organoids. Conclusion: The pathogenic *B. mandrillaris* KM-

20 strain is phylogenetically diversified. Nitroxoline is the most potent amoebicidal drug without cyst induction. The use of human cerebral organoids allows for dissecting pathogenicity, identifying biomarkers for brain injury, and testing a potential amoebicidal drug.

Or6. Hiding in plain sight comparative genomic evidence for the presence of peroxisomes in various heterolobosean

Mónica González-Lázaro[1,*] and Karla Acosta[1].

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Naegleria fowleri is an opportunistic human pathogen that can produce a highly lethal infection of the central nervous system. This free-living amoeba belongs to the Heterolobosea, which together with the Jakobida, Tsukubamonadida, and Euglenozoa form the Discoba, one of the three clades of the Excavates (Adl et al., 2019). To date, *N. fowleri* is the only Heterolobosean known to contain peroxisomes, which were identified by performing ultrastructural, cytochemical and comparative genomic analyses (González-Robles et al., 2020). The functions and enzymatic content of peroxisomes can vary greatly, but the biogenesis of these organelles is carried out by a group of conserved proteins known as peroxins. Using the putative *N. fowleri* peroxin sequences as queries for comparative genomic analyses, a complete set of putative peroxins was identified in *Neovahlkampfia damariscottae, Willaertia magna, Acrasis kona, Percolomonas cosmopolitus* (Tetramitia, Heterolobosea, Discoba, Excavates) and *Pharyngomonas kirbyi* (Pharyngomonada, Heterolobosea, Discoba, Excavates). This set of putative peroxins (Pex1, 2, 3, 4, 5, 6, 7, 10, 11, 12, 16, 19 and 22) is probably sufficient for the biogenesis and maintenance of functional peroxisomes. Further comparative genomic analyses were also performed to identify putative peroxisomal enzymes, which were analyzed *in silico* for the presence of peroxisomal targeting signals (PTS1/PTS2). As expected, all of these Heterolobosean have at least one putative catalase enzyme with a PTS1 sequence. Other putative peroxisomal enzymes such as 2, 4-dienoyl-CoA reductase (PTS1), 3-ketoacyl-CoA thiolase (PTS2), acyl-CoA-oxidase (PTS1 and PTS2) and urate oxidase (PTS1) were identified in all or almost all of these organisms. The structure of these predicted proteins was determined using AlphaFold2 to corroborate that the C-terminus (for PTS1) or the N-terminus (for PTS2) were free to interact with the peroxisomal targeting receptors. Taken together, these results provide additional bioinformatic evidence for the presence of peroxisomes in these Heterolobosean.

-Adl, S. M. et al. (2019). Revisions to the classification, nomenclature, and diversity of eukaryotes. *J Eukaryot Microbiol* 66, 4–119. PMID: 30257078.

-González-Robles, A. et al. (2020). Ultrastructural, cytochemical, and comparative genomic evidence of peroxisomes in three genera of pathogenic free-living amoebae, including the first morphological data for the presence of this organelle in Heterolobosean. *Genome Biol Evol* 12, 1734–1750. PMID: 32602891.

SESSION II
MOLECULAR BIOLOGY

Chair: Dr. Donald Munson, Dr. Lissette Retana Moreira

Or7. A matter of life and death: contrasting alternative splicing during encystment and programmed cell death in *Acanthamoeba castellanii*

Alvaro de Obeso Fernández del Valle[1,*] and Jesús Gómez Montalvo[1].

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Alternative splicing (AS) is a central post-transcriptional mechanism shaping gene expression and phenotypic plasticity across eukaryotes. In the free-living amoeba *Acanthamoeba castellanii*, AS governs two diametrically opposed outcomes: survival through encystment and elimination via programmed cell death (PCD). Here, we contrast these two transcriptional programs, representing alternative strategies that determine the organism's fate. In both processes, intron retention (IR) emerges as a key AS event, influencing genes involved in proteolysis, autophagy, oxidative stress, and RNA processing. During encystment, IR progressively accumulates, contributing to metabolic downregulation and dormancy. During PCD, our analysis revealed not only widespread IR but we also looked at exon skipping, alternative splice sites, and mutually exclusive exons, which were present particularly within pathways central to cell dismantling such as autophagy, proteasome activity, the ubiquitin system, and oxidative phosphorylation. Despite these differences in splicing dynamics, striking commonalities emerge. Retained introns in *Acanthamoeba* share conserved features, such as higher GC content and positional enrichment toward the 3′ end of genes. During PCD, however, this pattern shifts to a more uniform distribution across gene bodies and displays an inverse correlation with transcript abundance, underscoring distinct regulatory logics in survival versus death pathways. Together, these findings highlight IR as a central, yet context-dependent, regulator of *Acanthamoeba* fate. By orchestrating transcriptome reprogramming, AS enables the amoeba either to silence metabolism and persist as a cyst or to undergo programmed self-elimination. This comparative perspective underscores splicing as an intriguing field in protist biology and suggests novel targets for therapeutic strategies against pathogenic *Acanthamoeba*.

Or8. Multi-omics analysis of *Acanthamoeba* after contact with human corneal epithelial cells

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Acanthamoeba spp. are among the most prevalent protists in the environment and the causative agents of *Acanthamoeba* keratitis and granulomatous amoebic encephalitis. Although these amoebae are considered emerging pathogens due to the difficulties of treating infections caused by these microorganisms, and also to their capacity to harbour other pathogenic microorganisms, little is known about their pathogenesis. To fill this knowledge gap, we performed comprehensive, detailed proteomics, transcriptomics, and genomics analyses during the early phase of interaction between the amoebae and human cells. In the present study, we investigated the proteomic, secretomic, and transcriptomic landscapes before and after cell-cell contact using RNA sequencing and tandem mass tag (TMT) technology. The exploration of transcript abundances revealed 12,208 differentially expressed genes, with 2,366 upregulated and 1,705 downregulated. 95% of the expression profiles from 20 transcripts involved in the pathogenicity and randomly selected, displayed comparable trends to those obtained by RNA sequencing. Through TMT-based analysis, we identified roughly 14,000 proteins in the proteome; among these, 3,828 were significantly upregulated, and 2,834 were significantly downregulated. In the case of the secretome, 422 proteins were significantly upregulated and 474 were significantly downregulated. The generated proteomic, secretomic and transcriptomic datasets provide a valuable overview of the regulatory events that occur in the specific context of the interaction between *Acanthamoeba* and HCECs.

Or9. Development of a novel diagnostic method, loop-mediated isothermal amplification (LAMP) for diseases caused by *Balamuthia mandrillaris*

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Balamuthia mandrillaris is a free-living amoeba that can cause cutaneous skin lesions, systemic disease, and a severe, life-threatening central nervous system (CNS) infection known as *Balamuthia* amoebic encephalitis (BAE). BAE currently has a high mortality rate ~92% indicating detection methods and treatment protocols are inadequate. The current diagnostic methods for *B. mandrillaris* have many limitations including the long detection time, low sensitivity and specific diagnostic materials that are only available in highly specialized centers. We believe the early stage of diagnosis is important for successful therapy because of the rapid development of CNS *B. mandrillaris*-infection. Develop, optimise and evaluate a novel loop-mediated isothermal amplification (LAMP) assay for *B. mandrillaris.* This novel diagnostic assay can detect five different *B. mandrillaris* strains with no cross-reactivity with the DNA of other free-living amoeba (*Acanthamoeba* sp. or *Naegleria fowleri*), protozoa (*Toxoplasma gondii* or *Sarcocystis neurona*) or bacteria (*Escherichia coli*, *Serratia marcescens*, and *Pseudomonas aeruginosa*). The positive readout can be observed by the naked eye within 30 minutes at 65°C. The lower limit of detection for a positive signal was 10 fg/μL of extracted *B. mandrillaris* DNA, 10x *B. mandrillaris* trophozoites/100 μL of media, or 1x heated trophozoite/100 μL of media, which displays ~10-100-fold higher sensitivity than conventional *B. mandrillaris* PCR. Due to its simplicity, speed, and higher sensitivity, our LAMP method developed here may provide more applications for quickly detecting and diagnosing *B. mandrillaris*-infections to potentially increase the cure rate from BAE.

Or10. *Acanthamoeba castellanii* genome annotation and transcriptomic and proteomic profiling of cyst formation for five days

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Nutrient deprivation can trigger encystation of *Acanthamoeba castellanii* trophozoites, and the resultant cysts are thought to be central to pathology during human eye infections by this amoeba. Proteins found in the cyst wall could be diagnostic and/or therapeutic targets. However, efforts to systematically characterize cyst wall proteins have been hindered by poor annotations of the *Acanthamoeba castellanii* genome, which is challenging because of numerous introns and short intergenic distances. We used long-read RNA sequencing (MAS-ISO-seq) across a complete encystation time course (including mature cysts) to improve genome annotation accuracy from 52% to 98%. Annotated genes increased from 15,019 to 15,944. Unique peptides observed by mass spectrometry proteomics across the encystation time course increased from 65,565 to 84,124. Genes with predicted signal peptides, which are frequently found in cyst wall proteins, increased from 1,362 to 1,877. We used this new "ACANB" (*Acanthamoeba*, Broad Institute) annotation and an integrated analysis of deep encystation transcriptomics and proteomics to uncover multiple new cyst wall proteins, which were confirmed by confocal fluorescence microscopy. We also used these cyst wall proteins as confocal microscopy markers to demonstrate trophozoites encyst in a human epicorneal tissue model. More broadly, the ACANB annotation sets a new foundation for biology in *Acanthamoeba.*

Or11. *Acanthamoeba* genotype T4 complexity at the molecular level

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Acanthamoeba castellanii (Douglas) Page, 1967 is the type species of the genus *Acanthamoeba* and by far the most commonly isolated *Acanthamoeba* species, from environmental samples as well as from clinical samples. Most isolates that morphologically belong to this species show genotype T4 in 18S rRNA gene phylogenies. Currently, eight T4 subtypes have been described, T4A-T4H, which largely correspond to the ten mitochondrial SSU rDNA subtypes, T4a-T4j. In a recent study, we demonstrated that the Neff strain, one of the most widely used strains in biological research, forms a distinct lineage together with the type strain of *Acanthamoeba terricola* Pussard, 1964 and provided morphological and molecular evidence to validate the species *A. terricola*. Here, we now analysed coding and non-coding regions of different nuclear and mitochondrial genes of all T4 subtypes, including 18S rDNA, ITS2, 16S rDNA, and cytochrome c oxidase subunit 1. Our results corroborate the subtypes within genotype T4. Moreover, the subtypes are also confirmed by the secondary structure of ITS2, a unique shape characterizing each T4 subtype, except T4A and T4B, which appear to share the same structure.

Or12. A reference genome for *Acanthamoeba* genotype T6 & high-throughput genotyping of full-length 18S rRNA directly from *Acanthamoeba* keratitis clinical samples

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The London School of Hygiene & Tropical Medicine (LSHTM) Diagnostic Parasitology Laboratory (DPL) maintains an isolate and DNA archive from hundreds of samples derived from patients with *Acanthamoeba* keratitis (AK). As part of ongoing characterization of these samples, one cloned isolate, most closely matching the morphological description of *A. palestinensis*, was found to have 18S rRNA genotype T6, for which there was no reference genome. Hybrid whole genome assemblies were produced using Oxford Nanopore Technologies (ONT) and Illumina sequence data. Three final genomes were produced: all three were highly complete (87-94.1% estimated using BUSCO) but no single assembly represented the best genome using all metrics. For example, we found that the more complete assemblies also contain more duplicate genes, suggesting the requirement for proper phasing in the next version of the T6 reference genome. A representative T6 genome diverged significantly from other available genomes, for example, we found a gap-excluded divergence of 17.49% compared against *A. castellanii* C3 (T4) and 10.8% against *A. palestinensis* Reich (T2). These assemblies provide a valuable T6 genome resource: we have already identified orthologues for the virulence-associated genes serine proteinase, laminin-binding protein, and mannose-binding protein. Additionally, a new nested PCR, sequencing, and bioinformatics pipeline (targeting full-length 18S rRNA sequences) was also developed, primarily built around the open-source software NGSpeciesID. The pipeline was designed to exclude 18S rRNA sequence from non-*Acanthamoeba* eukaryotic sources and to be robust against samples containing more than one *Acanthamoeba* spp. The pipeline produced accurate results when compared with exemplar Sanger sequences. In a single ONT run 66 unique isolates were successfully sequenced, including 31 derived directly from DNA extracted from clinical samples. This pipeline represents a promising improvement to genotyping capabilities for diagnostic laboratories and could alleviate the limitation of using the short *Acanthamoeba*-specific ASA.S1 amplicon for diagnostics by enabling full-length 18S rRNA sequence be obtained even from samples containing other eukaryotic DNA sources that

co-amplify using non-*Acanthamoeba* specific primers. This approach also has scope to be applicable to sequence endosymbionts and additional *Acanthamoeba* genes with diagnostic potential.

Or13. Simultaneous detection of causative agents of amoebic keratitis by qPCR from Schirmer strips

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Keratitis is a serious corneal infection caused by various microorganisms, including bacteria, viruses, or some species of free-living amebae (FLA), primarily *Acanthamoeba* spp. Recently, *Vermamoeba vermiformis* has gained prominence as an emerging pathogen and has been reported to occur in coinfections with *Acanthamoeba* spp., among other microorganisms. Amebic keratitis presents a wide range of symptoms, including eye redness, excessive tearing, light sensitivity, etc, and is often confused with other conditions that share similar symptoms. Differential diagnosis is challenging due to the lack of quick and effective methods for identifying amoebas as the causative agents at the early stages of the disease. This study aimed to establish an accurate DNA isolation protocol of FLA from Schirmer strips infected with different concentrations of cysts derived from cultures of *Acanthamoeba castellanii* and *V. vermiformis* (10^5 -10^3 cysts/10µl). The infections were performed individually for each FLA and as a coinfection. DNA isolation was performed using a semi-automated system, followed by molecular identification of these protozoa, performing a double-plex qPCR reaction. For the validation of the procedure, 15 clinical samples belonging to patients with dry eye symptoms were analyzed. The results showed specific and reproducible amplification at all concentrations tested, with fluorescence signal detection of *A. castellanii* at 28-34 replication cycles (Ct), and Ct = 19-32 for *V. vermiformis*, individually. The coinfected strips exhibited similar correlations between the pathogen's DNA concentration and Ct, indicating high sensitivity and

specificity. Furthermore, from the clinical samples analyzed, *A. castellani* was detected in four samples, while *V. vermiformis* was detected within the amplification limits in two strips. Simultaneous detection of these FLA was reported in one clinical sample. However, more research is necessary to confirm these results. In conclusion, the methodology developed in this study was effective for the simultaneous identification of these amoebae. It could be applicable in real clinical contexts of amoebic keratitis, even in asymptomatic patients, as it has been proven to be a quick, non-invasive, and sensitive protocol.

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SESSION III
ENVIROMENTAL I

Chair: Dr. Ascel Samba, M.Sc. Itzel Citlalli Rubio-Gutierrez

Or14. *Lophomonas* spp and *Acanthamoeba* sp a protozoan in common cockroaches, as a possible symbiosis or a passive vector carrying non-tuberculous mycobacteria and *Mycobacterium tuberculosis*

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Lophomonas spp., is an emerging protozoan parasite belonging to the Parabasalids (Parabasalia, lophomonadida) which infects the respiratory tracts of humans. Besides a few reports of human lophomoniasis, the true burden of *Lophomonas* infection is still unknown. There is no clear understanding of its pathophysiology in humans. Cockroaches are a group of arthropods that are closely associated with an anthropogenic environment, which can adversely impact human health. The presence of the multi-flagellated protozoon *L. blattarum*, in the gut of *B. germanica* and *Periplaneta americana* and other Cockroaches has been described. Few studies have observed and analyzed the presence of protozoa in the fecal droppings of cockroaches, but several studies have examined the external body surface and/or the intestinal contents of cockroaches for protozoa. *Periplaneta americana* cockroaches are considered vectors that transmit bacteriological, viral and fungal diseases, as well as carriers of parasites. Protists. It is believed that *Periplaneta americana* may be part of a dissemination system, in which *Lophomonas* spp or *Acanthamoeba* sp acts as a transient or symbiotic intracellular host for *Non-tuberculous mycobacteria* and *Mycobacterium tuberculosis.*

Or15. Culture enrichment and metabarcoding as a powerful integrated approach to detect pathogenic free-living amoebae in freshwater systems

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Free-living amoebae (FLA) such as *Naegleria* and *Acanthamoeba* are opportunistic pathogens increasingly associated with fatal and severe human infections, particularly in environments with inadequate water sanitation, limited hygiene, and restricted diagnostic infrastructure. In this pilot study, we applied an integrated approach that combines filtration, pelleting, and culture enrichment followed by metabarcoding to investigate the diversity of potentially pathogenic FLA in sectors of the Kakum River Basin near Cape Coast, Ghana. This combined strategy was particularly effective in detecting amoebae that may exist in cyst stages or at low abundance, which are often missed by direct DNA-based methods. Our results revealed a wide diversity of FLA across Amoebozoa and Heterolobosea clades, including taxa of clinical relevance such as *Acanthamoeba*, *Vermamoeba*, *Balamuthia*, and *Paravahlkampfia*. Notably, several species of Naegleria and other opportunistic amoebae were also detected and morphologically confirmed. The favorable environmental conditions at the sampling sites appear to support the persistence and proliferation of these potentially pathogenic organisms, raising important public health concerns. Our findings demonstrate that culture enrichment, when integrated with high-throughput sequencing, greatly enhances detection sensitivity and taxonomic resolution. This underscores the importance of using multi-pronged molecular tools to uncover neglected pathogens in vulnerable communities. The study provides a critical baseline for broader epidemiological efforts and informs the development of cost-effective surveillance tools for monitoring FLA-related health risks in freshwater systems in sub-Saharan Africa. This integrated approach offers a sensitive and scalable framework for detecting clinically important FLA in environmental samples where traditional methods may fail.

Or16. Isolation and molecular characterization of potentially pathogenic free-living amoebae from various aquatic sources

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Free-living amoebae (FLA), thanks to their diversity and ubiquity, we can study from different perspectives. They can survive host-independent and play a crucial role in microbial ecosystems by regulating bacterial populations; some genera are pathogenic and can cause infections in humans and animals; FLA can act as reservoirs and vectors for other clinically significant microorganisms. Our monitoring focused on detecting potentially pathogenic FLA in water distribution systems and recreational water sources. A total of 55 samples, primarily from European countries, were analyzed using cultivation, microscopy, and molecular techniques. Amoebae were detected in 83% of the samples. Molecular typing, targeted at FLA and *Acanthamoeba*, confirmed the predominance of *Acanthamoeba* isolates, and also identified *Vermamoeba vermiformis*. The amoebae strains were isolated from both engineered and natural water systems used for recreational or everyday human activities. Our work will continue by detecting the presence of the genus *Naegleria*, also with an emphasis on the fact that the first case of Primary Amoebic Meningoencephalitis has recently appeared in Slovakia, where the source of infection has not yet been confirmed. A phylogenetic analysis will be conducted to further characterize the genetic relationships of the isolated strains. These results underscore the importance of continuous monitoring and raise public health concerns regarding the potential risks posed by FLA. Furthermore, we aim to emphasize the importance of water quality control, specifically focusing on free-living amoebae, highlighting the need for effective monitoring and increased public awareness of the associated health risks.

Or17. Geographic distribution and genetic diversity of pathogenic free-living amoebae in the northern region of Mexico. Part II

Luis Fernando Lares-Jiménez[1,*], Héctor Manuel Acuña-Ramírez[1], Libia Zulema Rodriguez-Anaya[2], Manuel Alejandro Borquez-Román[1], Ana Laura Soto-Camacho[1], José Reyes Gonzalez-Galaviz[2], Isidra Teresita Ayala-Montenegro[3], Ramón Casillas-Hernández[1], José Cuauhtémoc Ibarra-Gámez[1] and Fernando Lares-Villa[1].

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The presence and genetic diversity of free-living amoebae in Mexico are poorly documented, and in some states, they are even nonexistent. For this reason, we decided to initiate the search for these organisms in the northern region, taking into account the sampling season and natural water bodies. Between 2020 and 2022, 32 water samples were taken from eight northern states, including Baja California, Baja California Sur, Sonora, Chihuahua, Durango, Coahuila, Nuevo León, and Tamaulipas. One-liter samples from river water, canals, lagoons, streams, dams, waterfalls, and oases were concentrated by centrifugation, and the sediment was plated in duplicate on non-nutrient agar plates spiked with live *Escherichia coli*, which were incubated at 37 °C and 42 °C for primary isolation of the amoebae. The following day, using an inverted microscope, small groups of amoebae separated from each other were selected and cloned, taking into account the different morphologies of the cysts and trophozoites found. Although 299 strains were obtained from the various water bodies in primary isolation, 172 at 37°C and 127 at 42°C, the lack of adaptation in some cultures, fungal contamination and excessive growth of certain aquatic bacteria reduced the amoeba population, which was subjected to molecular identification and bioinformatics. DNA was extracted from each strain maintained in culture and used for amplification and pre-identification by PCR, using specific primers for *Acanthamoeba*, *Naegleria* spp., *Naegleria fowleri*, and universal primers for other amoeba genera. Final identification is then performed by sequencing the PCR products. Molecular results reveal free-living amoebae of the genera *Naegleria*, *Acanthamoeba*, and *Vermamoeba*, among others, including *Naegleria fowleri* and *Acanthamoeba culbertsoni*, now classified as genotype T10. Furthermore, the selection of isolation temperatures, chosen to search for pathogenic free-living amoebae described to date, influenced the genetic diversity of the amoebae found and reported for the first time at the sampled sites.

SESSION IV
ENVIROMENTAL II

Chair: Dr. Fernando Lares-Villa, Dr. Carlos Bethencourt-Estrella

Or18. Detection and characterization of free-living amoebae using qPCR and culture-based methods in Tenerife, Canary Islands

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Free-living amoebae (FLA) are widespread protozoa found in soil and water, some of which can cause serious human infections. In this study, we examined 62 environmental samples from Tenerife, including soils, playgrounds, and public drinking water. Two molecular approaches were used: direct qPCR targeting four potentially pathogenic FLA (*Acanthamoeba* spp., *Vermamoeba vermiformis*, *Naegleria fowleri*, and *Balamuthia mandrillaris*), and culture-based isolation followed by standard PCR and phylogenetic analysis. We found at least one kind of FLA in 73% of samples, most commonly *Vermamoeba vermiformis* (60%) and *Acanthamoeba* spp. (55%). *Balamuthia mandrillaris* was found in 10%, its first report in the Canary Islands. *Naegleria fowleri* was not detected. Cultures revealed broader diversity, including *Vahlkampfia, Vannella,* and *Rhogostoma.* Thermotolerance and osmotolerance tests showed *Acanthamoeba* spp. and *V.* vermiformis survived at 37°C and 0.5 M mannitol, suggesting pathogenic potential, though growth declined at 42°C and 1 M mannitol. These findings underscore the widespread occurrence of FLA and highlight their potential risk to public health. Their ability to act as carriers of pathogenic bacteria further reinforces the need for routine surveillance and preventive measures in high-risk environments.

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Or19. Protist-bacteria partnerships across ecosystems: a One Health view of *Acanthamoeba* and *Neoparamoeba* species as microbial reservoirs

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Free-living amoebae (FLA) are increasingly recognized as key environmental sentinels and microbial reservoirs with implications for human, animal, and ecosystem health. Herein, we synthesise recent findings across diverse ecosystems to explore the ecological versatility and biomedical relevance of these protists through a One Health lens.

Field studies in the Puna salt plains of Argentina and coastal lagoons in Australia revealed diverse *Acanthamoeba* populations harboring a range of bacterial endosymbionts, including potential pathogens. In parallel, *Neoparamoeba* spp., commonly associated with marine environments and aquaculture, have been shown to engage in dynamic interactions with *Vibrio* species—some of which are known to cause disease in fish and humans. These relationships may enhance bacterial persistence and virulence, with implications for aquaculture biosecurity and zoonotic risk.

In clinical environments, *Acanthamoeba* has been identified as a protective reservoir for *Pseudomonas aeruginosa,* facilitating its survival and resistance to disinfection. This underscores the role of FLA in bridging environmental and healthcare-associated microbial ecosystems.

A systematic review of intracellular microorganisms within *Acanthamoeba* highlights a complex network of symbiotic interactions, many of which influence microbial evolution, antimicrobial resistance, and host-pathogen dynamics. Comparative analyses with other protists, such as *Trichomonas vaginalis*, suggest conserved mechanisms of microbial accommodation and immune modulation.

Together, these findings position *Acanthamoeba* and *Neoparamoeba* as critical nodes in the One Health triad—linking environmental, animal, and human health. Their ubiquity, resilience, and capacity to host diverse microbes make them valuable models for understanding microbial ecology, pathogen emergence, and cross-domain transmission.

This work advocates for the integration of FLA into environmental surveillance and infection prevention strategies, particularly in high-risk or resource-limited settings. Recognizing their dual role as microbial reservoirs and ecological indicators enhances our ability to anticipate and mitigate emerging health threats across interconnected systems.

Or20. Molecular evidence of *Acanthamoeba* genotypes T2 and T4 in nasal mucosa of gardeners as healthy carriers in Lima, Peru

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The nasal mucosa, an important and complex structure of the face, has a diverse microbiome that changes with age and lifestyle. It is considered the first point of contact against microbial attacks from the outside world and therefore plays an important role in immunity. Gardeners are constantly exposed to damp soil and standing water, so their microbial load, which we now know includes free-living amoebae, comes into contact with the nasal mucosa. Free-living amoebas cause granulomatous amoebic encephalitis (GAS), which is a disease of the nervous system whose development is often associated with comorbidities; however, it has also been reported in immunocompetent patients. Peruvian and foreign studies report the presence of free-living amoebas in the nasal mucosa of healthy individuals. It has been established that the route of infection is cutaneous and/or by inhalation through the nasal passages, subsequently reaching the central nervous system via the bloodstream or through the olfactory epithelium. These amoebae are potentially pathogenic free-living protists such as the T4, T12, and T18 genotypes of *Acanthamoeba* spp. However, *Balamuthia mandrillaris* has not yet been detected in healthy individuals.

The present study sought to demonstrate the presence of free-living amoebae in the nasal mucosa of gardeners as healthy carriers. 96 nasal swab samples were obtained as part of the study, of which 42 (43.75%) were positive for *Acanthamoeba* spp. in monoxenic cultures. Clean sequences were obtained in 35 of the 42 positive samples.

PCR and sequencing analysis resulted in *Acanthamoeba* sp. Blast analysis confirms similarity with *Acanthamoeba* sp, mainly genotype T-4 and T-2. This would be the first time that the existence of genotype T-2 has been confirmed in Peru. Currently, 12 strains correspond to genotype T-4, one to genotype T-2. The others are still to be determined through phylogeny. However, we know that we have strains related to: *Acanthamoeba polyphaga, Acanthamoeba castellani, Acanthamoeba culbertsonii,* and *Acanthamoeba quina.*

Or21. Morphological and molecular studies recover different communities of free-living amoebae from the same environmental samples

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A study of amoebae diversity from urban park ponds in Xiamen, China, revealed significant differences between the amoebae community recovered from enrichment cultures and the set of amoebae-associated barcodes detected in environmental DNA (e-DNA), collected from the same site. Enrichment cultivation recovered 42 amoebae morphospecies from three ponds in a city park in Xiamen. Morphological and molecular studies of the isolated strains showed that most of them are new to science. This indicates that amoebae diversity is still poorly understood and highlights the need for molecular data for correct amoebae identification. For the e-DNA samples, we amplified the V4 region of the 18S rRNA gene. To compare the morphological results with the results of e-DNA sequencing, we amplified DNA extracted from cultures with the primers used for e-DNA studies. Comparison of these datasets shows that only a small proportion of cultured species were recovered among the e-DNA sequences. There are entire clades that are not represented in the e-DNA (e.g., vanellids). However, DNA from cultured representatives of these clades isolated from the same samples was successfully amplified with the primers used in the eDNA study. At the same time, the e-DNA data contained large clades of sequences without morphologically recovered species. We found a very rich diversity of Variosea, *Nolandella*-related and *Vermamoeba*-related sequences in the e-DNA data, significantly exceeding the known number of species in these groups. We performed an analysis of the entire EukBank database of V4 region sequences belonging to Amoebozoa and found the same bias in favour of these taxa. These results indicate that molecular ecology results should still be interpreted with caution and that culture-based studies remain a valuable tool for recovering amoebae diversity. At the same time, molecular data provide valuable guidance for a targeted search for representatives of sequence-rich clades not yet recovered at the morphological level. Supported by RSF project 24-44-00096 and the National Natural Science Foundation of China (32361133557).

Or22. Evolutionary origin of Amoebozoa from an ecological point of view

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Current views on Amoebozoa evolution suggest the origin of Amoebozoa from a flagellate ancestor. It implies independent loss of kinetosomes and flagella in two main evolutionary lineages of Amoebozoa and numerous modifications and losses in the remaining one. The reason for such large-scale losses remains unclear. In this talk, I will try to prove that the origin of Amoebozoa was triggered by the environmental conditions of the mid-Proterozoic Earth and related with the single suppression of flagellum formation at the base of this evolutionary lineage. Probably, the place of origin of Amoebozoa was a photosynthetic microbial mat that covered the bottom of a shallow sea, which at that time constituted most of the surface of the Earth. The low oxygen content of the Earth's atmosphere at that time (0.1- 4 % of today's O_2 concentration) limited the maximal size of aerobic organisms due to the low rate of oxygen diffusion. Photosynthetic microbial mats provided an oxygen-rich environment, allowing single-celled organisms to evolve towards increasing size. The microbial mat is a complex and highly organized structure, and there are almost no individual filaments that could serve as food for small protist species. However, the increase in size allowed amoebozoans to explore the mats, break them down, and use the cyanobacteria that make up most of these mats as food. This type of feeding behaviour is still seen among modern amoebozoan species. Increasing size made the flagellated locomotion low efficient because of Reinolds number limitations. As a result, Amoebozoa developed acto-myosin mode of locomotion and became a truly “amoeboid” lineage. Both basal amoebozoan lineages (Tubulinea and Discosea) lack flagellated stages or any flagellum remnants. However, the flagellum-associated genetic toolkit (as well as many other basic gene families) has been successfully conserved, and since the basal Evosea lineage, flagellated stages have reappeared in amoebozoan life cycles. This was likely enhanced by increasing environmental complexity. Apparently, the recapitulation of flagellated stages was the event that triggered the formation of complex life cycles, completely absent in Tubulinea and Discosea, but present in Variosea, Archamoebea and reaching maximum complexity in Eumycetozoea. Supported with RSF project 23-74-00050.

SESSION V

Naegleria fowleri

Chair: Dr. Maritza Omaña Molina, Prof. Adriana Trujano Rodríguez

Or23. A comprehensive molecular analysis of virulence factor regulation in *Naegleria fowleri* and related species

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Naegleria fowleri, a ubiquitous free-living amoeba, is the etiological agent of primary amoebic meningoencephalitis (PAM), a rare but rapidly fatal infection of the central nervous system with a mortality rate exceeding 95%. The existence of non-pathogenic species within *Naegleria,* such as *Naegleria lovaniensis,* which are thermotolerant, suggests that virulence is determined not only by thermotolerance but also by the differential regulation of specific virulence factors. This study aimed to comparatively evaluate the transcriptional (mRNA) and protein expression of four key virulence factors—Nfa1, Naegleriapore A (NPA), Cathepsin B (CatB), and Mp2CL5—across *Naegleria* species with distinct pathogenic potentials to comprehend the regulatory mechanisms underlying *N. fowleri* pathogenesis. A multi-species comparative approach was employed, utilizing high- and low-virulence strains of *N. fowleri,* alongside *N. australiensis, N. lovaniensis,* and *N. gruberi.* Transcriptional expression was assessed using semi-quantitative RT-PCR with densitometric analysis, while protein profiles were preliminarily examined via SDS-PAGE. Preliminary results revealed distinct transcriptional profiles: pathogenic *N. fowleri* and *N. australiensis* express a broad array of the virulence factors, whereas non-pathogenic species exhibit restricted expression patterns, with *N. lovaniensis* transcribing Nfa1 and NPA, and *N. gruberi* transcribing Nfa1 and CatB. The highvirulence *N. fowleri* strain showed elevated mRNA levels for NPA and Mp2CL5 compared to the low-virulence strain. A discrepancy was observed in *N. lovaniensis,* which exhibited robust NPA mRNA expression but had diminished corresponding protein bands, suggesting a significant posttranscriptional or translational regulatory modification. The pathogenicity of *Naegleria fowleri* appears to be driven by a complex, multi-layered regulatory network that controls the expression and ultimate abundance of key effector proteins, rather than simply the presence of their corresponding genes. PAPIIT-UNAM-IN222923 funded this project.

Or24. Cytokine expression, nitric oxide and reactive oxygen species production during primary amoebic meningoencephalitis in vivo

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Primary amoebic meningoencephalitis (PAM) is an acute and fulminant infection caused by the freeliving amoeba *Naegleria fowleri*, this amoeba produces a severe inflammatory response in the olfactory bulbs (OBs) and it has been related to the pathophysiology of the disease. The objective of this work was to evaluate the inflammatory response given in the OBs by the expression of some proinflammatory cytokines such as TNF-α, IL-6 and IL-1β as well as the production of nitric oxide (NO) and reactive oxygen species (ROS). In the present study, we used Balb/c mice as an animal model where we instilled 25,000 *N. fowleri* trophozoites through the nose and from day 3 to day 7 postinstillation, we performed a RT-PCR and Western blots of pro-inflammatory cytokines as well as immunohistochemistry analysis. We also measured the expression of NO and ROS. Interestingly, both RT-PCR and Western blot assays revealed an increase of TNF-α, IL-1β and IL-6 at the third day postinfection, which increased in a time dependent manner. Moreover, immunohistochemistry analysis of cytokine production correlated with the presence of inflammatory cells in the OBs. Additionally, a significant increase of ROS and NO production was also observed during PAM progression. Based on these results, we demonstrated for the first time, that *N. fowleri* induces local production of inflammatory cytokines and promoting an oxidative milieu in an *in vivo* study; these mediators, together with the presence of inflammatory cells could be associated with the extensive damage in the OBs, leading to eventual host death. This work was supported by CONACyT grant number 237523.

Or25. Immune response to intranasal immunization with *Naegleria fowleri*-derived immunogenic peptides in a mouse model of primary amoebic meningoencephalitis

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Naegleria fowleri is a free-living amphizoic amoeba and the causative agent of primary amebic meningoencephalitis (PAM), an acute and fatal disease of the central nervous system. To infect the olfactory neuroepithelium, *N. fowleri* employs different pathogenicity mechanisms, including: Mp2CL5, Nfa1, cathepsin B, sphingomyelinase, and phospholipase; as well as cytoskeletal components such as: actin, profilin, and septin. Based on these factors, our research group designed in silico peptides (Smp145, SPCB-NF, and SpNfa1) to evaluate their potential immunoprotective effects in a murine meningitis model. In a study by Gutiérrez et al. (2020), mass spectrometry analysis of spot 9 from the 19 kDa band of *N. fowleri* revealed a peptide sequence corresponding to septin, a key cytoskeletal protein involved in cell migration and amoeboid motility through its interactions with actin filaments, microtubules, and membrane phospholipids. Notably, this sequence was absent in the homologous 19 kDa band of *N. lovaniensis*, suggesting that septin may play a critical role in *N. fowleri* amoeboid movement during epithelial adhesion. Thus, this study aimed to design an immunogenic peptide derived from septin using immunoinformatic tools and to evaluate its immune response when co-administered with other *N. fowleri* immunogenic peptides. The peptide was computationally assessed for its binding affinity to murine and human MHC-II, as well as to BCR. Additionally, its antigenicity, allergenicity, and toxicity were determined. The selected peptide was synthesized and intranasally co-administered with Smp145, SPCB-NF, and SpNfa1 peptides to BALB/c mice, followed by challenge with a lethal dose of *N. fowleri* trophozoites. Immune responses were then analyzed. Western blot results revealed recognition of 150, 100, 60, and 19 kDa bands in *N. fowleri* total extract using anti-septin antibodies. Immunocytochemistry localized septin in the cytoplasm, membrane, and nucleus of trophozoites. Elevated IgG and IgA levels were detected in serum and nasal washes of immunized mice. These results suggest that septin contributes to PAM pathogenesis and may serve as a promising vaccine candidate against *N. fowleri* infection when combined with Smp145, SPCB-NF, and SpNfa1 peptides.

Or26. Characterization of the ubiquitin proteasome pathway in trophozoites of *Naegleria* genus

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Free-living amoebae are part of the Protozoa kingdom. These organisms are gaining popularity due to the clinical importance of some species, such as the genus *Naegleria*. Here the amoebae *N. fowleri* is the only pathogenic species for humans, causing a highly fatal disease that develops in the central nervous system, called primary amoebic meningoencephalitis (PAM). Also worth mentioning is the study of non-pathogenic species *N. gruberi* used as a model for the study of cell differentiation and *N. lovaniensis* the closest relative to *N. fowleri,* but with the inability to cause PAM. Otherwise the ubiquitin-proteasome pathway (UPP) is essential for the degradation of misfolded, defective, and short-lived regulatory proteins. This pathway is composed of the 26S proteasome, the most important catalytic complex for the recycling and degradation of intracellular proteins. It is divided into the catalytic core (20S) and the regulatory particle (19S). Ubiquitin is a small, highly conserved protein in eukaryotic organisms that tags the proteins for degradation. The aim of this project was to demonstrate the presence of 19S subunits and ubiquitin protein. Additionally, to know whether is co-localized with the 20S subunits (which have already been described) to form the 26S proteasome in trophozoites of *N. fowleri*, *N. lovaniensis* and *N. gruberi.* Comparative bioinformatics analysis, considering model sequences, determined the presence and homology of 19S subunits and ubiquitin. Experimental techniques confirmed the presence and localization of these proteins, as well as the co-localization of both 26S proteasome complexes in these amoebae. Therefore, it is concluded that amoebae of the genus *Naegleria* have 19S subunits that, together with 20S subunits, make up the 26S. They also have the protein ubiquitin, with which they form the ubiquitin-proteasome pathway. Our acknowledgement to CONACYT for financial support (CVU: 997938) provided during the project.

Or27. Characterization and role in contact-independent pathogenic mechanisms of *Naegleria fowleri* extracellular vesicles

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Extracellular vesicles (EVs) are lipid bilayer-enclosed spherical particles released by cells, primarily involved in intercellular communication. Recent studies have identified *Naegleria fowleri* extracellular vesicles (EVs) that exhibit proteolytic activity and exert a proinflammatory effect on immune cells. However, their potential to induce host cell damage remains unclear. This study aimed to characterize EVs secreted by *N. fowleri* and evaluate their involvement in contact-independent pathogenic mechanisms. Trophozoites were cultured for 24 h at 37°C in bactocasitone medium without fetal bovine serum. EVs were isolated from the supernatant by filtration through 1.2 µm pore and differential centrifugation, then characterized using transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA). Proteomic profiling was performed by mass spectrometry. Proteolytic activity was evaluated using zymography, and hemolytic activity was determined photometrically. Additionally, EV internalization, changes in transepithelial electrical resistance (TER), and induction of cell death were assessed in Madin-Darby canine kidney (MDCK) epithelial cells. TEM and NTA revealed that EVs are spherical and have a size range of 80 to 500 nm. Proteomic analysis identified 1006 proteins with diverse functional profiles. Zymography demonstrated serine protease activity with molecular weights above 100 kDa. EVs induced hemolysis, were internalized by MDCK cells, disrupted TER, and triggered necrosis. These findings suggest that *N. fowleri* trophozoites release heterogeneous EVs with active proteolytic enzymes capable of inducing host cell damage through contact-independent mechanisms, supporting their involvement in amoebic pathogenesis. Funding for this research was provided by PAPIIT-UNAM (Grant No. IN209824) and Programa de Investigadoras e Investigadores COMECYT 2025 (Folio: EESP2025-0002).

SESSION VI

Naegleria fowleri II

Chair: Dr. Jesús Serrano Luna, Prof. Maryam Niyyati

Or28. Characterization and functional roles of extracellular vesicles secreted by *Naegleria fowleri*

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Extracellular vesicles (EVs) are small lipid vesicles released by prokaryotic and eukaryotic cells, involved in intercellular communication, immunomodulation, and pathogenesis. Until 2022, the secretion of EVs by *Naegleria fowleri* was not reported. In this work, we present a complete characterization of EVs produced by trophozoites of *N. fowleri,* including size distribution, zeta potential, protein profiles, and protease activity. Besides, we evaluated the immunomodulatory effect of EVs secreted by two clinic isolates of *Naegleria fowleri* in primary cultures from mouse cell microglia and BV-2 cells using gene transcription analyses, morphological changes induced in primary culture microglia cells after the incubation of cells with EVs, and the presence of nucleic acids of *N. fowleri* in the EV fractions. Under our incubation conditions, EVs of different sizes were observed. SDS-PAGE revealed protein bands of 25 - 260 KDa, and Western blots confirmed the presence of antigenic proteins, which evidenced the strongest recognition by rat polyclonal antibodies raised against *N. fowleri* in the ~80 KDa region. A zeta potential mean value of −12.228 ± 4.843 mV was obtained, and proteomic profiles of the EVs identified at least 184 proteins as the main part of the vesicles' cargo; protease activity assays revealed the predominance of serine proteases. Regarding immunomodulation assays, results revealed increased expression of NOS, IL-6, TNF-α, and IL-23, and the regulatory cytokine IL-10 in primary cultures of microglia, as well as increased expression of NOS and IL-13 in BV-2 cell cultures. Morphologic changes from homeostatic microglia to a more amoeboid morphology were also observed. Finally, specific *N. fowleri* DNA that could be amplified using both conventional and qPCR was confirmed in the EV fractions. The present characterization uncovers the complexity of EVs produced by *N. fowleri,* suggesting a potential relevance in the release of virulence factors involved in pathogenicity and their potential role as biomarkers of primary acute meningoencephalitis.

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Or29. Establishment of a transfection system in *Naegleria fowleri* using ectopic expression vectors

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The pathogenic free-living amoeba *Naegleria fowleri* is the causative agent of primary amoebic meningoencephalitis (PAM), an often (97%) fatal infection. This high mortality rate is due, in part, to the limited availability of effective drugs – a situation exacerbated by the lack of molecular tools to study this organism. Such tools are essential for trans-gene expression, providing vital insight into gene function and enabling genetic validation of potential drug targets. We have developed expression vectors using UTRs of predicted actin and ubiquitin genes from the *N. fowleri* TY reference strain to drive expression of a codon-optimized puromycin N-acetyltransferase (PAC) resistance gene together with either a codon-optimized enhanced yellow fluorescent protein (eYFP) or non-codon-optimized mCherry reporter, respectively. Plasmids were transfected using a 40 kDa polyethylenimine (PEI-40), a cationic polymer which binds to the negatively charged phosphates on DNA to form complex nanoparticles that enhance plasmid uptake. We have observed both eYFP and mCherry expression in a portion of the transfected cells, with mCherry detected at a higher frequency than eYFP. Clonal population isolation followed by puromycin selection produced stable antibioticresistant lines; however, fluorescence expression was lost over time. Overall, we have established a technique for DNA integration into cells using PEI-40, and transgene expression driven by both actin and ubiquitin UTRs. Efforts towards optimization of plasmid architecture to enhance stable expression of both PAC and various fluorescent markers are underway. These advances will provide powerful tools to allow resolution of fundamental questions about amoeba biology and to validate potential therapeutic targets.

Or30. Beta-hydroxylactones as anti-*Naegleria fowleri* agents: insights into cell death mechanisms

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Naegleria fowleri is a thermophilic free-living amoeba that causes primary amoebic meningoencephalitis, a rare but highly lethal brain infection for which current treatments remain limited in efficacy and are often associated with toxicity. This study evaluated a library of fifteen synthetic compounds, chemicals classified as beta-hydroxylactones, selected based on their favorable physicochemical properties, low cytotoxic potential, and previously reported anti-protozoan activity against other genera. *In vitro* assays were conducted using trophozoites of two clinical strains of *N. fowleri* (KUL and Nf69) and murine macrophages (J774A.1) to assess anti-amoebic activity and host cell toxicity through a resazurin-based viability assay. Furthermore, fluorescence microscopy was employed to determine the mode of cell death triggered by selected compounds, assessing chromatin condensation, plasma membrane integrity, mitochondrial dysfunction, oxidative stress and autophagic vacuole formation. Two compounds, SET-61 and SET-64, emerged as particularly effective, displaying strong amoebicidal activity against trophozoites and cysts of the two strains. Moreover, distinct morphological and biochemical alterations were determined, supporting by different analyses the induction of programmed cell death through different pathways. These findings suggest that synthetic beta-hydroxylactones represent promising therapeutic candidates for the development of effective treatments for primary amoebic meningoencephalitis.

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Or31. Bio-guided isolation of compounds from *Argyranthemum frutescens* and *Salvia canariensis* and their efficacy against *Naegleria fowleri*

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Primary amoebic meningoencephalitis (PAM) is a disease caused by *Naegleria fowleri*, a free-living parasitic protozoan known as the brain-eating amoeba, which can be found in warm freshwater. Infection occurs through nasal inhalation of contaminated water, with symptoms appearing within days and often being lethal. Current treatments are based on the combination of amphotericin B with other drugs like miltefosine, though causing severe side effects due to their high toxicity, besides, there are no standardised protocols. Nowadays, plant-derived compounds are being studied as an alternative for the treatment of parasitic diseases such as PAM. Therefore, this study investigates the activity of compounds isolated through bio-guided fractionation from the roots of two Canary Islands-endemic plants: Salvia canariensis and *Argyranthemum frutescens* (both wild and cultivated forms). For the activity assays, fluorescence levels were measured using the alamarBlue reagent, being the most active compounds being those from cultivated *A. frutescensand*, which bio-guided fractionation led to the isolation of SU-29, the most selective one against trophozoites of *N. fowleri*, outperforming miltefosine. These results highlight the potential of *A.frutescens*-derived compounds as promising candidates for the development of novel treatments against *N. fowleri* infections.

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Or32. Enolase inhibitors as therapeutic agents for *Naegleria fowleri* infection

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Current treatments for *Naegleria fowleri* infections are inefficacious and mortality rates remain over 95%. We have previously demonstrated the importance of glycolysis to the amoebae (Milanes, 2018), suggesting inhibitors of glycolytic enzymes could be potent therapeutic agents. Supporting this postulate, we have found that human enolase 2 (ENO2) phosphonate inhibitors are potent inhibitors of recombinant *Nf*ENO, with the most potent, (1-hydroxy-2-oxopiperidin-3-yl) phosphonic acid (HEX) having an IC_{50} value of 0.14 ± 0.04 µM. Molecular docking studies with *Nf*ENO (PDB 7UGH) revealed the phosphonate agents bind to the *Nf*ENO active site with varying affinities (-8.6 to -6.2 kcal/mol), mirroring potency. HEX was also a potent amebicide, with an EC_{50} value of 0.21 ± 0.02 µM while the CC_{50} value was >300 µM (Milanes, 2024). Gradual exposure to the compound in culture over several months generated slow growing amoebae with EC_{50} values 10-fold higher than parental lines. RNA sequencing of the resistant line revealed upregulation of transcripts involved in metabolic processes, including glucose 6-phosphate isomerase and fructose-bisphosphate aldolase, genes upstream of enolase. To assess the potential of HEX as a monotherapy for amoebae infection, infected rodents were treated by intranasal HEX instillation. This treatment increased longevity, with eight of 12 HEX-treated rodents surviving the experimental period (resulting in an indeterminable median survival time). Only 1 of 12 vehicle-treated rodents remained at the end of the experimental period, yielding a median survival time of 10.9 days. Brain extraction of the surviving infected animals revealed that six of the eight survivors remained infected, indicating that HEX suppressed the infection but did not eliminate it (Milanes, 2024). To assess the potential of HEX as a partner therapeutic, we have assessed synergy in combination with amphotericin B and miltefosine, with results suggesting an additive effect of the agents. In summary, the phosphonate based ENO2 inhibitors are potent *Nf*ENO inhibitors, toxic to *Naegleria* in culture, and have promising activity in a rodent model of disease, suggesting these compounds could be further developed for use in treatment of infections either alone or in combination with other therapies.

Or33. The impact of drug pressure on life cycle progression and extrachromosomal DNA copy number in *Naegleria fowleri*

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Naegleria fowleri is an amphizoic pathogenic free-living amoeba that is the causative agent of primary amoebic meningoencephalitis (PAM), a lethal infection of the brain with a mortality rate of >97%. The high mortality is a result of ineffective therapeutic interventions and a limited understanding of pathobiology. *Naegleria fowleri* has three life cycle stages: trophozoites, the feeding and reproductive stage; cysts, the dormant and stress-resistant stage; and flagellates, the temporary and highly motile stage. Currently, it remains unresolved how drug pressure influences the amoeba to convert between these stages. While in the brain, the presence of drugs may trigger an encystment response, causing the amoeba to be dormant and less sensitive to treatment until drug pressure is removed. We aim to improve understanding of how drug pressure during infection may drive the amoeba to dormancy and impact their ability to excyst. The ability of these amoebas to encyst and excyst in the presence of sub-toxic levels of the potent anti-amebic enolase inhibitor, (1-hydroxy-2-oxopiperidin-3-yl) phosphonic acid (HEX), will be tested to shed light on the response of amoebas to drug pressure during infection. Further, we hypothesize that metabolic changes occur during encystation and excystation, which are partially influenced by regulating rRNA abundance. To assess this, we have measured changes in the copy number of the closed Circular Extrachromosomal Ribosomal DNA Element (CERE) in response to drug treatment and developmental changes. CERE is a unique DNA element present in approximately 4000 copies per cell that encodes the rDNA. Improved insight into cyst pathobiology will shed light on potential new therapeutic targets for enhanced intervention.

SESSION VII
TREATMENT I

Chair: Dr. Jacob Lorenzo-Morales, Prof. Alvie Loufouma Mbouaka

Or34. Therapeutic potential of withaferin A derivatives against *Acanthamoeba* spp.: in vitro assays

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Acanthamoeba are ubiquitous free-living protozoa that have been isolated from diverse environmental sources such as soil, air, and water. These amoebae are recognized as opportunistic pathogens capable of causing severe human diseases, including *Acanthamoeba* keratitis, granulomatous amoebic encephalitis, and cutaneous infections. Beyond their direct pathogenicity, *Acanthamoeba* can also serve as a reservoir or vehicle for other microorganisms, including bacteria and viruses, contributing to their environmental persistence and transmission. Despite the problematic pathology that can be caused by this parasite, there is currently no established treatment protocol, and those that are used are not fully effective and have side effects. Withaferin A has demonstrated several biological properties, including antiparasitic activity. In recent studies, its ability to inhibit the growth of protozoa has been reported, including some preliminary evidence of activity against *Acanthamoeba* spp. has been reported. For this reason, the activity of 18 withaferin derivatives against different strains of the *Acanthamoeba* genus was studied in the present work, including a study of the cell death induced by these derivatives in the parasites.

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Or35. Drug dose-response variability across *Acanthamoeba* strains in different media conditions

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Acanthamoeba spp. are ubiquitous free-living amoebae present in a diverse range of habitats and exist either as actively feeding trophozoites or dormant cysts. Pathogenic *Acanthamoeba* is responsible for causing severe and multifaceted infections in humans. *Acanthamoeba* may cause ulcerative keratitis, which is usually related to poor handling and maintenance of contact lenses; granulomatous amoebic encephalitis (GAE), cutaneous or disseminated infections. *Acanthamoeba* has been classified into 23 genotypes (T1-T23), with T4 being the most predominant genotype, and phylogenetically similar strains are found demonstrating different responses to the same test formulations. Numerous drugs demonstrating anti-*Acanthamoeba* effects are reported; however, their effectiveness in treating *Acanthamoeba* infections in clinical settings remains unviable, challenging, and difficult. Additionally, substantial differences are reported in the testing methodology, conditions, and non-consistent use of strains and media has been observed throughout our field. With this background, this study aimed to assess drug susceptibility under diverse conditions to develop more effective, tailored treatments that address drug variance and improve patient outcomes. The optimal seeding density of all the *Acanthamoeba* strains was optimized in each media condition, at 27°C for 3 days. Then the trophocidal activity of 19 recommended/suggested drugs was evaluated across nine *Acanthamoeba* strains in PG vs. Ac6 media, and currently for four strains in PG vs. Ac6 vs. M20 media, using the CellTiter-Glo v2.0 (CTG) assay. The compiled data from all three media conditions (PG, Ac6, and M20) provide critical insights into strain-specific drug susceptibility profiles, enhancing the clinical relevance of our findings and informing optimal drug dosage, microbial breakpoints, and suggesting personalized treatment strategies. To further advance research and clinical application, we established the first open-source Repository of Amoeba Drug Activity Records (RADAR)—a comprehensive database for tracking and analyzing drug efficacy against *Acanthamoeba* strains *in vitro*.

Or36. Amoebicidal effect of synthetic peptides against *Acanthamoeba castellanii*: a study of cell death using fluorescence imaging systems

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Antimicrobial cationic peptides have been recently cited as promising substitutes for antibiotics to address various infectious diseases and mainly against resistant bacteria. Within the cells, peptides have been reported to affect various cell compartments including nucleus, mitochondria, and plasma membrane. Although their action mode is still unclear, in general they induce Programmed Cell Death via the plasma membrane. In the present study, the amoebicidal activity of various peptides on *Acanthamoeba castellanii* Neff was conducted using the viability assay alamarBlue™ colorimetric assay. Later, the impact of the most active peptide on a cell's events was studied in a clinical case of *Acanthamoeba castellanii* using a fluorescence imaging system. We could demonstrate that the peptide OH_KR34 could affect various cell's clinical features inducing cytoskeleton damage and programmed cell death via the disruption of cell membrane permeability, mitochondrial dysfunction and DNA fragmentation. Our findings confirm once again that synthetic peptides might constitute a rich source to develop new amoebicidal drugs.

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Or37. Amoebicidal agent nitroxoline impairs functional encystment and induces transcriptional collapse in *Balamuthia mandrillaris* via copper and iron chelation

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Central nervous system infection with the free-living amoeba, *Balamuthia mandrillaris*, results in granulomatous amebic encephalitis (GAE) with mortality rates up to 90%. Until recently, treatment relied on a six-drug cocktail with limited efficacy and high toxicity. Our lab previously identified the antibiotic nitroxoline as a potent inhibitor of *Balamuthia* trophozoites and inducer of encystment. Following case reports of patient survival with nitroxoline treatment in the United States, nitroxoline is now available for GAE treatment through the Centers for Disease Control. However, the mechanism of action of nitroxoline remained unclear. To address this, we used a combination of genomics, transcriptomics, chemical complementation, electron microscopy, and recrudescence assays. We generated a high-quality annotated draft genome of *B. mandrillaris* strain CDC:V039 using PacBio HiFi, Illumina, and Hi-C reads, significantly surpassing existing reference genomes with telomere-to-telomere coverage for most chromosomes. This resource enabled us to characterize the molecular response to stress-induced encystment triggered by nitroxoline, galactose, and hypoxia. Our data reveal that encystment is a dynamic, stress-specific process rather a universal encystment program. Nitroxoline induced cysts uniquely exhibited transcriptional collapse and structural damage, phenotypes that were reversible with copper and iron supplementation. Nitroxoline treatment also inhibited excystment of galactose-induced cysts, suggesting that it may disrupt the viability of both trophozoites and cysts, which are likely to coexist in patient tissue. Together, these findings deepen our molecular understanding of *Balamuthia* encystment, offer clues for further therapeutic intervention, and support the continued use of nitroxoline in combating this deadly infection.

Or38. Amebicidal effects of adamantane-azole gold(I) complexes: a study of cell death pathway in *Acanthamoeba castellanii* and toxicity profile

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Acanthamoeba spp. are widely distributed free-living protozoa and anfizoic organisms capable of causing severe infections, including amoebic keratitis and granulomatous amoebic encephalitis. The lack of selective and effective treatments highlights the urgent need for new bioactive molecules targeting both trophozoite and cyst forms. This study investigated the antiAcanthamoeba activity of adamantane-azole gold(I) complexes (C1-C4), which inhibit selenoenzymes like thioredoxin reductase (TrxR) involved in *Acanthamoeba* redox balance. Axenic cultures of *Acanthamoeba castellanii* (ATCC 50492) were tested to determine their viability and explore mechanisms of cell death. An ultrastructure study of amoeba was carried out. The potential synergy between these complexes and chlorhexidine was evaluated, and a toxicity profile against in vitro and in vivo models was accomplished. Molecular docking was employed to predict the inhibitory activity of complexes against TrxR. C2 and C4 showed greater amoebicidal activity, with inhibitory concentration of 50% (IC_{50}) values of 0.12 and 6.23 µM, respectively. C2, C3 and C4 caused changes in the cell cycle and induced phosphatidylserine exposure, while C4 also induced mitochondrial depolarization. None of the complexes induced toxic effects in the in vivo or in vitro models evaluated. Overall, the gold(I) complexes C2, C3, and C4 demonstrated potent antiamoebic activity, highlighting their potential as promising therapeutic candidates for Acanthamoeba-related infections, with minimal toxicity and promising mechanisms of action that warrant further investigation.

SESSION VIII
TREATMENT II

Chair: Dr. Fiona Henriquez, Dr. Álvaro de Obeso Fernández del Valle

Or39. Hylo® intense eye drops as a potential amoebicidal agent against *Acanthamoeba* spp.: induction of programmed cell death and anti-adherent effect

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The treatment of *Acanthamoeba* keratitis remains a major clinical challenge due to the limited efficacy, high toxicity, and poor tolerability of many currently available ophthalmic solutions. Most eye drops used in clinical practice are not specifically designed to target *Acanthamoeba* and often require prolonged administration, leading to ocular side effects. This study aimed to evaluate the amoebicidal activity and anti-adherent properties of HYLO® INTENSE against *Acanthamoeba castellanii* Neff, *A. griffini*, *A. polyphaga*, and a clinical strain of *A. castellanii*. Amoebicidal activity was assessed using the alamarBlue™ colorimetric assay to determine IC_{50} values, while mechanisms of action were explored through the use of specific cellular markers. Additionally, the anti-adherent effect of HYLO® INTENSE was tested on protein-coated surfaces using Laminin and Corning™ Matriz Matrigel™ matrices to assess the ability of *A. polyphaga* trophozoites to adhere in the presence of the eye drops. The results demonstrated that HYLO® INTENSE significantly inhibits *Acanthamoeba* growth in all tested strains and induces programmed cell death in *A. polyphaga* trophozoites through mitochondrial dysfunction, chromatin condensation, oxidative stress, membrane permeabilization, cytoskeletal disorganization, and triggered autophagy process. Moreover, the formulation also exhibited a pronounced anti-adherent effect, notably reducing the adhesion of *A. polyphaga* trophozoites to protein-coated surfaces. These findings suggest that HYLO® INTENSE eye drops could represent a promising low-toxicity therapeutic candidate for the management of *Acanthamoeba* keratitis, offering both amoebicidal and anti-adherent effects in a formulation suitable for ophthalmic use.

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Or40. Small molecules secreted by *Pseudomonas aeruginosa* kill *Acanthamoeba castellanii*

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Acanthamoeba castellanii is a pathogenic free-living amoeba (FLA) that causes fatal central nervous system infections in immunocompromised hosts, as well as sight-threatening amebic keratitis (AK) in healthy contact lens wearers. *Pseudomonas aeruginosa* are ubiquitous environmental Gramnegative bacteria that co-exist with *A. castellanii* and are prey for these bacteriovorous FLAs. Given the strong evolutionary pressure placed on *P. aeruginosa* by *A. castellanii,* we hypothesized that *P. aeruginosa* secretes amoebicidal compounds to defend itself against amoebal grazing. Using several orthogonal assays to measure trophozoite and cyst viability, we demonstrated that the cell-free supernatants of several *P. aeruginosa* strains are lethal to *A. castellanii* (Neff) and that <3 kDa small molecules are specifically responsible for trophocidal activity. To identify genes required for amoebicidal activity, we individually screened supernatants prepared from an arrayed PA14 transposon library and identified a hit in FabY, a non-canonical ketosynthase which initiates fatty acid synthesis in *P. aeruginosa.* FabY activity influences the production of rhamnolipids, quinolones, and phenazines; these secondary metabolites have been broadly implicated in inter-kingdom signaling and toxicity. Mutants disrupting enzymes in rhamnolipid, quinolone, and phenazine biosynthetic pathways were constructed and tested (singly and in combination) to identify FabY-dependent amoebicidal molecules. Although PA14 ΔfabY supernatant is severely attenuated for trophozoite killing, PA14 ΔphzA1-G1ΔphzA2-G2ΔrhlRΔrhlIΔpqsA supernatant retains trophocidal activity. Efforts are currently underway to identify metabolites differentially present in active vs. inactive supernatants, using MS, while a bioassay-guided fractionation pipeline is being used to identify active molecules in the PA14 ΔphzA1-G1ΔphzA2-G2ΔrhlRΔrhlIΔpqsA supernatant. Through these complementary approaches, we hope to identify and understand how compounds secreted by *P. aeruginosa* are lethal to *A. castellanii.* Knowledge of such natural compounds and their mechanism of action both illuminates ecological predator-prey interactions and may lead to the discovery of natural products with important clinical indications.

Or41. History and progress towards standardization of *Acanthamoeba* methods for contact lens care products

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Acanthamoeba trophozoites and cysts are known to cause serious corneal infections, highly associated with contact lens wear. Development and optimization of testing methods for contact lens disinfecting solutions are needed to protect contact lens users from *Acanthamoeba* keratitis. In 2015 ISO 19045 was published to assess a contact lens solutions ability to cause encystment of *Acanthamoeba* trophozoites. Since publication, multiple concerns have been raised with methodology performance in new contact lens care products. ISO 19045-2 was published in 2024 and outlines a method to assess disinfection of *Acanthamoeba* trophozoites by contact lens care products. Active work is ongoing to optimize a standard method to assess disinfection of *Acanthamoeba* cysts by contact lens care products in ISO 19045-3. Optimization of methods such as *Acanthamoeba* cyst culture and organism recovery methods and food source after disinfection are ongoing. Historical perspectives on *Acanthamoeba* standard development will be presented.

Or42. Efficacy of octenidine-containing mouthwashes on *Entamoeba* spp. and other protists in the oral cavity

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Entamoeba gingivalis is an amoeba inhabiting the oral cavity of humans. Transmission is supposed to happen via direct contact and droplets praying and no cyst stage has been identified yet. The clinical relevance of *E. gingivalis* is still under investigation, however, a significantly higher detection rate in periodontitis sites strongly points towards a pathogenic potential. In a future project we intend to elucidate this aspect in more detail as first data clearly indicate a correlation between *E. gingivalis* and clinical symptoms and shifts in the oral microbiome. Indeed, proper oral hygiene measures generally represent a crucial parameter for the prevention and control of common dental problems. In that regard, antiseptic mouthrinses are widely used to support (daily) oral biofilm management, especially in otherwise inaccessible areas of the oral cavity or when mechanical plaque control by the use of a toothbrush is temporally or even permanently impaired. Therefore, the aim of this study is to evaluate the effect of commercially available octenidine-containing mouthwashes (OCMWs) octenimed® and octenident® against *Entamoeba spp.* Both antiseptic OCMWs have been proven in various clinical trials to effectively inhibit plaque formation, gingivitis and oral microbial growth (bacteria and fungi) and octenidine itself has been recently shown to be highly active against amoebae of the genus *Acanthamoeba,* too. In detail, we already investigated the effect of both OCMWs on *Entamoeba* spp. *in vitro* resulting in a total elimination of entamoebae after 30 seconds, exhibiting a significantly stronger effect than the control (3% H_2O_2). Furthermore, preliminary tests showed the potential of octenident® to eliminate *E. gingivalis* DNA in the oral cavity of carriers as determined by comparative quantitative real-time PCR. Additionally, the *in vitro* effect of OCMWs on *Trichomonas tenax* and *Acanthamoeba* spp. will be evaluated in the course of this study. Altogether, those data indicate that OCMWs demonstrate a strong effect on *Entamoeba* spp. *in vitro,* indicating promising clinical results in the oral cavity. The regular use of OCMWs might also be a potent strategy for eliminating further protists in the oral cavity

SESSION IX
DIVERSITY

Chair: Dr. Julia Walochnik, M.Sc. Miguel Ángel Ramírez-Flores

Or43. Diversity and systematics of sibling species within the genus *Thecamoeba* (Amoebozoa: Discosea)

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Members of the genus *Thecamoeba* Fromentel, 1874 are widely distributed in the environment and can be isolated from marine and freshwater habitats, soil, and leaf litter. These amoebae have notable morphological features, such as dorsal folds and ridges, as well as diverse of nucleoli organisation. Until recently, the genus *Thecamoeba* was considered one of the few taxa of amoebae whose species could be relatively easily identified by light microscopy. However, recent molecular studies have demonstrated significant genetic diversity within the "classical" morphological species of *Thecamoeba*. Known sibling species of *Thecamoeba* have minor morphological differences. Some of them can be differentiated by statistical analysis of morphometric data obtained in cloned culture, while others have no reliable distinguishing features. Also, in some cases, genetically identical cultures can have significant differences in the ranges of morphometric characteristics. This makes species identification impossible without molecular data.

We have conducted a morphological study and sequenced the SSU and COI genes of more than 150 *Thecamoeba* strains collected worldwide, mainly in various cities of Eurasia. Based on these results, we propose to distinguish four species groups: *Thecamoeba quadrilineata, T. striata, T. similis*, and *T. aesculea*, each containing a number of sibling species that are virtually indistinguishable morphologically. Diversity in some species groups is high; for example, up to twenty gene variants were detected in the species groups of *Thecamoeba similis*. Nearly identical sequence variants were repeatedly obtained for isolates from geographically distant locations, indicating a high stability of this molecular marker. By analysing the COI gene (amino acid and nucleotide sequences) and the variable regions of the 18S rRNA gene, we identified molecular signatures that can be used to identify members of clades at different taxonomic levels and visualized them as heatmaps. This analytical approach may provide a better understanding of the boundaries between “species” of asexual organisms and may form the basis for a convenient systematization of sibling species. Supported by RSF project 24-44-00096 and National Natural Science Foundation of China 32361133557.

Or44. Clinical presentation, genotypic diversity, and intracellular bacteria in *Acanthamoeba* keratitis patients treated at a quaternary referral eye hospital in Sydney, Australia

Nicole Carnt[1*], Mark Willcox[1], Fiona L. Henriquez[2], Gauri Sr Shrestha[3,1], Uday Narayan Yadav[4], Yalewayker Asrat[1], Constantinos Petsoglou[5,6] and Binod Rayamajhee[1]

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Acanthamoeba keratitis (AK) is an emerging corneal infection that can lead to sight loss. This study aimed to evaluate the incidence of AK, assess circulating *Acanthamoeba* genotypes, investigate intracellular microbes, and identify potential infection sources at a quaternary eye referral hospital in Sydney, Australia. A prospective case series study was conducted at the Sydney Eye Hospital, Australia, from June 2021 and October 2022. AK patients underwent corneal debridement for culture, and their domestic tap water was analysed to trace infection sources. Microscopy, cyst morphology, PCR, and Sanger sequencing of the amoebal 18S rRNA were performed to confirm the *Acanthamoeba* isolates. Sanger sequencing and high-resolution microscopy were used to identify intracellular bacteria within amoebal cells. A total of 21 AK patients were recruited in this study (40.7±12.3 years of age, 52% female). Six (28.6%) corneal and four (44.4%) water samples tested positive for *Acanthamoeba* genotype T4. One corneal and one water isolate harboured intracellular bacteria; the corneal isolate contained *Staphylococcus epidermidis* and the water isolate contained *Pseudomonas aeruginosa.* Eight patients were confirmed contact lens wearers and two reported trauma related risk factors. Eye pain was the primary symptom (66.7%), followed by red eye. The median symptoms duration was 24.5 days (IQR: 13–95 days). Polyhexamethylene biguanide (PHMB) was the main therapy, followed by chlorhexidine, with 16 patients also receiving antibiotics. The median treatment duration was 12 weeks (IQR: 8–26 weeks). One patient required corneal grafting, and another had a recurrent ulcer 78 weeks after the initial infection. In Sydney, the prevalent genotype of *Acanthamoeba* among AK patients and tap water was T4, confirming previous studies. However, this study suggests a higher annual incidence of AK than previously reported in retrospective studies (17 vs 4 new cases per year). Prospective studies of longer duration are required to confirm if there is an increasing incidence of AK in Sydney and associated factors such as climate.

Or45. Diversity of free-living amoebae in soil samples from Northwestern Spain revealed by culture and molecular techniques

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Free-living amoebae (FLA), including *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, *Sappinia* spp., *Vahlkampfia* spp., and *Vermamoeba vermiformis*, are widely distributed in natural environments and are known to cause rare but potentially fatal or debilitating infections in humans. Despite increasing interest in their ecological and epidemiological relevance, data on their presence in Spanish soils remain limited. This study presents the first comprehensive, year-long assessment of FLA diversity in soils from four provinces in the autonomous community of Castilla y León, Spain. A total of 87 soil samples were collected during three different seasons from various environments. Samples were processed using culture techniques on non-nutrient agar and analysed by conventional PCR followed by Sanger sequencing, and quantitative real-time PCR (qPCR). All samples showed amoebic growth in culture, and 93% were confirmed positive by molecular methods. *Acanthamoeba* spp. (mainly T4 genotype) was the most frequently identified genus, followed by *V. vermiformis.* Additionally, other genera and species of FLA were identified, indicating a high level of microbial diversity in the soils of Castilla y León. This study provides novel data on FLA diversity in Spanish soils and supports future research on their ecology and public health relevance.

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Or46. Free-living amoebae as reservoirs of other microorganisms

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In addition to their role as parasites, free-living amoebae (FLA) can act as hosts of and vehicles for phylogentically diverse microorganisms while some of them replicate intracellularly. These microorganisms are adapted to the intracellular conditions in the amoeba, find suitable conditions and protection from negative environmental influences and take advantage of the dispersal in the environment by their amoebic host. It is expedient to call these organisms "endocytobionts", at least during the initial steps of any studies. By doing so, it is not necessary to go into potential characteristics of these relationships such as parasitism, phoresy, zoochory, or mutualism at an early stage of study. Among those organisms resisting the lysis within their amoebic host, there are obligate and facultative pathogenic microorganisms affecting the health of humans or animals. FLA-endocytobiont relationships are not only important for the tenacity of the involved microorganisms. Especially if FLA are present in biofilms and there are close ties with many other microorganisms, the odds are for some of these microorganisms to develop human pathogenic properties. Here, the amoebic passage seems to be a prerequisite for the development of virulence factors and it may have an impact on evolutionary processes.

Or47. A long journey until the secrets were revealed: parasitic keratitis amoebae as vectors of the scarcely described "Pandoraviruses" to humans

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Here, the results of a long effort to derive valuable phylogenetic data about an extraordinary spore-like infectious particle (endocytobiont) within host amoebae (*Acanthamoeba* sp.) recently isolated from the contact lens and the inflamed eye of a patient with keratitis are presented. The development of these endocytobionts has already been demonstrated with electron microscopic photo sequences, leading to a relevant model of its development presented here. The molecular biological investigation following the discovery of two other *Pandoravirus* species within aquatic sediments in 2013 led to the taxonomic affiliation of our endocytobiont with the genus *Pandoravirus*. A range of endocytobionts (intracellular biofilms) have been found in recent years, among which are several viruses that obligatorily proliferate within freeliving amoebae. In human medicine, foreign objects which are placed in or on humans cause problems with microorganisms in biofilms. Contact lenses are especially important, because they are known as a source of a rapid formation of biofilm. These were the first Pandoraviruses described, and because this is additionally the first documented association with humans, we have clearly demonstrated how easily such (viral) endocytobionts can be transferred to humans. This case counts as an example of parasites acting as vectors of phylogenetically different microorganisms especially when living sympatric within their biocoenosis of biofilms. Finally we succeded in revealing the phylogenetic nature of these "extraordinary endocytobionts" within Acanthamoebae: Pandoraviruses!

Or48. Role of free-living amoebae in the environmental persistence of pathogenic mycobacteria: a mesocosm-based approach

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Mycobacterium avium subsp. *paratuberculosis* (Map), the bacterium that causes paratuberculosis, is known to persist in a variety of environmental conditions, which contributes to the continued transmission of the disease despite control efforts. Recent studies suggest that freeliving amoebae (FLA) may act as environmental reservoirs and potential vectors for Map, yet their role in natural environments remains poorly understood. This study aims to better understand the contribution of FLAs to Map persistence by using controlled mesocosm systems. Water from cow troughs was spiked with the environmental Map strain PICSAR at 106 CFU/ml, either alone or in combination with one FLA isolate at 105 amoeba/ml, either *Acanthamoeba* sp. or *Rosculus vilicus.* Over three months, bacterial persistence is longitudinally monitored using quantitative PCR to assess the impact of amoebae on the long-term survival of Map in these mesocosms. Preliminary data covering 0 to 15 days post-inoculation reveal slight instability in the mycobacterial signal in mesocosms inoculated with *Acanthamoeba sp.,* whereas water spiked with *Rosculus vilicus* show a more sustained Map signal. This is the first mesocosm-based experimental approach to investigate FLA–mycobacteria interactions in a realistic environmental context. The results are expected to provide new insights into the ecological role of amoebae in maintaining and transmitting pathogenic mycobacteria within agroecosystems. Ultimately, these findings could guide the development of targeted biosecurity strategies to mitigate environmental reservoirs and manage or reduce infection risks.

Or49. The microbial Trojan horse: *Acanthamoeba* as a selective reservoir for multidrug resistant bacteria in polluted estuarine sediments

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Environmental drivers of antimicrobial resistance (AMR) are increasingly recognised, yet the role of microbial predators such as Acanthamoeba in shaping resistance remains underexplored. This study investigates the intracellular microbiome of *Acanthamoeba* isolated from the heavily polluted Vashi Creek estuary in Mumbai and its association with multidrug resistant bacteria. All *Acanthamoeba* isolates belonged to the resilient T4 genotype. Bacteria recovered from *Acanthamoeba* exhibited significantly higher resistance to multiple antibiotic classes including aminoglycosides, macrolides, fluoroquinolones, and beta lactams when compared to sediment associated bacteria, with MAR index values averaging 0.45 versus 0.08. Crucially, AAB resistance correlated with specific potentially toxic elements such as arsenic, vanadium, and calcium. These elements are implicated in phagocyte survival and suggest selective intracellular pressures that promote resistance. This is the first study to directly link exposure to potentially toxic elements with the intracellular resistome of *Acanthamoeba* associated bacteria. The findings highlight *Acanthamoeba* as both a reservoir and an incubator of antimicrobial resistance, reinforcing the need for One Health informed environmental monitoring, particularly in low- and middle-income countries where surveillance is limited but antimicrobial resistance burden is high.

POSTER PRESENTATIONS

P1. Identification of amoebic strains in contact lenses and clinical cases of keratitis in Mexico City

David Segura Cobos[1,*], Virginia Vanzzini Zago[2], Rubén L. Rodríguez Expósito[3], Marco Luis Gómez Piña[1], Ismael Castelan Ramirez[1], Dolores Hernández Martínez[1], Jacob Lorenzo Morales[3] and Maritza Omaña Molina[1].

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Acanthamoeba keratitis (AK) is a challenging-to-treat ocular infection caused by *Acanthamoeba* spp., which is usually unilateral, with contact lens use being a major risk factor. The clinical presentation includes severe ocular pain, photophobia, blurred vision, and, in severe cases, vision loss. Diagnosis can be difficult, as it is often mistaken for herpetic keratitis. Treatment involves antifungal and antibiotic medications, and in some cases, additional interventions such as corneal debridement or corneal transplantation. In the present study, amoebic strains were identified from a contact lens, unilateral keratitis cases (including one following insertion of an intrastromal ring [IR]), and a case of bilateral AK in a contact lens wearer, all treated at the "Hospital para Evitar la Ceguera en México; Luis Sánchez Bulnes" in Mexico City, Mexico. Samples were received in monoxenic culture, axenized in 2% bactocasitone medium supplemented with fetal bovine serum. Growth curves were performed in axenic cultures at different temperatures (21, 30, and 36 °C) to determine optimal growth temperature. Strains were identified morphologically and by molecular biology. Most isolates corresponded to *Acanthamoeba castellanii*, genotype T4, with an average size of 19–22 μm and optimal growth between 21 and 30 °C. The amoeba isolated from the contact lens belonged to the family Vahlkampfiidae, which could not be axenized and grew at room temperature. The IR strain also could not be axenized, showing limited trophozoite growth and early cyst formation at room temperature. Amoebae from the bilateral case were identified as *Acanthamoeba castellanii*, growing at both 21 and 30 °C, in which more than one nucleus was observed along with evidence of cannibalistic behavior. These findings indicate the prevalence of the amphizoic amoeba *Acanthamoeba castellanii* in clinical keratitis cases and its presence as a contaminant in contact lenses, representing a risk factor for keratitis. This report documents the first case of bilateral AK in Mexico City.

P2. Characterization of the first two clinical isolates of *Naegleria fowleri* in Costa Rica

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During the first trimester of 2020, Costa Rica reported the first three clinical cases of primary amoebic meningoencephalitis (PAM); from these cases, two of them resulted fatal, but also an early diagnosis allowed the survival of one patient. Moreover, the Free-Living Amoeba Research Laboratory (AViLab) achieved the axenic culture of *Naegleria fowleri* from cerebrospinal fluid samples in two of the three cases, a fact that has allowed the study and characterization of the first two autochthonous isolates of *N. fowleri* in the country. In this study, we characterize and compare these two clinical isolates of *N. fowleri,* as well as their excretion/secretion products (including extracellular vesicles, EVs), using several approaches. Analyses of protein profiles, protease activity by zymography, proteomics, cytopathic effect and drug susceptibility assays were included, among others. The potential role of EVs as biomarkers and their immunomodulatory role during an infection has also been evaluated *in vitro*. The results obtained show a clear similarity between both isolates, with slight differences in proteases profiles and cytopathic effect. In addition, both isolates resulted sensitive to miltefosine and amphotericin at concentrations similar to those reported in literature. Regarding EVs, the presence of immunogenic components as part of the protein cargo and an immunostimulatory effect on microglia cells has been demonstrated. Moreover, the presence of bioactive DNA in these vesicles and their successful amplification through polymerase chain reaction has been confirmed, making them a promising target for the development of new diagnostic tools.
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P3. Adaptation of free-living amoebae to textile wastewater

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Free-living amoebae are nude amoebae that are characterized by the absence of a protective structure in its vegetative form; they are cosmopolitan and have a wide distribution in nature, they are mainly in the soil and aquatic environments. They have been found in different kinds of water, even in wastewater; however, wastewater from the textile industry contains a variety of chemical substances such as dyes, which may inhibit the presence of free-living amoebae. The objective of the study was to determine the adaptation of free-living amoebae to textile wastewater (TW). To reach the objective were made mixtures of domestic wastewater (DW) with textile wastewater (TW) in different proportions. The mixtures of wastewater were from 100% DW and 0% of TW up to 30% of DW and 70% TW. The samples of wastewater mixtures were inoculated in non-nutritive agar with the bacterium *Enterobacter aerogenes* (NNE), the cultures were incubated at 30 °C and cultures were observed with an inverted microscope. The isolated amoebae were identified morphologically. Ten genera of free-living amoebae were identified: *Acanthamoeba, Mayorella, Polychaos, Rosculus, Saccamoeba, Thecamoeba, Valhkampfia, Vannella, Vermamoeba* and *Vexillifera.* In 100% DW, and in the mixture of 10% DW and 90% TW were found 8 genera, but the number of genera decreased drastically to 2 when the proportion of TW increases to 20%; however, when the proportion of TW went of 40 to 70%, the number of genera increased slightly and remained between 4 and 5. The most frequent genera and therefore the best adapted to textile wastewater were *Vannella, Acanthamoeba, Polychaos* and *Rosculus.* The gradual increase in textile wastewater allowed some free-living amoebae to adapt to this type of water.

P4. Comparative genomics and proteomics reveal strain-specific features in *Naegleria fowleri* clinical isolates from Costa Rica

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Naegleria fowleri, the causative agent of primary amoebic meningoencephalitis (PAM), is a free-living amoeba responsible for a rare but almost always fatal infection. In Costa Rica, two clinical isolates, NF_Limon and NF_Guanacaste, were obtained from fatal PAM cases and display differential virulence in murine models. To investigate the basis of this variation, we conducted whole-genome sequencing, de novo assembly, and comparative genomic and proteomic analyses.

Both isolates possess ~28 Mbp diploid genomes encoding approximately 9,000 predicted proteins, with a largely conserved core genome containing known virulence factors such as *nfa1*, and genes encoding for Naegleriapore A/B, various proteases, kinases, and cytoskeletal components. Proteomic profiling revealed a shared core proteome involved in metabolism and cytoskeletal maintenance, along with strain-specific proteins. Notably, NF_Limon exhibited unique proteins associated with translation, motility, including myosin II, and hemerythrin-like proteins homologous to Nfa1. However, the limited number of strain-specific genes suggests that differences in virulence are likely driven by differential gene expression and/or host interaction dynamics rather than by gene content alone. This study provides the first genomic and proteomic characterization of *N. fowleri* isolates from Costa Rica and offers novel insights into strain-dependent pathogenicity in this lethal amoeba.

P5. Characterization of the natural bacterial microbiota in *Acanthamoeba* spp. and *Naegleria fowleri* isolated from rivers and tap water in Guadeloupe

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Acanthamoeba spp. and *Naegleria fowleri* are free-living amoebae (FLA) known to cause severe infections in humans, including *Acanthamoeba* keratitis and life-threatening forms of meningoencephalitis. While these FLA can harbor amoeba-resistant bacteria (ARB) under laboratory conditions, the composition and characteristics of their natural bacterial microbiota remain largely unexplored. Our work aimed at characterizing the natural bacterial microbiota of *Naegleria fowleri*, *Acanthamoeba castellanii*, *Acanthamoeba lenticulata* and *Acanthamoeba* sp (T17 genotype) isolated from rivers and tap water in Guadeloupe. The whole bacterial microbiota of the water source and the FLA grown with *E. coli* and under axenic culture conditions, during successive passages, were characterized using 16S rRNA gene metabarcoding. The culturable subset of ARB was analyzed by mass spectrometry (MALDI-TOF MS), conventional 16S PCR, and disk diffusion method. Transmission electron microscopy was used to locate the ARB inside the amoebae. The metabarcoding analyses revealed that while *Enterobacter*, *Klebsiella* and *Salmonella* genera were detected in water, the most common ARB belonged to *Bosea, Escherichia/Shigella*, *Microbacterium* and *Pseudomonas* genera. The amoebal hosts showed temporary and permanent associations with different bacterial genera (including the genera *Legionella* and *Bordetella* in Ac. T17 and *A. lenticulata*, respectively), depending on the number of passages and culture conditions. The *Pseudomonas* species isolated from distinct FLA exhibited resistance to key antibiotics. The ARB were detected in the cytoplasm of the trophozoites. The presence of pathogenic FLA and ARB in treated and non-treated waters in drinking water distribution systems in Guadeloupe poses a potential health risk. Our results emphasize the need to regularly control the FLA and their associated ARB to ensure water safety and improve knowledge on amoebae-bacteria interactions to establish better water management protocols. The natural presence of ARB in pathogenic FLA also raises key questions regarding the host immune response during amoeba infection.

P6. Physicochemical and geoenvironmental characterization of freshwater bodies with capacity to host and promote the presence of potentially pathogenic free-living amoebae

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Within the group of free-living amoebae (FLA), the genus *Naegleria* stands out for including species adapted to high temperatures, such as the facultative amoeba N. fowleri, the causative agent of primary amoebic meningoencephalitis (PAM). *Naegleria* spp. can be found in various terrestrial and aquatic environments, easily coming into contact with humans. Characterizing and assessing water bodies is essential to determine the presence of pathogenic microorganisms and the risk of infection. This study proposed an environmental survey to identify physicochemical and geospatial parameters related to the presence of *N. fowleri* in natural freshwater sources used for tourism and recreation in Costa Rica during 2023. Water and sediment samples were collected from 24 locations, and parameters such as temperature, pH, electrical conductivity, and dissolved oxygen in water were measured. Additionally, water samples were cultured, isolated, and subjected to molecular tests, revealing that seven sites (29,2 %) tested positive for *Naegleria*, but all were negative for *N. fowleri*. In sediment samples, metal, cation and texture analyses were performed. Although dissolved oxygen, water conductivity, copper concentration and clay percentage in sediment were statistically associated ($p < 0,05$) to the presence of *Naegleria* spp., epidemiological association was not demonstrated. The sites predominantly featured forest cover and were located in subclimatic regions with significant rainfall patterns. This study represents an initial monitoring effort to determine the frequency of potentially pathogenic AVL in surface waters of Costa Rica.

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P7. Amplification, cloning and analysis of the gene encoding HSP70 of *Naegleria fowleri*

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Free-living amoebae (FLA) are amphizoic protists that are dispersed in the environment: water, soil, and air. Certain genera of ALVs, in addition to living in the environment, produce various types of diseases in humans. Such is the case of *Naegleria fowleri*, the causative agent of primary amebic meningoencephalitis (PAM), an acute disease of the central nervous system characterized by a fulminant, necrotizing, and hemorrhagic condition. The mortality rate of this pathology is over 95%, making it a public health problem. It has been demonstrated that the hsp70 protein of *N. fowleri* may be a factor involved in the pathogenicity of the amoeba, since it plays an important role in proliferation and cytotoxicity *in vitro*. Also, its involvement in the protection against PAM in a murine model of infection has been suggested. Therefore, the objective of this project was to amplify, clone, analyze and purify the hsp70 protein from *N. fowleri* strain ATCC30808. The gene of interest was identified, restriction enzyme analysis was performed, and two non-cutting enzymes were selected, being XhoI and HindIII the selected enzymes. Subsequently, specific oligonucleotides were designed with the recognition sites for each restriction enzyme at the 5' and 3' ends, respectively. The approximately 2,000 bp product was amplified and introduced into the pGEM-T easy cloning vector, and DH5α chemically competent bacteria were transformed. Subsequently, the insert of interest was introduced into the expression vector pColdI and BL21 DE3 competent bacteria were transformed, thus obtaining positive bacterial colonies. Once the correct reading frame was confirmed, induction of expression with IPTG and protein purification were carried out. The techniques performed allowed obtaining the recombinant Hsp70 protein from *N. fowleri* strain ATCC30808. Although the project is at an advanced stage, it is expected that the proposed strategy will allow, in future stages of the project, the intranasal immunization of BALB/c mice with the recombinant Hsp70 protein + choleric toxin following an

immunization schedule comprising 4 immunizations with 7-day intervals between each one. Suggesting that such immunization will confer significant protection against PAM.

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P8. Bilateral *Acanthamoeba* keratitis caused by two different amoebae of the genus *Acanthamoeba*

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Acanthamoeba spp. are etiological agents of granulomatous amoebic encephalitis (GAE), cutaneous infections, and *Acanthamoeba* keratitis, a sight-threatening, typically unilateral corneal infection. This study describes the identification and characterization of amoebae isolated from a clinical case of bilateral *Acanthamoeba* keratitis treated at the "Hospital para Evitar la Ceguera en México; Luis Sánchez Bulnes" in Mexico City.

Corneal scrapings from both eyes were cultured in NNE medium with heat-inactivated *E. coli*. The left-eye (LE) strain exhibited faster proliferation. In axenic media, both strains grew in PYG and bactocasitone with fetal bovine serum at room temperature, 30 °C, and 37 °C, but not in Chang medium. Growth curves revealed differential behavior: the LE strain reached growth rates of 2.8 %/h (room temperature), 2.9 %/h (30 °C), and 1.3 %/h (37 °C), whereas the right-eye (RE) strain showed 2.6 %/h, 1.9 %/h, and 1.4 %/h, respectively.

Morphologically, LE trophozoites measured on average 27.4 × 19.4 μm, with a prominent hyaline pseudopodium, and cysts with six arms (14 μm), consistent with *Acanthamoeba castellanii* var. gigantea. RE trophozoites measured 30 × 17.3 μm, with less-defined pseudopodia, and cysts with five arms (14 μm), preliminarily consistent with *Acanthamoeba palestinensis.*

Molecularly, both strains belonged to genotype T4; LE was identified as *A. castellanii* and RE as *Acanthamoeba* sp. Studies conducted at the Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias confirmed mesophilic physiology and the presence of multidrug-resistant endosymbiotic bacteria.

Pathogenicity was assessed using a murine GAE model with BALB/c mice (4 animals per group) intranasally inoculated with 1×10^6 viable trophozoites. No mice died; however, two in the RE group exhibited transient hyperactivity around day 10. At day 21, mice were euthanized, and amoebae were recovered from the brain, liver, kidney, and lungs in 3/4 RE animals; in the LE group, they were recovered from the kidney and brain in at least 3 animals.

These findings suggest the presence of two distinct strains, possibly different species, involved in this bilateral keratitis case.

P9. Molecular identification of free-living amoeba isolated from medical devices and hospital environments: evidence from hospitals in Northwestern Iran

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Free-living amoebae (FLAs) are ubiquitous protozoa in soil, air, and artificial systems, including hospital environments. Some genera of free-living amoebae, such as *Acanthamoeba*, can cause serious health complications such as *Acanthamoeba* keratitis and granulomatous amoebic encephalitis. This study investigated the presence of FLAs in hospital environments and ready-to-use medical devices, including beds and gowns, which were examined for the first time. In this crosssectional study, 45 environmental and medical device samples were collected from two hospitals in Northwestern Iran. After filtration, the samples were cultured in a 1.5% non-nutrient agar medium enriched with *Escherichia coli*. The growth of the FLAs and their genus was determined through microscopic analysis. Positive samples were submitted for PCR analysis targeting the 18S rRNA gene, followed by sequencing and phylogenetic analysis. Also, the pathogenicity of *Acanthamoeba* isolates was evaluated through osmo- and thermotolerance tests. FLAs were detected in 22.22% (10/45) of samples using microscopy. The isolated microorganisms were identified as belonging to the genera *Vermamoeba* (9), *Acanthamoeba* (7), and *Vahlkampfia* (7). Among the positive samples, 5 were obtained from environmental sources, 4 from equipment, and 1 from surgical gowns. Most *Acanthamoeba* isolates demonstrated resistance to osmolarity and heat shock. Sequence analysis identified *Acanthamoeba* T4 genotype (5), *Vahlkampfia* sp. (3), and *V. vermiformis* (6). In this study, FLAs were isolated from patients' beds and surgical gowns for the first time, emphasizing new infection risks within an ophthalmology hospital, which highlights the need for improved disinfection protocols for sterile equipment.

P10. Deciphering the molecular insights into amoeba-*Mycobacterium* interactions

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Recent studies have provided initial evidence to suggest that a diverse range of free-living amoebae (FLAs) could serve as environmental hosts for *Mycobacterium avium* subsp. *paratuberculosis* (Map). These findings highlight the potential role of FLAs in the environmental persistence and dissemination of Map, the causative agent of Johne's disease. However, the molecular mechanisms underlying FLA-Map interactions remain largely unexplored. To address this, timeresolved transcriptomic (RNA-seq) and proteomic (nLC-MS/MS) analyses are being conducted to investigate changes in the expression of Map genes and proteins during infection of a permissive environmental *Acanthamoeba* sp. (MOI 100), which was isolated from contaminated farm environments. By profiling bacterial gene expression at multiple key time points (0-, 2-, 6-, and 24-hours post-infection), this study aims to identify genes and proteins associated with virulence that are modulated during intracellular residency within FLAs. Furthermore, by comparing the molecular signatures of FLA-associated Map with those expressed during infection of macrophages, insights will be gained into environmental adaptation strategies, persistence mechanisms and potential enhancement of virulence. While these analyses are ongoing, this work represents a promising step towards elucidating the complex molecular dialogue between environmental amoebae and pathogenic mycobacteria.

P11. First identification of *Balamuthia mandrillaris* encephalitis in primates in Spain: Two case reports

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In this study, we report a case of a 4-year-old female Bornean orangutan (*Pongo pygmaeus*) in a zoological centre that exhibited neurological symptoms for several days. After unsuccessful treatments and a worsening in her condition, euthanasia was deemed necessary. Additionally, we describe the case of a 4-year-old male chimpanzee (*Pan troglodytes verus*) who died suddenly in a different zoo. Postmortem analysis revealed brain lesions with multiple hemorrhages, oedema, and inflammation in various organs in both cases. Histology showed the presence of *Balamuthia mandrillaris* trophozoites in necrotic and inflamed brain tissues, consistent with granulomatous amoebic meningoencephalitis. The diagnosis was confirmed using a multiplex qPCR assay on brain tissue samples from both animals water and soil samples from the chimpanzee's and orangutan's enclosure tested positive for *B. mandrillaris* DNA by qPCR, confirming environmental exposure. An immunofluorescent antibody (IFA) assay detected *B. mandrillaris* in chimpanzee brain slices. According to the authors' knowledge, this report documents the first known cases of *Balamuthia*

amoebic encephalitis in non-human primates in Spain and the first case in *Pan troglodytes verus*. Funding: This work was supported by CIBERINFEC (CB21/13/00100) and MePRAM Project (PMP22/00092) from the Instituto de Salud Carlos III, Madrid, Spain; Ministerio de Sanidad, Consumo y Bienestar Social, The Cabildo Insular de Tenerife CC20230222, CABILDO.23 and Ministerio de Sanidad, Spain. R. L. R. E., J. C. P. and C. J. B. E. contracts were funded by the Cabildo Insular de Tenerife 2023-2028 PROJECT CC20230222, CABILDO.23. I.S. was funded by a postdoctoral fellowship from ULL-Fundación Bancaria La Caixa-Fundación Bancaria Cajacanarias 2024. P. P. P. (TESIS2021010070) was funded by the ACIISI, 85% of which was co-financed by Fondo Social Europeo (FSE). M.B.Y, A.O and S.M.H.A were funded by Fundación Canaria para el Control de las Enfermedades Tropicales (FUNCCET) and Área de Acción Exterior from the Cabildo Insular de Tenerife.

P12. Review on the importance of pathogenic *Acanthamoeba* and *Balamuthia* in soil communities of free-living amoebae

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Free-living Amoebae found after infections or postmortems are usually traced back to where patients likely contracted the pathogen. Pathogenic amoebae need some human assistance to get rid of competence from other amoebae and become infective, as low numbers of individuals can invade healthy patients, and even lower numbers are needed to develop an amoebic infection in immuno-compromised ones. Pathogenic amoebae in soil communities show extremely low numbers (they are rare species), while voracious bacterivorous species like *Vahlkampfia avara* and *V. inornata* may be the dominant ones. Isolation or even detection of pathogenic species of *Acanthamoeba*, *Naegleria fowleri,* and *Balamuthia* comes mostly from stressed or perturbed environments where fast growing bacteria have been favored, such as garbage deposits on soil surface, sewage, or any other excess of organic matter, allowing the omnivorous amoebae to thrive and reaching infective numbers. Other factors reduce competence by increasing temperature (selecting thermotolerance of *Naegleria*), biocides such as chlorine (*Legionella* avoids chlorine by entering *Acanthamoeba* cysts), or maintaining wet soil long enough to support a community where hunters can thrive (*Balamuthia* hunts smaller amoebae, nematodes, and rotifers). The presence of omnivorous amoeba in agroecosystems or undisturbed soils indicates that the protist community has achieved stability. Additionally, detecting hunter amoebae signifies that the micro food web is linked to the soil mesofauna food web, and plant-microbe productivity is approaching a stable state. Instead of being supervillains searching for victims, these pathogens require human intervention to become problematic. Investigations into their infective cycles and metabolisms are crucial for identifying methods to eliminate them. Additionally, understanding their existence within the environment may provide further insights into managing these pathogens effectively. Ultimately, prevention remains the most effective strategy.

P13. Seasonal distribution and biodiversity of free-living amoebae and their associated bacteria in a model of drinking water storage tower

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Free-Living Amoebae (FLA) are single-celled eukaryotes found in many natural (soils, rivers, …) and artificial (pools, Drinking Water Storage Towers (DWST)) ecosystems. Some of these FLA can cause serious cerebral or ocular infections, in addition to be potential reservoirs of diverse pathogens. Previously in our laboratory, a study in 3 different Drinking Water Storage Towers (DWSTs) during 5 seasons showed that several FLA were detected with a greater density in surface biofilm samples in comparison to bottom ones. These results suggested a role of the tidal range within the DWST in the enrichment of FLA in the surface biofilm of these DWST due to the exposure of biofilm supports to immersion-emersion cycles (tidal range), allowing organic matter deposition and FLA adherence. The aim of this work consisted of analyzing the role of the tidal range in the distribution and the biodiversity of FLA in the biofilm of a DWST model specially designed for this study. Several cages, each containing a triplicate of glass plates, were placed in the water column: 3 cages placed at 3 different levels of the tidal range (surface, middle, bottom), besides one settled to float on the water surface. Additionally, one cage was placed before the entrance in the water column of the DWST model. The samples were collected at each season from April 2021 to January 2022, each time after 3 months of incubation. They were analyzed for FLA and bacteria quantification and identification by microscopy and molecular biology. Our results showed a higher density of FLA in the biofilm surface samples, within the tidal range, compared to the ones collected at the bottom of the water column which are always immersed. Several bacteria genera were also predominantly identified in surface samples suggesting an interaction with FLA and a potential role in their installation and/or proliferation in biofilms. These results confirm the role of the tidal range in the unequal distribution of FLA in our DWST model. This model could then be further used to investigate more in details the role of water level variation in the enrichment of FLA in surface biofilm.

P14. Pathogenesis review of *Naegleria fowleri* NFA1 associated with lectins

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Naegleria fowleri, a brain-eating amoeba, mainly causes primary amoebic meningoencephalitis (PAM) through the nose while swimming in lakes and rivers. Recently, there was a case of *N. fowleri* infection from tap water in Houston, USA. The biggest problem is that the mortality rate due to amoeba infection is extremely high 98%, with only about 4 out of 200 patient cases surviving. If a vaccine were developed, it could be a way to prevent such infections in advance, but the current research manpower pool and other problems make research quite difficult. However, through efforts so far, several proteins have been confirmed to be related to the pathogenicity of *N. fowleri*, and their potential as targets for vaccines or therapeutics has been evaluated. One of them is Nfa1, which is strongly expressed in pseudopodia involved in the movement of *N. fowleri* and participates in phagocytosis by attaching to target cells. It has been known that Nfa1 is closely related to the pathogenicity of amoeba through various methods such as functional analysis by polyclonal/monoclonal antibodies, knock-down, and overexpression. In particular, the fact that Nfa1 is expressed significantly in the pseudopodia suggests the possibility that contact-dependent lectins and glycoproteins may be involved. The results of analyzing the interaction between bacteria or target cells by adding various monosaccharides to *N. fowleri* have proven the relevance of lectins and glycoproteins. Therefore, the gene or protein level interrelationship between Nfa1 and lectins or glycoproteins will be studied further.

P15. Cyclooxygenase-like activity is a potential target in *Acanthamoeba castellanii* pathogenesis

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Previous studies have shown that nonsteroidal anti-inflammatory drugs (NSAIDs) affect the growth and encystment of *Acanthamoeba castellanii*, suggesting the presence of a cyclooxygenase (COX)-like activity in this parasite. More recently, COX-like activity has also been reported in *Leishmania mexicana*, with the gp63 protein identified as the enzyme responsible. Additionally, the D12 monoclonal antibody, which recognizes the gp63 protein in *L. mexicana*, has been shown to cross-react with several clinically significant parasites, including *A. castellanii*. Supporting this observation, COX-like activity has been demonstrated in both cells extracts and extracellular vesicles of *A. castellanii*. A bioinformatics analysis further revealed the presence of a gp63 ortholog in *A. castellanii* Neff, referred to as putative leishmanolysin (XP_004337275.1), suggesting a conserved function across species. In this study, we evaluated the effect of various COX-2 inhibitors (Aspirin, Sodium diclofenac, Ibuprofen, and Celecoxib) on cell migration and on the cytopathic effect of *A. castellanii*. Using light microscopy, we monitored trophozoite migration on brain tissue micropatterns and found that Aspirin had the most pronounced inhibitory effect on cell migration. The cytopathic effect of cyclooxygenase inhibitors on *A. castellanii* trophozoites co-cultured with MDCK cells was evaluated using confocal microscopy. Cells were treated with 50 μM inhibitors and stained with Rodhamine phalloidin to stain the actin cytoskeleton. Notably, Aspirin preserved the structure of the actin cytoskeleton in MDCK cells more effectively than the other compounds tested. Treatment with these NSAIDs resulted in a 60% to 90% reduction in the cytopathic effect. Furthermore, the encystment process was significantly inhibited by Celecoxib and Ibuprofen. Immunolocalization studies showed that gp63 is present on the surface of both trophozoites and mature cysts. In trophozoites, stimulation with brain extract enhanced the association of gp63 with actin. Overall, NSAID treatments reduced cytopathic effects, inhibited trophozoite migration, and impaired

encystment. These findings suggest that COX-like activity plays a role in the pathogenic mechanisms of *A. castellanii*.

P16. Towards a genomic framework for understanding *Acanthamoeba* pathogenicity

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Acanthamoeba are protozoa commonly found in water, soil and household dust. Most *Acanthamoeba* are free-living microbivores but pathogenic Acanthamoeba species are opportunistic parasites. Pathogenic species can be causative agents of CNS infection granulomatous amoebic encephalitis (GAE) in immunocompromised individuals and *Acanthamoeba* keratitis (AK) in immunocompetent individuals.

The efficacy of AK treatment ranges from 35%-86%, with treatment failure in 39% of cases due to the formation of resistant cysts. Treatment failure leads to a reduction in visual acuity necessitating a corneal transplant and surgical enucleation in approximately 25% and 5% of patients, respectively. In the case of GAE, there is a mortality rate of over 90% and the optimal treatment for this infection has not been described.

To better understand the genetic underpinnings of pathogenicity and treatment resistance in *Acanthamoeba*, we have generated high-quality, chromosome-level genome assemblies from a representative set of both environmental and clinical isolates using PacBio HiFi long-read sequencing and Illumina Hi-C chromosomal scaffolding. This effort is being complemented by ongoing collaborations to resequence clinical isolates from UK patients. These genomic resources are being used to investigate sequence and structural polymorphisms associated with virulence potential and cyst resilience.

Our work lays a foundation for future comparative and functional genomic studies aimed at identifying conserved and strain-specific mechanisms of pathogenesis. These efforts will ultimately inform the development of more effective diagnostics and therapeutic strategies for both AK and GAE.

P17. Induction of apoptosis and inhibition of cyst formation in *Naegleria fowleri* by pan-histone deacetylase inhibitors: Vorinostat (SAHA) and quisinostat (JNJ-26481585)

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Infection by *Naegleria fowleri* results in primary amoebic meningoencephalitis (PAM), a highly lethal disease whose global incidence is increasingly prevalent. Despite this escalation, the development of a fully effective treatment remains elusive, primarily due to the limitations inherent in current pharmacological approaches. In this study, we investigated the amoebicidal effects of histone deacetylase inhibitors (HDACis) on both trophozoites and cysts of *N. fowleri.* HDACis were evaluated for their anti-proliferative and cytotoxic effects on *N. fowleri* using the MTT assay and the lactate dehydrogenase (LDH) assay, respectively. Apoptosis induced in *N. fowleri* by HDACis was analyzed using fluorescence-activated cell sorting (FACS) analysis. The MTT assay indicated that class-specific HDACis, including class I HDACis (MS275 and MGCD0103) and class II HDACi (MC1568), had minimal impact on the viability of *N. fowleri* trophozoites. In contrast, pan-HDACis (SAHA and JNJ) markedly inhibited the viability of *N. fowleri* trophozoites in a dose- and time-dependent manner. The LDH assay demonstrated that 10 μM concentrations of SAHA and JNJ significantly heightened cytotoxicity against *N. fowleri* over a 48-h period. Corroborating the increase in cytotoxicity, FACS analysis revealed a notable elevation in the apoptosis rate of *N. fowleri* post-treatment with SAHA and JNJ. Although 10 μM concentrations of SAHA and JNJ did not exhibit a lethal effect on the cysts of *N. fowleri*, they were found to completely inhibit the transformation of *N. fowleri* into cysts. These cumulative findings suggest that SAHA and JNJ have promising potential as pharmacological therapeutic agents against *N. fowleri* infection.

P18. Repurposing NSAIDs against *Acanthamoeba castellanii*: molecular docking insights into leishmanolysin inhibition

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Drug repurposing—the identification of new therapeutic uses for approved compounds—offers a cost-effective and time-efficient strategy, particularly valuable for rare or neglected diseases. *Acanthamoeba castellanii,* a free-living amoeba, causes serious infections such as Acanthamoeba keratitis and granulomatous amoebic encephalitis, both of which are difficult to treat due to limited therapeutic options and resistance to conventional drugs. Nonsteroidal anti-inflammatory drugs (NSAIDs) are promising repurposing candidates, given their established pharmacological profiles. Previous experimental findings by our research group have demonstrated that NSAIDs can interfere with key pathogenic processes in *A. castellanii.* In this study, we explored the molecular interactions between three NSAIDs—aspirin, ibuprofen, and sodium diclofenac—and a putative *A. castellanii* virulence factor: a leishmanolysin-like metalloprotease (gp63). Three-dimensional models of *A. castellanii* gp63 and *Leishmania major* leishmanolysin were generated using ColabFold (AlphaFold2-MMseqs2). Model quality was validated via MolProbity, ProSA-web, and ResQ, with pLDDT scores above 85 indicating high confidence. Structures were refined and visualized in ChimeraX, and the active site was completed by aligning with the crystal structure template (PDB: 1ML1) to incorporate the essential Zn^{2+} cofactor. Ligand geometries were optimized using ORCA and validated through MolGC. Docking simulations were performed in AutoDock using 150 runs of the Lamarckian Genetic Algorithm. Protonation states at physiological pH (7.2) were set using Avogadro and PDB2PQR, and charges were assigned appropriately. Our results revealed that ibuprofen and sodium diclofenac exhibited strong binding affinities for the gp63 active site of *A. castellanii,* involving key interactions with the Zn^{2+} cofactor and conserved catalytic residues. Aspirin showed moderate interaction. These findings support further investigation of NSAIDs as potential repurposed therapeutics against *A. castellanii* infections.

P19. From anti-inflammatory to antiparasitic: *in silico* drug development prospects of NSAIDs targeting *Acanthamoeba castellanii*

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Acanthamoeba castellanii is a free-living amoeba commonly found in the environment and also can cause severe human infections, including Acanthamoeba keratitis and granulomatous amoebic encephalitis. Current treatments are often toxic, limited in efficacy, and lack specificity, creating an urgent need for safer and more effective therapeutic alternatives. Drug repurposing —re-evaluating existing pharmaceuticals for new therapeutic uses— offers a cost-effective and time-efficient approach to identifying novel treatments. Non-steroidal anti-inflammatory drugs (NSAIDs) like ibuprofen, aspirin, diclofenac, and celecoxib are widely used for their anti-inflammatory, analgesic, and antipyretic properties. Emerging evidence suggests that some NSAIDs may also exhibit antiparasitic activity, including protozoan pathogens. In this study, a bioinformatic strategy was employed to assess the potential of selected NSAIDs for repurposing their effects against *A. castellanii*. The objectives were to predict possible molecular targets in the parasite and to evaluate potential off-target interactions in the human host that could result in adverse effects. All four NSAIDs met key drug-likeness criteria, but their predicted efficacy and safety profiles varied. Diclofenac and celecoxib displayed higher toxicity levels and potential for endocrine disruption. Target prediction analysis identified common interactions with oxidoreductases, G-protein coupled receptors (GPCRs), and other enzymes, with diclofenac exhibiting the most complex interaction profile. Additionally, structural similarity assessments revealed that these compounds resemble known toxic industrial chemicals, indicating potential health risks. Overall, the findings support the potential of NSAIDs as candidate antiparasitic agents against *A. castellanii* but also underscore important safety considerations. This study contributes to a growing understanding of NSAID-pathogen interactions and establishes a foundation for future experimental validation. Continued research is necessary to

confirm these predictions and assess the therapeutic viability of NSAID repurposing for treating infections caused by *A. castellanii*.

P20. The 4-aminomethylphenoxy-benzoxaborole AN3057 as a potential treatment option for primary amoebic meningoencephalitis

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Primary amoebic meningoencephalitis (PAM) is a rare but fatal disease of the central nervous system (CNS) caused by the "brain-eating amoeba," *Naegleria fowleri.* There are several requirements for an effective drug against PAM: it must be fast-acting and able to cross the bloodbrain barrier. Currently, PAM treatment relies on repurposed, brain penetrable antimicrobial drugs, deployed at extremely high doses, which is associated with severe side effects and remains largely ineffective (with a case fatality rate of greater than 97%).

Benzoxaboroles are a unique class of compounds with boron heterocyclic scaffolds that showed considerable promise as drugs against pathogenic fungi and a range of protist parasites. We discovered that the 4-aminomethylphenoxy-benzoxaborole (AN3057) exhibits nanomolar potency against *N. fowleri in vitro,* and experimental treatment of infected mice significantly prolonged survival and demonstrated a 28% relapse-free cure rate. Based on proteomic analysis of resistant mutant strains, we identified and localized a mitochondrial pathway responsible for prodrug activation, which we were able to reconstitute *in vitro.*

P21. Antiamoebic activity of mexican propolis and its fractions on *Acanthamoeba* spp.

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Acanthamoeba spp. can cause severe pathologies such as amoebic keratitis and granulomatous amoebic encephalitis, for which there is no specific treatment of choice. The search for compounds with potential activity against these amoebae has gained interest, with natural products being one option. In this study, the antiamoebic effect of the methanolic extract of propolis and some of its chemical derivatives on *A. castellanii* and *A. culbertsoni* was evaluated. The methanolic extract was obtained by macerating propolis collected in Chihuahua, Mexico. A portion was subsequently fractionated by column chromatography (CC), analyzed by high-resolution mass spectrometry (ESIMS), and the fractions were characterized by nuclear magnetic resonance (NMR). The LC_{50} of the extract and the 11 formed fractions were obtained using the cell viability technique with crystal violet. LC_{50} values of 1.212 and 0.729 mg/mL were determined for *A. castellanii* and *A. culbertsoni,* respectively. The fractions with the highest activity against both species were F85-110 and F111-122, the latter with an LC_{50} of 0.014 and 0.012 mg/mL for each of these species under study, observing loss of trophic morphology, reduction in the size of the trophozoites, vacuolization, and presence of detritus; no precystic or cystic forms were observed. ESI-MS revealed the presence of phenolic compounds such as pinocembrin, biochanin A, baicalein, pinobanksin, chalcone, 5,8-dihydroxyflavanone, and rhamnetin, which have been reported to exhibit antimicrobial properties, possibly responsible for the amoebic activity. NMR analysis showed the presence of pinocembrin, 5,8-dihydroxyflavanone, and quercetin as the most abundant compounds in the fractions with the highest antiamoebic activity. This is the first report of the antimicrobial effect of Mexican propolis against *A. castellanii* and *A. culbertsoni.* Although the antiamoebic activity of propolis differs between the two species studied, the most active fraction produced a comparable effect, probably related to differences in sensitivity to the compounds identified in the extract, to the concentration of some chemical compounds in the resulting fractions, and the same action mechanism. PAPIIT-UNAM Project IN225124.

P22. Water treatment and seasonality shape the microbiome of drinking water distribution systems in Guadeloupe (French West Indies)

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Eukaryotic and bacterial microorganisms are commonly present in environmental waters, yet their distribution and dynamics within drinking water distribution systems (DWDS) remain underexplored. This study investigates the microbiomes of untreated (river and groundwater) and treated (storage and tap) waters from 17 DWDS across Guadeloupe (Lesser Antilles), with a focus on seasonal and treatment-related impacts.

A total of 90 water samples were collected over one year, covering both rainy and dry seasons. To assess microbial diversity, we conducted high-throughput 18S and 16S rRNA gene amplicon sequencing. For eukaryotic analysis, water samples were filtered, incubated with bacteria or yeast at 30, 37, and 44 °C to promote growth of culturable microorganisms, followed by DNA extraction, 18S PCR amplification, and Illumina sequencing. Bacterial communities were analyzed from filters directly, using 16S rRNA gene amplification and sequencing. A similar protocol was applied to explore bacteria associated with eukaryotes. Conventional microbiological and physicochemical analyses were also performed for each sample.

The 18S metabarcoding results revealed distinct eukaryotic community profiles depending on water source and season. Free-living amoebae (FLA) were predominant, with *Vermamoeba*, *Naegleria*, and *Echinamoeba* being the most abundant genera. Pathogenic FLA, such as *Naegleria fowleri* and *Acanthamoeba* spp. were also detected, including in treated waters. The 16S analyses showed significant differences between free-living and FLA-associated bacterial communities. While free-living bacterial populations were relatively consistent across DWDS (dominated by *Salmonella*, *Klebsiella*, and other *Enterobacteriaceae*), FLA-associated bacteria displayed greater variability, with *Pseudomonas* and *Stenotrophomonas* being most prevalent.

Our findings highlight the influence of seasonality and disinfection on the composition and abundance of both eukaryotic and bacterial communities in drinking water systems. The detection of pathogenic FLA in treated water raises important public health concerns and emphasizes the need for improved monitoring and control strategies. This study provides novel insights into DWDS microbiomes in tropical island settings and underscores the importance of integrated approaches to ensure microbiological safety in drinking water distribution.

P23. Knowledge, attitudes and practices regarding *Naegleria fowleri* infection among visitors to freshwater bodies with high prevalence of vahlkampfiids

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Free-living amoebae (FLA) of the Vahlkampfiidae family (VAHL) are widely distributed in nature, occupying aquatic and terrestrial habitats. The group includes amphizoic organisms such as *Naegleria fowleri*, the causative agent of primary amoebic meningoencephalitis. This study aims to determine the presence of FLA of this family capable of growing at elevated temperatures and thethe genus *Naegleria* in different recreational water sources in Costa Rica during 2023, as well as people´s knowledge-attitude-practices (KAP) profile in relation to the infection by *N. fowleri*. Water samples were taken in 24 locations and cultured at 42 °C, isolation and molecular tests were performed, resulting in 20 locations positive for VAHL and seven positives for Naegleria spp., but sequencing identification detected only *Tetramitus* spp. predominantly, followed by *Vahlkampfia* sp. and *Neovahlkampfia* sp. A KAP survey was applied to 72 people between 18 and 66 years old, of which 37,5 % had never heard about *N. fowleri*, and average scores of 35,8% for knowledge and 3,01 and 2,16 for attitudes and practices were obtained, being five the favorable or lower risk value. The statistical tests showed significant differences ($p < 0,05$) between the level of knowledge and gender/residence origin of the person. These results indicate a high frequency of thermotolerant FLA in non-thermal surface waters in Costa Rica with the ability to proliferate at high temperatures. Whilst KAP survey showed a low level of knowledge and deficiencies in the management and prevention of PAM risk in the population exposed to natural freshwater reservoirs.

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P24. Analysis of virulence factors in extracellular vesicles secreted by *Naegleria fowleri*

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Naegleria fowleri is the etiologic agent of Primary Amoebic Meningoencephalitis, an acute and fulminant infection of rapid progression that affects the Central Nervous System, mainly in children and young adults with a history of having performed aquatic activities in natural or artificial freshwater bodies. This disease has a mortality rate greater than 95%. One of the main problems is its symptomatic similarity with other meningitis caused by viruses or bacteria, which makes it difficult to make a rapid and timely diagnosis that prevents the progression of this infection. Therefore, it is necessary to know the antigenic determinants as well as the pathogenicity mechanisms of this amoeba to implement strategies that allow better therapeutic targets and antiamoebic diagnoses. Therefore, the aim of this work was to analyze some virulence factors as part of the extracellular vesicle (EVs) cargo secreted by *N. fowleri.* The secretion as well as the identification of EVs cargo was performed by immunocytochemistry, SDS-PAGE, Western blot and RT-PCR. Our results showed that *N. fowleri* secretes a wide variety of vesicle sizes ranging from 0.2 µm to >2 µm. In addition, these EVs were recognized by anti-*N. fowleri,* anti-Naegleropore B, anti-19 kDa polypeptide band, anti-membrane protein Mp2CL5, anti-cathepsin B and anti-actin antibodies. Specifically in relation to small vesicles, our purified exosomes were recognized by CD63 and Hsp70 markers, along with the previously mentioned proteins. RT-PCR analysis was made through the isolation of EVs from *N. fowleri* trophozoite culture by concentration, filtration and ultracentrifugation. Interestingly, we obtained PCR products for Nfa1, Naegleropore B, Mp2CL5, and cathepsin B genes as part of exosomes cargo. This suggests that the molecules identified in this work could play an important role in communication as well as in infectious processes caused by this amoeba.

P25. Development of two vaccines based on the membrane protein Mp2CL5 in mice immunized intranasally in the *Naegleria fowleri* meningitis model

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Naegleria fowleri is an etiologic agent that causes primary amebic meningoencephalitis; unfortunately, there is currently no effective vaccine available for use in humans. Therefore, the objective of this work was to determine the immunoprotective response of two vaccine antigens: 1) the 19 kDa polypeptide band purified by the electroelution technique or 2) an immunogenic peptide designed using bioinformatics tools from the *N. fowleri* membrane protein MP2CL5. Both antigens were administered alone intranasally in mice or co-administered with cholera toxin (CT) as an adjuvant. The survival rate and immune response of mice immunized with both antigens and challenged with *N. fowleri* trophozoites were measured in nose-associated lymphoid tissue (NALT) and nasal passages (NP) by flow cytometry and enzyme-linked immunosorbent assay (ELISA). We also determined the immunolocalization of both antigens in *N. fowleri* trophozoites using confocal microscopy. Immunization with the 19 kDa polypeptide band alone or co-administered with CT was able to confer 80% and 100% protection, respectively, which was higher compared to immunization with Smp145 alone or adjuvanted with CT in which obtained 60% and 80% protection, respectively. Immunization with both antigens (alone or co-administered with CT) showed increased levels of IgA, IgG, and IgM in serum and nasal washes. The immunolocalization of both antigens in *N. fowleri* trophozoites was observed in the plasmatic membrane, being specifically found in pseudopod-like structures. The MP2CL5 antigens evaluated in this work were capable of conferring protection, which would lead us to consider them as potential candidates for vaccines against meningitis caused by *N. fowleri.* **Acknowledgment:** CBF2023-2024-175, PAPIIT UNAM IN224519, IPN-SIP 20250688.

P26. Long-term protective immune response in the mouse model of meningitis caused by *Naegleria fowleri*

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Naegleria fowleri is a free-living amoeba found mainly in warm freshwater and is the etiological agent of Primary Amoebic Meningoencephalitis (PAM), an acute and fatal disease with a mortality rate greater than 95% without timely diagnosis and treatment. Therefore, it would be of great importance to prevent it through the development of effective vaccines, allowing for long-term protection against PAM. Therefore, the objective of this work was to evaluate the long-term protective immune response in the nasal cavity of mice immunized intranasally with the 100 kDa band in the model of meningitis caused by *N.f.* Two groups of BALB/c mice were immunized four times intranasally with the 100 electroeluted band, one group received a booster immunization and was challenged with a lethal dose of *N.f.* trophozoites at 3 months; one group was analyzed for survival after the challenge and was observed for 60 days, while the other group for immune response analysis was sacrificed 72 hours after the challenge, where serum, nasal washes, nasal passages, and nasopharyngeal-associated lymphoid tissue were obtained. The samples were analyzed by the following techniques: the Enzyme-Linked Immunosorbent Assay (ELISA) was used to analyze the levels of specific antibodies for both antigens, while Immunoblot was used to analyze the antigens recognition by these antibodies and lymphocyte phenotyping by flow cytometry. The results obtained in this study showed a 60% of protection, as well as a statistically significant increase in the IgG and IgA antibodies levels. On the other hand, the results of flow cytometry showed a higher percentage of positive cells for both effector and memory B lymphocytes, as well as dendritic cells. Finally, the immunization schedule used in this study induced a long-term protective response that is essential for future vaccine development.

P27. Analysis of the humoral immune response against a synthetic peptide derived from the HSP70 protein of *Naegleria fowleri*

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We previously reported that intranasal administration of *Naegleria fowleri* lysates plus Cholera toxin (CT) confers protection against meningoencephalitis in mice. In that study, we identified immunogenic antigens from *N. fowleri* using the serum and nasal washes from protected animals. Interestingly, the immunogenic polypeptide bands found corresponded to 100, 70 and 50 kDa. Subsequently, we analyzed the sequences obtained by mass spectrometry of these polypeptide bands, resulting in nine polypeptides corresponding to the HSP70 protein. The sequences were then subjected to immunoinformatics analysis to identify their binding affinity to MHCII and B-cell. We also considered the lowest homology to human and mouse HSP70. Once the peptide with a high binding affinity for MHC class II and B cells was identified, it was sent for synthesis to evaluate the humoral immune response in Balb/c mice. The mice were intranasally immunized with the selected HSP 70 peptide, either alone or in combination with a modified cholera toxin (CTA) as an adjuvant. The immunization with both treatments induced IgG and IgA antibodies that moderately reacted with 100, 70 and 50 kDa bands of *N. fowleri*, showing a significant increase compared to the control group. Furthermore, serum IgG from mice immunized solely with the HSP70 peptide recognized structures of the trophozoite, including the membrane, pseudopodia, food cups, and exosomes. Finally, we propose that the robustness of the immune response may be further enhanced through multiepitope strategies that can effectively stimulate defenses against *N. fowleri* infection.

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P28. Analysis of the innate immune response in BALB/c and CD1 mice in the early stages of *Naegleria fowleri* infection

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Naegleria fowleri is the etiological agent of primary amebic meningoencephalitis (PAM), an acute infection of the central nervous system with a mortality rate of 98%. Although many people have been exposed to similar environments, few cases of human infection have been reported. The mechanisms of innate resistance to *N. fowleri* that may contribute to susceptibility to infection are currently not fully understood. Therefore, the aim of this study was to analyze the innate immune response in two mouse strains: BALB/c (inbred and susceptible to *N. fowleri* infection) and CD1 (outbred and resistant to *N. fowleri* infection) in the early stages of infection. For this purpose, BALB/c strain mice were infected with $5x10^4$ *N. fowleri* trophozoites, while CD1 strain mice were infected with $5x10^4$, $1x10^5$, or $2.5x10^5$ trophozoites. The survival rate of the mice was assessed 60 days after infection. In addition, two other groups of CD1 or BALB/c mice were infected i.n. with $5x10^4$ *N. fowleri* trophozoites and sacrificed at 12 and 24 h postinfection for analysis of the immune response. The results showed that CD1 mice infected with a dose of $5x10^4$ trophozoites had a 100% post-infection survival rate, in contrast to the BALB/c group of mice, which died within 6 to 8 days. CD1 mice challenged with a dose of $1x10^5$ trophozoites had a 60% survival rate; however, when infected with $2.5x10^5$ trophozoites, they had a 0% survival rate. In these two groups of infected CD1 or BALB/c mice, the immunolocalization of trophozoites in the olfactory epithelium, as well as of mucopolysaccharide, was found to be differential, as were the levels of IgA and IgG antibodies in serum and nasal washes. In addition, significant changes in the recognition of IL-6, IL-1β or TNF-α were found in proteins isolated from the nasal passages of infected mice. Therefore, this study allowed us to identify some of the factors involved in resistance and susceptibility to the development of PAM, which may provide important insights into the understanding of *N. fowleri* pathogenesis.

P29. Interaction of *Acanthamoeba castellanii* with bacteria by galactose and antibodies to a galactose-binding protein

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Acanthamoeba castellanii causes amoebic keratitis (AK), primarily in contact lens wearers, and severe lesions may require corneal transplantation. Direct contact between the amoeba and the cornea during AK induction is important for the pathogenic process of the amoeba. This direct contact is facilitated by galactose-binding protein (GBP) on the amoeba. In this study, the effect of treatments with galactose and monoclonal antibodies against GBP on the interactions between bacteria and *A. castellanii* were analyzed. *A. castellanii* trophozoites were first incubated with galactose, mannose, or monoclonal antibody to the GBP for 1 hr, followed by a mixed culture incubation with *Escherichia coli* O157:H7 for 1 hr, or with *E. coli* DH5α for 4 hr. The amoeba-bacteria association after the incubation treatment with mannose was 3.4 times lower than with no treatment. However, the association after the galactose treatment was about 0.7 times lower than with no treatment. In particular, incubation with monoclonal antibodies to GBP showed results very similar to those of mannose. *E. coli* O157:H7's invasion of the amoebas was reduced by about three times as compared to the association reaction. The effects of the incubation treatments with monoclonal antibodies against galactose, mannose, and GBP were so similar that they were almost incomparable. *E. coli* DH5α's association reaction with galactose was slightly lower than that in the monosaccharide-untreated (no treatment) group. However, it was confirmed that monoclonal antibodies against GBP could reduce the amoeba-bacteria association about 3.65 times more than no treatment. The incubation treatment with antibodies to GBP showed an increase in proteolytic enzyme expression to a degree very similar to that of the mannose treatment group. Based on the contact-dependent pathogenicity caused by interactions with monosaccharides and antibodies, there may be a need to molecularly analyze changes in protease activity and other antigenic proteins that occur within the *A. castellanii* cytoplasm.

P30. Novel targeted anti-amoebic strategies using nanobody technology

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Acanthamoeba keratitis (AK) is a serious eye infection that bears the risk of permanent blindness. The main causative pathogenic agent is *Acanthamoeba castellanii,* classified as an emerging parasite due to the global increase of AK cases. Safe and efficient compounds for AK prevention and treatment are lacking today. Current therapeutics require long-term treatment periods and are not specific, generating important toxicity for human corneal cells and resulting in permanent eye damage. Therefore, novel targeted and safe therapeutics against AK are needed, especially against the resilient cyst form. By generating Nanobodies (Nbs) that bind specifically on surface-exposed antigens of *A. castellanii*, a basis for new anti-amoebic strategies can be generated. A Nb library against whole cell trophozoites of *A. castellanii* has been generated and comprises 27 unique Nbs that bind specifically to *A. castellanii* trophozoites, shown by ELISA. These 27 Nbs are separated in 15 families, believed to target different antigens on the amoeba's surface. For some of these Nbs specific binding has been observed at single-cell level by fluorescent labelling and microscopy. These Nbs can form the basis of therapeutic solutions against AK by determining their targets via pull-down assays and screening for Nbs with inhibitory effects. Moreover, the binding Nbs discovered thus far could be fused to enzymes showing anti-amoebic activity, as such generating a targeted solution against AK. Thus far, Nbs specifically targeting the surface of *A. castellanii* trophozoites have been generated and binding by fluorescent labelling has been confirmed. This could potentially lead to a new detection method for diagnosis of AK, which is known to be very tricky as of to date.

P31. Extracellular vesicles secreted by *Acanthamoeba culbertsoni* have COX and proteolytic activity and induce hemolysis

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Several species of *Acanthamoeba* genus are potential pathogens and etiological agents of several diseases. The pathogenic mechanisms carried out by these amoebae in different target tissues have been documented, evidencing the relevant role of contact-dependent mechanisms. With the purpose of describing the pathogenic processes carried out by these protozoans more precisely, we considered it important to determine the emission of extracellular vesicles (EVs) as part of the contact-independent pathogenicity mechanisms of *A. culbertsoni,* a highly pathogenic strain. Through transmission electronic microscopy (TEM) and nanoparticle tracking analysis (NTA), EVs were characterized. EVs showed lipid membrane and a size between 60 and 855 nm. The secretion of large vesicles was corroborated by confocal and TEM microscopy. The SDS-PAGE of EVs showed proteins of 45 to 200 kDa. Antigenic recognition was determined by Western Blot, and the internalization of EVs by trophozoites was observed through Dil-labeled EVs. In addition, some EVs biological characteristics were determined, such as proteolytic, hemolytic and COX activity. Furthermore, we highlighted the presence of leishmanolysin in trophozoites and EVs. These results suggest that EVs are part of a contact-independent mechanism, which, together with contact-dependent ones, allow for a better understanding of the pathogenicity carried out by *Acanthamoeba culbertsoni.*

P32. Metal and redox homeostasis in *Acanthamoeba-Stenotrophomonas* interactions

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Acanthamoeba (Amoebozoa) are ubiquitous free-living amoebae that graze on other microbes via phagocytosis (Zurita-Artaloitia et al. 2023). However, some bacterial lineages evade digestion by phagosomes, establishing interactions with their hosts ranging from symbiosis to parasitism. We recently discovered that *Stenotrophomonas maltophilia* (Sm), an opportunistic multi drug-resistant pathogen (Gammaproteobacteria) of broad environmental occurrence, can replicate within acidified Rab7A-positive vacuoles (SCV) of *A. castellanii* Neff trophozoites (Rivera et al. 2024). After a lag phase, Sm18 bacteria initiate replication within the SCV, eventually returning to the extracellular environment via a non-lytic exocytic pathway. We are interested in learning how *Stenotrophomonas* manipulates membrane trafficking pathways and how it adapts to the bactericidal phagosomal milieu.

Here, we used structure-based deep-homology searches and phylogenomics to study the diversity and distribution of critical redox, Fe, Mn, Cu, and Zn metallostasis genes across Amorphea, and show that *Acanthamoeba* and other Amoebozoa possess homologs of human metal transporters involved in both nutritional immunity and metal intoxication in macrophages, suggesting that within amoebal phagosomes microbes are subjected to Fe and Mn limitation, Cu and Zn toxicity, and oxidative burst. We generated Sm18 mutants in multiple redox, Mn and Cu homeostasis genes, discovering that *katN* (Mn-dependent catalase), *sodC* (Mn-dependent superoxide dismutase), *mntH* (Mn importer), *actP* (Cu exporter), and *cueO* (periplasmic multicopper oxidase) are required for efficient replication in *Acanthamoeba* phagosomes. Live cell imaging revealed that transcriptional fusions of the *sodC*, *mntH* and *actP* promoter regions to mScarlet-I are expressed in the SCV.

We conclude that metal and redox homeostasis systems play crucial and ancestral roles in the phagocyte-bacteria interactions, from amoebae to humans.

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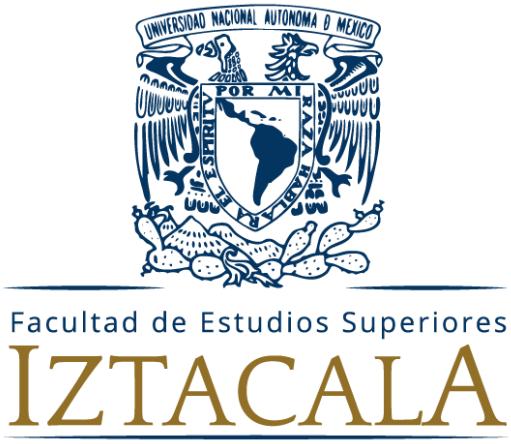
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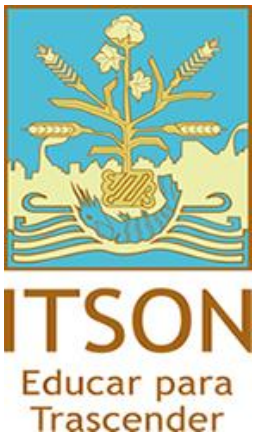
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