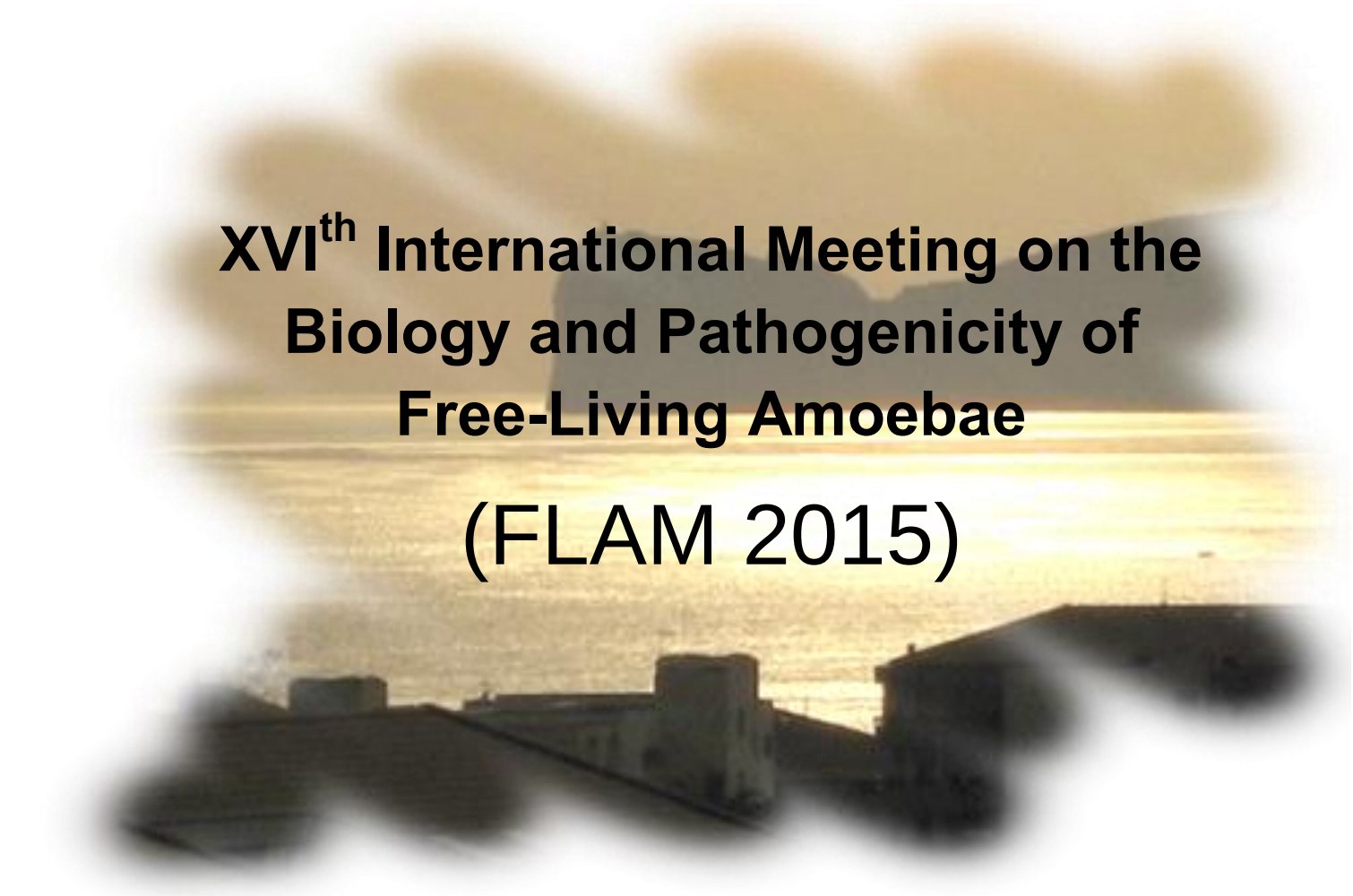

XVIth International Meeting on the Biology and Pathogenicity of Free-Living Amoebae

(FLAM 2015)

ABSTRACTS

18th-22nd May 2015

Alghero, Italy

XVITH International Meeting on the Biology and Pathogenicity of Free-living Amoebae (FLAM 2015)

May 18th-22nd 2015, Alghero Italy

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KEYNOTES

KEYNOTE 1

Chairs

Piero Capuccinelli and Pierluigi Fiori

Kn-1

Dr Govinda S. Visvesvara

Brain Eating Amoebas: Review of Recent Cases and Risk Analysis of Transmission to Recipients of Solid Organ Transplantation

CDC Foundation, Atlanta, GA

Acanthamoeba spp., *Balamuthia mandrillaris*, and *Naegleria fowleri*, popularly known as brain-eating amoebas, are free living amebae and occasionally cause devastating human disease leading to death. *Acanthamoeba* spp. and *B. mandrillaris* cause granulomatous amebic encephalitis (GAE), cutaneous and naso-pharyngeal as well as disseminated infection. *N. fowleri* causes a rare, but an acute, fulminating and rapidly fatal infection, primary amebic meningoencephalitis (PAM), in healthy children and young adults with a history of aquatic activities in fresh water. Diagnoses of these infections are challenging and antimicrobial therapy is empirical. Review of recent cases, diagnostic dilemmas and drug therapy will be discussed. Further research is needed to explore the possibility of a better drug delivery system, for example, designing drug-loaded nanoparticles that can cross the blood–brain barrier, so that the drugs can effectively reach the CNS and kill the amebae.

Recently, three transplant-associated clusters of encephalitis caused by *Balamuthia mandrillaris* have been described which prompted investigations of all the recipients and the donors through review of all medical records and testing of all available specimens by various diagnostic methodology. Drug regimens used in the surviving transplant recipients will be discussed. Infections with *Acanthamoeba* spp. also has occurred in transplant recipients. D'Auria et al (2012) described a case of *Acanthamoeba* GAE with cutaneous manifestation two months after receiving double lung transplantation. Further, more than 20 cases of *Acanthamoeba* infections in patients with solid organ transplantation have been described in the literature. Unfortunately, no comprehensive study was conducted to link the infections in the recipients with the respective donors. PAM due to *N. fowleri* is believed to be confined to the CNS. However, historical PAM case reports and animal studies suggest that *N. fowleri* might disseminate to other organs and thus may pose a risk of PAM for transplant recipients. Based on a literature review, during 1995 to 2012, five patients died of PAM and their solid organs were transplanted to 21 patients. This prompted an investigation of the suitability of solid organ transplantation from donors with PAM. Examination of postmortem tissue sections of lungs, liver, heart and spleen of the two donors revealed that *N. fowleri* amebae may be transported to other organs. Fortunately, none of the 21 recipients of solid organ transplantation developed PAM. However, the risks of transplantation with an organ possibly harboring the brain-eating amebae should be carefully weighed and risk assessed for each individual recipient before transplantation (Roy SL et al 2013).

KEYNOTE 2

Chair

Julia Walochnik

Kn-2

Innate Immunity to *Acanthamoeba*

Craig W. Roberts[a], Antonella Cano[a], Fiona L. Henriquez[b], Antonella Mattana[c], James Alexander[a]

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Acanthamoeba is ubiquitous and serological studies have demonstrated that the vast majority of people are seropositive. In immune-competent individuals overt disease is rare and is normally associated with contact lens (mis)usage where it manifests as keratitis. Thus the vast majority of humans develop an efficient, effective immune response to this facultative parasite. Consistent with these observations, in immune compromised individuals *Acanthamoeba* is an opportunistic parasite that causes frequently fatal granulomatous encephalitis. The immune response to *Acanthamoeba* is poorly understood and scarcely studied. However, macrophages have been demonstrated to play an important role in preventing *Acanthamoeba* keratitis. Thus our recent work has focused on determining the interaction of *Acanthamoeba* with macrophages. Co-cultures demonstrated that although macrophages physically interact with *Acanthamoeba* trophozoites of the Neff strain for prolonged periods of time, this does not result in their phagocytosis or destruction. In contrast, interaction of trophozoites of the T4 clinical isolate with macrophages through lamellipodia and filopodia resulted in their capture and destruction. Macrophages produced greater levels of IL-6 and IL-12 when cultured with trophozoites of the Neff strain rather than the clinical isolate. Trophozoites of the Neff strain, but not the clinical isolate also induced TNF-α production by macrophages. Cytokine production was largely dependent on MyD88, rather than TRIF mediated signalling events. The ability of protease inhibitors to reduce cytokine production in co-cultures indicated that *Acanthamoeba* proteases contribute to macrophage activation. Ongoing work aims to further dissect the phenotype of macrophages induced by *Acanthamoeba* and the mechanisms whereby this occurs. Such studies should provide insight into the pathogenesis and treatment of *Acanthamoeba* infections.

KEYNOTE 3

Chair

Iva Dyková

Kn-3

Dr Yann Héchard

Environmental factors shaping amoebal population into the wild

Vincent Delafont[1, 2], Didier Bouchon[1], Yann Héchard[1], Laurent Moulin[2]

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Very few studies have described the diversity of free living amoebae (FLA) in the environment and the role of environmental factors that could shape the amoebal populations. FLA might be pathogenic and they could bear pathogenic bacteria that resist FLA phagocytosis. Monitoring of FLA and intracellular bacteria interactions in drinking water network is therefore an important health concern.
In this study, we performed a wide sampling campaign into the drinking water network of Paris. The physicochemical parameters were monitored, FLA were isolated and identified, as well as intracellular bacteria, by 18S rRNA- and 16S rRNA-targeted pyrosequencing.
In this aim, water was sampled over one year from 4 reservoirs, weekly, and 33 end-point sites, monthly, onto the Paris water network. At the end, 398 samples were screened for the presence of migration front of FLA. Not surprisingly, the reservoir samples were less positive for FLA culture (ca 15-20%) than end-point sites (ca 70-75%). All migration fronts from positive plates were harvested and the total DNA isolated for metagenomics studies. The diversity of FLA was assessed by 18S-targeted metagenomics showing that 6 main genus were present and that there were high differences between the sites.
Statistical analyses show that the origin of water (surface vs ground) might explain partly the differences in FLA diversity between the sites. Besides, *Acanthamoeba* presence was positively correlated to pH and chlorine concentration while *Vermamoeba* was correlated to temperature.
A core microbiota was identified from the following bacterial genera found at all sites: *Pseudomonas*, *Escherichia*, *Bradyrhizobium*, *Stenotrophomonas* and *Mycobacterium*. Interestingly, analyzing their occurrence at the different sites, we were able to highlight specific interactions between specific bacterial species and amoebal species.
This study help to better understand the ecology of FLA and intracellular bacteria in a drinking water network, in order to better control their development.

ORAL SESSION 1

Chairs

Govinda S. Visvesvara

and

Jacob Lorenzo-Morales

Or-1

Typical and exceptional cases of *Acanthamoeba* infections

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In Austria, the first diagnosed case of AK was described in 1989, the causative agent being identified as *Acanthamoeba rhysodes* (Huber-Spitzy *et al*. 1989) and the first case of GAE was recorded in 2004 (Aichelburg *et al*. 2008). Since then, altogether 168 cases of AK and 3 cases of GAE have been diagnosed. The typical AK patient is a 20-40 years old contact lens wearer and has a unilateral genotype T4 *A canthamoeba* infection. The most important risk factor for AK in Austria is poor CL hygiene, contact lens cases often being heavily contaminated not only with amoebae and bacteria, but also with fungi, algae and nematodes. However, during the years we also observed cases in individuals below 10 and above 80 years old and also several cases in non-contact lens wearers. Non-contact lens wearers were typically very young or very old patients and usually either had an underlying eye disease or had experienced an injury of the cornea. Moreover, there were several complicated cases including cases needing multiple surgeries. Many cases had prolonged progressions, some patients being unable to work for several months. During the past years we also saw a significant increase of AK in the male population, in the year 2014, for the first time, we had more AK cases in males than in females. The three GAE cases diagnosed at our institution occurred in young male patients who were either severely immunocompromised or had other underlying predisposing conditions and involved genotypes T2, T4 and T5.

Aichelburg A, Walochnik J, Assadian O, Prosch H, Steuer A, Perneczky G, Visvesvara G, Aspock H, Vetter N. 2008. Successful treatment of disseminated *A canthamoeba* infection with miltefosine. Emerg Infect Dis 14:1743–1746.

Huber-Spitzy V, Grabner G, ArockerMettinger E, Baumgartner I, Skorpik F, Rappersberger C, Haddad R. 1989. *A canthamoeba* keratitis. An underdiagnosed entity? Klin Mbl Augenheilkd 194:454–457.

Or-2

Fatal disseminated *Acanthamoeba* infection in a lung transplant recipient from France

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Objective:
Infectious complications are responsible for significant morbidity and mortality in lung transplant recipients, but *Acanthamoeba* infections have rarely been reported in this setting [1]. We report here a new case of disseminated acanthamoebiasis in a woman with a history of lung transplantation.
Case report (Methods/Results):
A 25-year-old woman received bilateral lung transplantation in 2012 for cystic fibrosis, chronic renal insufficiency, diabetes mellitus, exocrine pancreatic insufficiency, and chronic sinusitis. She presented in July 2014 fever, acute sinusitis, a nodular lesion on the hard palate, and several nodules of the inferior limbs. Despite initiation of broad-spectrum antibiotics and intravenous liposomal amphotericin B, cutaneous nodules continued to spread, becoming inflammatory and necrotic; the oral lesion became necrotic and perforation of the hard palate occurred. The patient's condition deteriorated and resulted in death in September 2014.
Multiple skin biopsies were performed, revealing inflammatory reaction and necrotic tissues, with no bacterial or fungal agents. A biopsy of the lesion of the palate revealed ulcerated and necrotic tissues, with presence of protozoan parasites. Post-mortem, PCR conducted on a skin biopsy confirmed the diagnosis of disseminated *Acanthamoeba* infection.
Conclusions:
Early and sensitive diagnosis and on time treatment may prevent the poor outcome of *Acanthamoeba* infections. In consequence, a better and wider knowledge of these infections by the physicians, the clinical and surgical pathologists and the parasitologists will lead to an early diagnosis and on time treatment, improving the prognosis. Here, the cutaneous manifestations could have been the clues to the infection.

Reference:
[1] J.D. Christie, L.B. Edwards, P. Aurora P et al. J Heart Lung Transplant. Vol. 28, 2009, 1031

[2] G.S. Visvesvara. Curr Opin Infect Dis. Vol. 23, 2010, 590

Or-3

Isolation and genotyping of *Acanthamoeba* strains from corneal infections in Tuscany and Umbria (Italy)

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Objectives: *Acanthamoeba* spp. are free-living amoebae able to cause serious human diseases, including a painful and blinding infection of the cornea, the amoebic keratitis (AK), usually associated with contact lens wear. To date, the genus includes pathogenic and non-pathogenic genotypes (T1-T20) based on sequence analysis of the *18S* rRNA gene. This study reports 6 cases of AK diagnosed in Tuscany and Umbria (Italy).

Methods: Six corneal scrapings from symptomatic patients with suspected AK were collected at two Hospitals of Central Italy. The detection protocols included DNA extraction carried out using QIAamp DNA Micro Kit (Qiagen) followed by a real time PCR assay to detect *Acanthamoeba* spp. using the *Acanthamoeba* spp. genesig Easy Kit (Primer design Ltd). Real time negative samples were also cultivated onto Non Nutrient-agar. To assign genotypes, all positive samples were then subjected to conventional PCR targeting the *18S* rRNA gene by specific primers JDP1 and JDP2 and sequences were compared with those available in GenBankTM through a phylogenetic analysis performed by MEGA version 5.

Results: Three out of 6 samples resulted positive to real time PCR assay, while all the other samples resulted positive after cultivation. Five out of the six isolates were assigned to the genotype T4; one isolate was assigned to the genotype T3.

Conclusions: The study contributed to improve the understanding on the epidemiology of *Acanthamoeba* in Italian patients. The molecular characterization of AK was previously reported for 15 isolates from Northern and 69 isolates from Central and Southern Italy where the genotypes T4, T15, T3 and T11 were identified [1, 2, 3]. The molecular analysis conducted in the present study confirms the spread of the genotypes T3 and T4 associated with AK in Italy.

Reference:

[1] D. Di Cave, R. Monno, P. Bottalico, S. Guerriero, S. D'Amelio, C. D'Orazi, F. Berrilli. Eur. J. Clin. Microbiol. Infect. Dis. 28, 2009, 607.
[2] S. Gatti, P. Rama, S. Matuska, F. Berrilli, A. Cavallero, S. Carletti, A. Bruno, R. Maserati, D. Di Cave. J. Med. Microbiol. 59, 2010, 1324.
[3] D. Di Cave, R. D' Alfonso, K.A. Dussey Comlavi, C. D'Orazi, R. Monno, F. Berrilli. Exp. Parasitol. 2014, doi:10.1016/j.exppara.2014.05.009.

Or-4

Contributions from the Amoebiasis Laboratory of the Universidad Central de Venezuela, to the knowledge of Free-Living amoebae in Venezuela

Marìa Virginia. Pérez de Galindo[a], Mònica Galindo[a], Angelyseb Dorta[a], Carmen Guzmán de Rondòn[a], Carolina. Wagner[a], Marìa Alejandra Vethencourt[a], Anaibeth Nessi[a], Amada Bermúdez[b], Eva Pérez de Suárez[a]

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It has been demonstrated the presence of free-living amoebae (FLA) in all habitat where humans have access, and can also be parasites causing diseases. *Naegleria fowleri* produces Primary Amoebic Meningoencephalitis in immunocompetent individuals; several species of genus *Acanthamoeba* may cause Granulomatous Amoebic Encephalitis in immunosuppressed patients, meanwhile it can generate corneal ulcers, skin lesions and other organs affections in immunocompetents. Encephalitis and skin lesions can occur in immunocompetents infected with *Balamuthia mandrillaris* and *Sappinia pedata* was reported in an encephalitis case. In Venezuela, diseases caused by FLA have been reported and Amoebiasis Laboratory of the Universidad Central de Venezuela has designed a systematic protocol for samples taken from brain disorders, eye lesions, diarrhea, rhinitis, otitis, contact lenses, liquids used for care and water samples. We have identified the genera *Acanthamoeba*, *Naegleria* and *Valkhamphia*, in 47 of 574 samples by direct examination, flagellation test and growth in a biphasic culture medium, which has allowed its morphological and biological study and reached to genus identification, being sufficient to establish treatment. Recently, it was standardized the Polymerase Chain Reaction (PCR) for the investigation of *N. fowleri*, *Naegleria* sp and *Acanthamoeba* sp, which amplify part of the gene encoding the small ribosomal subunit 18S, using the primers referred by Qvarnstrom *et al,* 2006, Schild *et al,* 2006 and Kong and Chung, 1996, which originate amplified products of 153 pb, 186 pb and 910-930 pb, respectively. Tests were conducted to measure sensitivity of each PCR: 5 cells/ ml for *N. fowleri*, 50 cells/ml for *Naegleria* sp and 5.000 cells/ml for *Acanthamoeba* sp. A basic protocol for early diagnosis of these amoebae was set up, which we recommend to be used in diagnostic and research laboratories. It is important that ophthalmologists, neurologists, infectologist and parasitologists should be aware of FLA as possible etiologic agents of diversal pathologies and Laboratory Services to be prepared for its early identification.

Or-5

Primary amoebic meningoencephalitis caused by *Naegleria fowleri*: an old enemy presenting new challenges

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Naegleria fowleri is a protist pathogen, known to infect the central nervous system and produce primary amoebic meningoencephalitis. The most distressing aspect is that the fatality rate has remained more than 95%, despite our advances in antimicrobial chemotherapy and supportive care. Although rare worldwide, most cases have been reported in the United States, Australia and Europe (France). A large number of cases in developing countries go unnoticed. In particular, religious, recreational, and cultural practices such as ritual ablution and/or purifications, Ayurveda, and the use of neti pots for nasal irrigation can contribute to this devastating infection. With increasing water scarcity and public reliance on water storage, here we debate the need for increased awareness of primary amoebic meningoencephalitis and the associated risk factors, particularly in developing countries.

Or-6

First molecular characterization of clinical *Acanthamoeba* strains at the genotype level in Venezuela

***Carolina Wagner*[a,b] , María Reyes-Batlle[a], María Vethencourt[b], Mónica Galindo[b], Anaibeth Nessi[b], Angelyseb Dorta[b], Carmen M[a] Martín-Navarro[a], Atteneri López-Arencibia[a], Alexis Dorta-Gorrín[a], Basilio Valladares[a], Enrique Martínez-Carretero[a], José E. Piñero, Carmen Guzmán de Rondón[b]., Maria Pérez de Galindo[b], Jacob Lorenzo-Morales[a]**

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[b]Universidad Central de Venezuela, Caracas, Venezuela

Pathogenic strains of *Acanthamoeba* are causative agents of a sight-threating infection of the cornea known as *Acanthamoeba* keratitis (AK) which is often associated with the misuse of contact lenses. *Acanthamoeba* strains are also causative agents of lethal encephalitis, known as Granulomatous Amoebic Encephalitis (GAE).

In this study, 5 clinical isolates of *Acanthamoeba* previously isolated in the Amoebiasis Laboratory of the Universidad Central de Venezuela, were characterized at the genotype level. For this purpose, the diagnostic fragment 3 (DF3) of *Acanthamoeba* 18S rDNA gene was amplified and sequenced. The obtained sequences were aligned and homology and phylogenetic analyses revealed that all the tested isolates belonged to genotype T4.

Up to date, this is the first molecular characterization of *Acanthamoeba* strains at the genotype level in Venezuela and the first report of genotype T4 in this country.

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ORAL SESSION 2

Chairs

Melissa Jamerson

and

Julia Walochnik

Or-7

First report of *Naegleria fowleri* in Costa Rica

Lissette Retana-Moreira [a,b], ***Elizabeth Abrahams-Sandí*** [a,b], ***Alfredo Castro-Castillo, María Reyes-Batlle*** [c], ***Jacob Lorenzo-Morales*** [c]

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The first isolation of *Naegleria fowleri* from hot springs is reported. This isolation is associated to a fatal PAM case occurred on july 2014, in an 11-year old child from Florida who visited a resort in La Fortuna de San Carlos, Costa Rica. Water samples were collected from different sources of the resort visited by the child, filtered and filters were the placed onto 1.5% non-nutrient agar supplemented with *Escherichia coli.* After 3-4 days of incubation, trophozoites and cysts with morphological characteristics similar to *Naegleria* were observed. Thermotolerance and exflagelation assays were performed, with positive results. Samples were sent to Universidad de La Laguna for molecular confirmation of species. A specific PCR that amplifies the 18S rDNA gene of *N. fowleri* was performed, as well as sequencing of the PCR products. This analysis revealed 97-98% homology with other *N. fowleri* strains available in GenBank.

Funded by: This study was supported by project 803B4050, Vicerrectoría de Investigación, University of Costa Rica. J.L.M. was supported by the Ramón y Cajal Subprogramme of the Spanish Ministry of Economy and Competitivity RYC201108863. MRB was supported by Obra Social La Caixa Fundación Cajacanarias 2014.

Or-8

Assessing the presence of *Acanthamoeba* in ocular surface in non-keratitis patients using the Schirmer strip test

***María Reyes-Batlle*[a], Pedro Rocha-Cabrera[a,b] , Carmen Mª Martín-Navarro[a], Alexis Dorta-Gorrín[a], Atteneri López-Arencibia[a], Ines Sifaoui[a], Javier Rodríguez Martín[b], Enrique Martínez-Carretero[a], Basilio Valladares[a], Fernando Martín-Barrera[b], José E. Piñero[a], Jacob Lorenzo-Morales[a].**

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[b]*Department of Ophthalmology, Hospital Universitario de Canarias, Tenerife, Spain.*

Pathogenic strains of *Acanthamoeba* are causative agents of a sight-threating infection of the cornea known as *Acanthamoeba* keratitis (AK) which is often associated with the misuse of contact lenses. However, there is a still a question remaining to be answered which is whether these microorganisms are present in the ocular surface of healthy individuals. Therefore, the aim of this study was to determine the presence of Acanthamoeba in the in ocular surface in healthy patients and also in those with other ocular surface infections. Sterile Schirmer strip tests were collected from a group of individuals attending a local ophthalmology consultation.

The collected samples were cultured in 2% Non-Nutrient Agar (NNA) plates and positive plates were then cultured in axenic conditions for further analyses. Molecular analysis classified all isolated strains belonged to *Acanthamoeba* genotype T4 and osmotolerance and thermotolerance assays revealed that all strains were potentially pathogenic. Furthermore, all strains were assayed for sensitivity against voriconazole and chlorhexidine. Assays showed that both drugs were active against the tested strains. In conclusion, the Schirmer strip test is proposed as an effective tool for the detection of *Acanthamoeba* in ocular surface.

Funded by grants: RICET/ RD12/0018/0012, Project PI13/00490 , Project ref. AGUA3 ALA and MRB were funded by Becas de Investigación Obra Social La Caixa-Fundación Cajacanarias para Postgraduados 2014". JLM was supported by the Ramón y Cajal Subprogramme RYC-2011-08863.

Or-9

Identification and typing of *Acanthamoeba* spp. by MALDI-TOF MS.

Cristel Accardi[a], Maristella Santoro[b], Federica Del Chierico[a], Lorenza Putignani[b], Federica Berrilli[c], Rossella D'Alfonso[d], David Di Cave[c,e]

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Objectives: The present study reports on the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) profiling for the identification and typing of *Acanthamoeba* strains compared to PCR-based genotyping assay.

Methods: Thirteen *Acanthamoeba* strains were collected at the Laboratory of Parasitology, Foundation Polyclinic Tor Vergata from clinical samples and cultured onto Non Nutrient-Agar containing *Escherichia coli* for 15 days at 37°. Each strain was genotypied by conventional PCR assay for the amplification of the ASA.S1 region of *18S* rRNA gene using the genus specific primers JDP1 and JDP2.

For the MALDI-ToF-MS based assay, cysts were mixed thoroughly and added by absolute ethanol. Recovered pellets were air dried after centrifugation and mixed with 70% formic acid (Sigma-Aldrich , Milan , Italy) and with the same volume of acetonitrile (Sigma-Aldrich). One µl was placed on a polished steel target MSP 96 (Bruker Daltonik GmbH, Bremen, Germany) and dried at RT. Each sample was overlaid with 1 µl of the α-Cyano-4-hydroxycinnamic acid. The matrix-sample was co-crystallized drying at RT. Twenty-four spectral measurements/sample (500 laser shot) were performed with a Microflex LT mass spectrometer (Bruker Daltonics), using FlexControl software (version 3.0, Bruker Daltonics). The entire set of the 240 spectra replicas was analyzed by MALDI biotyper 3.0 for clustering and PCA analyses.

Results: All spectra replicates of each samples were employed for the creation of a mean spectra (MSP), by standard MSP creation method (FlexAnalysis software). Final MSP spectra were assigned to the correct taxon node of the taxonomy tree into the MALDI-TOF MS library and used to perform analytical ID of *Acanthamoeba* isolates (MALDI Biotyper 2.0, Bruker Daltonics). All the strains were correctly identified with a score comprised from 1.7 to 2.0.

In MSP spectra dendrogram we can observe that the strains with the same genotype clusters are separated: the T3 and T15 genotypes appear to be similar, while the T4 genotype seems to be more variable.

Conclusions: The MALDI-TOF MS-based analysis for the identification and typing of *Acanthamoeba* represents a substantial improvement in diagnostic parasitology. The strenght of this method is, indeed, to overcome PCR-based genotyping, allowing peptide fingerprinting-based typing.

Or-10

Epidemiology of Amoebic Keratitis in Northern Sardinia, Italy, 2008 to 2014

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Acanthamoeba keratitis is a rare but sight-threatening corneal infection occurring in otherwise healthy immuno-competent individuals. The main risk factor is poor contact lens hygiene or ineffective contact lens disinfection. Its incidence in the US has been estimated at 1-2 cases/1,000,000 contact lens users. Recent evidence suggests that *Acanthamoeba* is not a unique cause of amebic keratitis, as this condition can also be caused by other free living amoebae, such as *Hartmannella* and *Vahlkampfia*. The different amoebic genera share indistinguishable clinical corneal findings. As amoebic keratitis often mimic several other types of keratitis, including viral, bacterial, or fungal keratitis, laboratory investigation is essential to make the correct diagnosis. Aggressive treatment with polyhexamethylene biguanide (PHMB) 0.02% and/or chlorhexidine 0.02% eye-drops is successful, as long as the diagnosis is made early.

Although amoebic keratitis is rare, this condition appears to be relatively more common in Northern Sardinia, Italy. In this presentation, data about the cases of amoebic keratitis diagnosed at the University of Sassari, Sassari, Italy, during the last 7 years are discussed.

ORAL SESSION 3

Chairs

Dennis Kyle

and

Naveed A. Khan

Or-11

Development of High-Throughput Screens used to Discover Potential Treatments for *Naegleria fowleri*

Christopher A. Rice[a], Beatrice L. Colon[b], Danielle Demers[c], Bill J. Baker[c], Jim Leahy[c], Roman Manetsch[d], Cedric Pearce[e], Marc A. Giulianotti[f], David W. Boykin[g] & Dennis E. Kyle[a].

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Naegleria fowleri causes a deadly brain disease called primary amoebic meningoencephalitis. Since the first documented infection in 1965, 142 cases have been reported in the US with only 3 successfully treated survivors. *Naegleria* infections have been linked to warm fresh water, poorly chlorinated swimming pools and nasal irrigation using Neti pots. The major problem for infections with *Naegleria* is the lack of effective and quickly acting therapeutics. The aim of this study was to develop and validate high throughput *in vitro* screening methods against *N. fowleri* and use these methods to screen experimental compounds to discover active compounds that can be pursued as additive(s) to the current treatment regimen. Drug susceptibility assays were performed using axenic *Naegleria fowleri* (ATCC 30215). The alamarBlue colorimetric microtiter plate assay was adapted from McBride *et al*.,[1]. The second CellTiter-Glo 2.0 luminescent cell viability plate assay was adapted from Debnath *et al*.,[2]. All compounds were screened from 24 ng/ml to 50 μg/ml. We report the development of two *in vitro* drug susceptibility assays for *N. fowleri* established at 72 hrs, since we sought hits that act rapidly on the amoebae. Optimal seeding densities were standardised at 100,000 cells/well and 4,000 cells/well in 96-well plate formats for alamarBlue and CellTiter-Glo, respectively and 3,000 cells/well for both 384-well plate assay type [3]. Both assays provided similar quantitative dose response data. Using these methods we screened and validated over 15,000 synthetic and natural products using both methods, identifying multiple compounds for future lead optimisation. Synthetic libraries combined show 33% inhibition rate. Natural product libraries combined show 2.5% hit rate screened at 5 μg/ml. All hits from these and future studies will be followed up to elucidate IC50 data against *Naegleria fowleri* and cytotoxicity on J774 murine macrophage cell line. We developed and validated two methods for HTS drug susceptibility assays against *N. fowleri*. Several of these experimental compounds look promising by inhibiting growth and development of *N. fowleri*. Although the mechanisms of action are unknown, structure-activity relationship studies will enable us to elucidate compounds that are blood brain barrier permeable, selective, and rapidly inhibit *N. fowleri*. This research was supported by a grant from the National Institute of Allergy and Infectious Diseases (R21AI103664).

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Or-12

High Throughput Screen Identifies Bis-Benzimidazole Hits Against *Naegleria fowleri*

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Abstract: Primary amoebic meningoencephalitis (PAM) is a neurodegenerative disease caused by the pathogenic free-living amoeba, *Naegleria fowleri*. PAM is rare, yet a uniformly fatal disease that culminates 5-12 days after symptom onset. The high fatality rates (97%) may be due to a variety of factors including the acute pathology induced, the lack of early diagnosis, and less than optimal therapeutics. Given the lack of robust drug discovery for PAM, our primary objective was to develop medium to high throughput assays and use them for structure activity relationship studies to identify new chemotypes with an IC_{50} <1 μM and selectivity index >10. Drug susceptibility assays used axenic logarithmic phase *N. fowleri* (ATCC 30215) trophozoites. We developed high throughput screening methods adapted from the alamarBlue and CellTiter-Glo assays that were previously described for *Acanthamoeba* and *N. gruberi*, respectively [1, 2]. Results from both *in vitro* assays showed similar IC_{50} values for all compounds tested, thus validating our methods. Cytotoxicity was tested against J774 murine macrophages. We screened more than 225 compounds, representing 6 structural classes of amidino compounds and identified two classes with nanomolar potency [3]. We continued lead optimization studies and have identified >10 compounds with low nanomolar potency and high selectivity index (> 10). The most active amidino analogs are >5000 fold more effective than pentamidine. The compounds identified with these assays are the first to be described with nanomolar potency against *N. fowleri*. Current PAM treatments have originally been developed for other pathogens and no therapies have derived from novel drug discovery programs for *N. fowleri*. With the development of these high throughput screening methods, we now have the ability to screen large libraries to identify additional novel chemotypes to accelerate drug discovery for pathogenic free-living amoebae. This study was supported by a grant from the National Institute of Allergy and Infectious Diseases (R21AI103664).

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[2]. Debnath, A., Tunac, J. B., Galindo-Gomez, S., Silva-Olivares, A., Shibayama, M., & McKerrow, J. H. (2012). Corifungin, a new drug lead against *Naegleria*, identified from a high-throughput screen. *Antimicrob Agents Chemother,* 56(11), 5450.

[3]. Rice, C. A., Colon, B. L., Alp, M., Goker, H., Boykin, D. W., & Kyle, D. E. (2015). Bis-Benzimidazole Hits against *Naegleria fowleri* Discovered with New High-Throughput Screens. *Antimicrob Agents Chemother,* 59(4), 2037.

Or-13

Activity assessment of three aromatic Tunisian plants against the trophozoite stage of Acanthamoeba

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Medicinal and Aromatic Plants (MAP) have been used since prehistoric times in folk medicine, and they still at the present the most important health care source for several countries around the world [1]. Extracts and essentials oils, obtained from those plants have been widely used in the pharmaceutical, foods and cosmetic industries. Recently, Investigations on the evaluation of the biological activities some MAP species have confirmed the interesting activities that some of them exhibited such as antimicrobial [2],antiprotozoal [3], antitumoral [4]. Several studies focused the extraction of high-added value compounds from MAP, although, a few papers have been focus on their amoebicidal activity. In the present communication, extracts and essentials oils from three different plants namely the *Rosmarinus officinalis*, *Thymus capitatus* and *Artemisia herba alba* were screened for their activity against *Acanthamoeba castellanii* Neff. The IC_{50}/96 h was chosen as the appropriate and comparable data. The parasite have been inhibit by the hexane extracts and the essentials oils with an IC_{50} ranged from 2.73 ±0.58 µg/ml for the essential oil of the thyme to 60.44 ± 9.3 µg/ml for the hexane extract of the rosemary. The essential oils of the three plants were more active than the hexane extracts. Bio-guided fractionation of the hexane extract of the thyme was conducted and led to the identification of three compounds namely the Thymol, the 2,3-dihydroxy-p-cymene and the taxifolin. The MAP evaluated in this study could be used for the development of novel therapeutic approaches against *Acanthamoeba* infections. Meanwhile, further studied to investigate the mode of action of this compound against the parasite tested as well as the synergy effects between them are required.

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Or-14

Evaluation of the *in vitro* activity of voriconazole and chlorhexidine against clinical strains of *Acanthamoeba* cases in Mexico

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Acanthamoeba is an opportunistic pathogen which is the causal agent of a sight-threatening ulceration of the cornea known as Acanthamoeba keratitis (AK) and, more rarely, an infection of the central nervous system called "granulomatous amoebic encephalitis" (GAE). The symptoms of AK are non-specific, and so it can be misdiagnosed as a viral, bacterial, or fungal keratitis. Furthermore, current therapeutic measures against AK are arduous, and show limited efficacy against the cyst stage of *Acanthamoeba*.

In the present study, five clinical strains of *Acanthamoeba* isolated from AK cases in contact lens wearers of Mexico were assayed for their susceptibility to two active compounds currently used in AK treatment, The sensitivity of the strains to voriconazole and chlorhexidine was evaluated using a colorimetric assay base on Alamar Blue.

Both compounds were active against the *Acanthamoeba* strains tested in this study. However, voriconazole showed to be more active than chlorhexidine against all tested strains.

In conclusion, voriconazole could be used against AK as a first-line treatment against AK cases in Mexico.

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Or-15

Formulative strategies for improving the antiamoebic activity of rokitamycin

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Rokitamycin (RK) is a semisynthetic macrolide of the leucomycin group, which is actually studied as an alternative drug to the analogous macrolide antibiotics. It is strongly inhibitory, *in vitro*, for *Acanthamoeba* castellanii [1], which are the causative agents of granulomatous amoebic encephalitis (GAE) and amoebic keratitis. Due to its low water solubility, the use of pure drug is limited. To hypothesize its use in the therapeutic treatment of *Acanthamoeba* infections, formulative studies were needed. Formulations could be useful both in the case of ocular application to treat amoebic keratitis and in nasal administration as an alternative route for the drug administration to the brain in GAE therapy.

With this aim, primarily, mucoadhesive microspheres based on chitosan, its salt and derivatives, were designed as carriers to obtain a controlled release of RK, able to improve the antiamoebic activity of this drug and with characteristics suitable for ocular and nasal administration [2,3]. Afterwards, new o/w ophthalmic emulsions were developed to control RK local release and improve ocular drug bioavailability.

Microspheres containing RK were prepared by spray-drying and *in vitro* characterized. *In vivo* RK absorption, after nasal administration, was evaluated [4]. Emulsions were prepared by high-shear homogenization methods; dimensional, morphological and rheological characterizations were performed. *In vitro* microbiological studies were carried out to verify the effect of formulations on *Acanthamoeba* castellanii.

Compared with the free drug, the loading of RK in chitosan microspheres improves and prolongs the *in vitro* antiamoebic activity of RK. *In vivo* studies showed that, after IV administration, RK is not able to cross the blood–brain barrier and to reach the CSF from the bloodstream. On the contrary, drug goes to the CSF and the bloodstream only after nasal administration of microparticles.

The leader o/w emulsions have mean diameter of about 6-7 μm which remains stable along 4 months. Formulations show typical physical-chemical properties of liquid ophthalmic dosage form. Emulsions decrease growth of *Acanthamoeba* with regard to dose and time. Particularly formulation included both substances resulted the most efficient.

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Or-16

First clinical data of *Acanthamoeba* keratitis treatment using statins

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Present therapeutic measures for *Acanthamoeba* keratitis rely on topical applications of antimicrobials, including the combination of propamidine isothionate and neomycin or chlorhexidine. Moreover, the length of these treatments makes the process arduous. Furthermore, as current treatments are poorly effective against the cyst form of these amoebae, residual infection often remains even after treatment.

Chlorhexidine and polyhexamethylene biguanide (PHMB) as monotherapy agents have been proven not to be sufficient against clinical or environmental strains of acanthamoebae, hence the importance of multiple-strain testing of drugs against *Acanthamoeba*, as their effectiveness might depend on the *Acanthamoeba* isolate. In a previous study, the *in vitro* activity of statins such as atorvastatin, simvastatin and fluvastatin was shown to be very high against both trophozoites and cysts of clinical strains of *Acanthamoeba,* causing low toxicity when tested in eukaryotic cell lines.

Therefore, in the present work and under informed consent, 4 patients suffering from *Acanthamoeba* keratitis that were admitted to the Cornea Unit of Hospital Ramón y Cajal in Madrid, Spain started a treatment combination that included atorvastatin, chlorhexidine and voriconazole. All patients reached remission of *Acanthamoeba* keratitis after 6 weeks of treatment.

To the best of our knowledge, this is the first report on the clinical application of statins (atorvastin in our case) for the treatment of patients suffering from *Acanthamoeba* keratitis and also the first report of total recovery of the patients treated with the combination of drugs mentioned above which included atorvastatin.

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Or-17

Photochemotherapeutic strategy against *Acanthamoeba* infections

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Acanthamoeba is a protist pathogen that can cause serious human infections including a blinding keratitis and a granulomatous amoebic encephalitis that almost always results in death. Current treatment includes a mixture of drugs and even then infection recurrence can occur. Photochemotherapy has shown promise in the treatment of *Acanthamoeba* infections, however selective targeting of pathogenic *Acanthamoeba* has remained a major concern. The mannose-binding protein is an important adhesin expressed on the surface membranes of pathogenic *Acanthamoeba*. To specifically target *Acanthamoeba*, the overall aim of this study was to synthesize photosensitising compound (porphyrin)-conjugated with mannose and test its efficacy *in vitro*. The synthesis of mannose-conjugated porphyrin was achieved by mixing benzaldehyde and pyrrole yielding tetra-phenyl porphyrin. Tetra-phenyl porphyrin was then converted into mono-nitro phenyl porphyrin by selectively nitrating the para position of phenyl rings as confirmed by NMR spectroscopy. The mono-nitro phenyl porphyrin was reduced to mono-amino phenyl porphyrin in the presence of tin dichloride and confirmed by peak at 629 m/z. Finally, mono-amino porphyrin was conjugated with mannose resulting in the formation of imine bond. Mannose-conjugated porphyrin was confirmed through spectroscopic analysis and showed that it absorbed light of wavelength ranging from 425-475nm. To determine antiacanthamoebic effects of the derived product, amoebae were incubated with mannose-conjugated porphyrin for 1 h, and washed 3X to remove extracellular compound. Next, amoebae were exposed to light of the appropriate wavelength for 1 h. The results revealed that mannose-conjugated porphyrin produced potent trophicidal effects and blocked excystation. In contrast, *A. castellanii* incubated with mannose alone and porphyrin alone did not exhibit anti-amoebic effect. Consistently, pre-treatment with mannose-conjugated porphyrin reduced *A. castellanii*-mediated host cell cytotoxicity from 97% to 4.9%. In contrast, treatment with porphyrin, mannose or solvent alone had no protective effects on host cells. These data suggest that mannose-conjugated porphyrin has application for the targeted photodynamic therapy of *Acanthamoeba* infections and may serve as a model in the rationale development of therapeutic interventions against other eukaryotic infections.

ORAL SESSION 4

Chairs

Fiona L. Henriquez

and

Craig W. Roberts

Or-18

Acanthamoeba castellanii induces macrophage pro-inflammatory and wound healing phenotypes

Antonella Cano[a], Fiona L. Henriquez[b], Antonella Mattana[c], James Alexander[a], Craig W. Roberts[a]

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Acanthamoeba castellanii is normally free-living and ubiquitous in nature, with a worldwide distribution. As facultative parasites they can cause a painful, sight threatening keratitis most frequently affecting contact lens wearers. *Acanthamoeba* can also be an opportunistic pathogen causing fatal granulomatous encephalitis in immune-compromised individuals. Dissecting the immunology of *Acanthamoeba* infections has been considered problematic due to the very low incidence of disease. Herein, the effect of *Acanthamoeba* trophozoites on the activation of resting macrophages was investigated. Towards this purpose bone marrow derived macrophages were co-cultured with either a laboratory strain of *A. castellanii*, named Neff, or *A. castellanii* isolated from a case of bilateral keratitis in Italy. *Acanthamoeba* was found to induce a pro-inflammatory macrophage phenotype following exposure to Neff strain, as measured by significant production of TNF-α, IL-12 and IL-6 from macrophages. In comparison the clinical isolate induced IL-12 and IL-6 to a significantly lesser degree than the Neff strain, but did not induce TNF-α. The utilization of macrophages derived from MyD88, TRIF, TLR2, TLR4, TLR2/4, and PAR_2 deficient mice along with a PAR_1 specific antagonist indicated that *Acanthamoeba*-induced pro-inflammatory cytokine production was through MyD88-dependent, TLR4-induced events, with a further contribution from PAR_1. Consistent with this latter observation, protease inhibitors significantly diminished IL-12 and IL-6 production by macrophages after challenge with *Acanthamoeba* trophozoites. *Acanthamoeba* trophozoites were also found to induce arginase activity in macrophages, a phenomenon normally associated with tissue-repair/wound healing or immune evasion mechanisms. Conversely, nitric oxide (NO), produced by the enzymatic activity of iNOS on L-arginine and associated with antimicrobial mechanisms was not detected in macrophages co-incubated with *Acanthamoeba*. Furthermore, *Acanthamoeba* trophozoites ablated macrophage NO production in response to Lipopolysaccharide (LPS). Overall our studies provide insights into the mechanisms by which *Acanthamoeba* induce and manipulate the macrophage activation state. These findings provide an improved understanding of the interaction of the immune system with *Acanthamoeba* and could have practical applications for the development of pharmaceuticals and treatments for *Acanthamoeba* infections.

Or-19

Acanthamoeba castellanii (Genotype T4) Stimulates the Production of Pro- and Anti-Inflammatory Cytokines in Human Monocytic Cell Line THP-1 and in Human Peripheral Blood Mononuclear Cells.

Antonella Mattana[1*], Manuela Sanna[1], Antonella Cano[2], Giuseppe Delogu[1], Giuseppe Erre[1], Craig W. Roberts[2], Fiona L. Henriquez[3], Pier Luigi Fiori[1] and Piero Cappuccinelli[1]

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Several species of *Acanthamoeba* can infect humans producing severe and sometimes fatal diseases. Human infections are generally site specific: corneal and neural tissues are the primary targets, although other tissues can be affected. The initial innate immune response and the formation of granulomas in the brain and other tissues are presumed to be essential events for impeding the invasion and dissemination of *Acanthamoeba* trophozoites. Cells of the monocyte/macrophage lineage orchestrate the immune response recognising pathogen associated molecular patterns (PAMPs) through membrane-associated molecules, including Toll Like Receptors (TLRs). This induces signalling events resulting in the synthesis and secretion of soluble cytokines and antimicrobial molecules.

The aim of this study is to understand the role that human monocytes/macrophages play during the early phase of *Acanthamoeba* infection in humans.

Therefore, the human myelomonocytic cell line THP-1, primary human monocytes isolated from peripheral blood and human monocyte-derived macrophages (MDMs) were stimulated with either trophozoites of a clinical isolate of *A. castellanii*, genotype T4, or with amoeba-derived cell free conditioned medium (cRPMI). The production of TNF-α, IL-6, IL-12, IL-10 and IL-8 was evaluated after 1.5, 3, 4, 5, 6, 18 and 23 hours post-stimulation. Both *Acanthamoeba* trophozoites and the cRPMI induced the production of TNF-α, IL-12 and IL-8, in all three cellular models and differences in the intensity and the kinetics of release could be observed. In contrast, IL-6 was only induced by cRPMI. THP-1 cells, monocytes and macrophages released IL-10 from 1.5 hours following stimulation with cRPMI, but not trophozoites.

These results suggest that the production of pro-inflammatory cytokines and chemokines by monocytes and macrophages plays a role in the inflammatory response during *Acanthamoeba* infections. In addition, it is demonstrated for the first time, that *Acanthamoeba* stimulates the production of IL-10 in these innate immune cells. As an anti-inflammatory, immune-suppressive cytokine, IL-10 might play a role in both promoting the immune evasion of *Acanthamoeba* and limiting the induced inflammatory response.

Study supported by the Regione Sardegna grant (CPR-59632, LR72012MATTANA).

ORAL SESSION 5

Chairs

Thelma Dunnebacke

and

Albrecht Kiderlen

Or-20

Granulomatous Amoebic Encephalitis: Ghost response of Immunocompromized Hosts?

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Karachi, Pakistan

Objective:
Naegleria fowleri, Acanthamoeba and Balamuthia mandrillaris spp., are known to cause fatal amoebic meningoencephalitis. Here, the author attempts to draw attention to these cases, which are reported as 'Granulomatous Amoebic Encephalitis' (GAE), and their occurrence in immunocompromised individuals and patients with AIDS [1, 2, 3,]. Newer contributions towards our understanding of immune responses resulting in granulomatous inflammatory lesions have led to a considerable increase in the number of studies that have reported the occurrence of GAE in a number of protist infections.

Methods:
Extensive reviews of manuscripts published over a period of 50 years on this topic and cytokine studies and/or morphological evidence provided in peer-reviewed published studies were analysed in detail by independent resources to evaluate the granulomatous inflammatory evidence provided to justify the title of GAE in this group of patients.

Results:
The evidence given in support of GAE did not appear to be convincing enough in the majority of published studies, and in particular its occurrence in patients with AIDS and other immunocompromised states was not justified.The distinction between the early development of type IV hypersensitivity (HSR) reactions and granuloma/granulomatous inflammation was found to be vague. The formation of a perivascular cuff by inflammatory cells occurs within 24–72 h in delayed type IV HSR and such a lesion presents as perivascular cellular cuffing at or around the lesion [4], while granulomatous inflammation tends to develop within 2–3 weeks; and both require a strong T-cell response to their development GAE [5], like any other granulomatous inflammation, can occur only in the presence of ample numbers of CD4+ T-lymphocytes.

Conclusions:
As our understanding of the immunological response to protist infections has grown, so too has the apprehension that some aspects of its true pathogenesis and morphology might be overlooked if use of the term GAE does persist for immunocompromised states such as patients with AIDS. It is therefore recommended that this terminology is used only when all the diagnostic criteria have been met, and use of a term such as 'granulomatoid' is suggested in cases where there remains an ambiguity in the diagnosis of similar lesions, especially in AIDS and related diseases.

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Or-21

Characterization of the experimental Primary Amoebic Meningoencephalitis produced by *Naegleria fowleri* in the rat model

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Naegleria fowleri is a free-living amoeba with a wide distribution in nature. This amoeba cause primary amoebic meningoencephalitis (PAM). The PAM occurs in healthy patients with a recent history of swimming activities. *Naegleria* enters by the nasal route through the olfactory neuroepithelium and migrating by the olfactory nerves until reach the olfactory bulbs in the central nervous system (CNS). Presently it is not known how *N. fowleri* interacts with the cerebral endothelium and activates it. In our laboratory we established the experimental PAM in a rat model with the aim to study the blood brain barrier (BBB). The main objective of the present work was to characterize the invasion process of *N. fowleri* from the olfactory neuroepithelium to the olfactory bulbs at different times post-intranasal inoculation; we evaluated IL-1β, IL-6 and TNF-α using histological and immunohistochemistry techniques. The results showed that infection at 7 days post-inoculation presented a high number of inflammatory cells such as neutrophils surrounding the trophozoites; also we can observed the production of these cytokines by the endothelial cells. We established a primary culture of brain endothelial cells and we analyzed the tight junction proteins such as claudin-5 and ZO-1. The results showed an increment in the phosphorylation of claudin-5 at 12 and 24 h post-inoculation. We conclude that *Naegleria fowleri* causes an acute and fulminant inflammation in the CNS in the rats and also provokes the activation of endothelial cells, as well as the modification of claudin-5. The knowledge of the blood brain barrier allows us the understanding of the pathogenesis of *N. fowleri* to disrupt the privilege of this barrier. This work was supported by Cinvestav (project funding and travel support) and by SEP-CONACYT grant 237523.

Or-22

In vitro growth, cytopathic effects and clearance of monolayers by clinical isolates of *Balamuthia mandrillaris* in human skin cell cultures.

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Objectives:
Balamuthia mandrillaris is a free-living ameba (FLA) that has been isolated or its DNA identified in soil, dust and water. It causes a fatal central nervous system infection in humans and animals [1]. Although it is environmental as *Acanthamoeba* and *Naegleria fowleri*, the two other free-living amebae that also cause CNS infections, *Balamuthia* does not feed on bacteria as the other FLA. In the laboratory, it can be grown on a variety of mammalian cell cultures [2]. Here we examined the ability of *Balamuthia* isolates to grow on different human skin cell cultures.
Materials:
Three different isolates of *B. mandrillaris* (two clinical responsible for fatal granulomatous amebic encephalitis and one environmental) were used. A corneal isolate of *Acanthamoeba castellanii* was used for comparison. Monolayers of three different human skin cell lines: human skin keratinocyte cells (WT/A), human skin microvascular endothelial cells (HMEC) and human foreskin fibroblast cells (HS 68) were used. Human lung fibroblast cells (HLF) monolayers were used as control. 20 amebae /mL were inoculated into T25 flasks of each human tissue culture and maintained at 37°C for 28 days. The amebae were counted every 4 days. Two-way RM ANOVA and Tukey's multiple comparison tests were used for statistical analysis.
Results:
Each of the three different strains of *B. mandrillaris* grew and multiplied in human fibroblast and microvascular endothelial cell cultures (HLF, HS 68 and HMEC). The amebae cleared the monolayer by ingesting the human cells. All three isolates did not significantly multiply in the human skin keratinocyte cell culture (WT/A).
Conclusions:
Balamuthia failed to significantly grow in and destroy the WT/A skin keratinocyte cell line, although the amebae remained in the trophozoite form and were still viable and infectious. The intact epidermis composed of keratinocytes is a barrier for *Balamuthia*.

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Or-23

Electron microcopy observations of *in vitro* cell injury produced by *Acanthamoeba culbertsoni*

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Amoebic keratitis (AK) is a chronic infection difficult to treat, caused primarily by *Acanthamoeba* genus, related particularly with the use of soft contact lenses. It had been observed that during AK infection the amoebae invade only the cornea; however recently, cases with amoebic extracorneal invasion had been reported. In this communication, some morphological mechanisms of damage of a strain isolated from a case of AK with extra corneal invasion, on MDCK (Madin Darby Cell Kidney) cells during 3, 6, 8 and 12 h interaction are described.

The strain belongs to T4 group but morphologically corresponds with *Acanthamoeba culbertsoni*. This species is related to infections in the central nervous system and cornea. Co-incubation of trophozoites and MDCK monolayer epithelial cells (1:1) was performed and described by transmission and scanning electron microscopy. We highlight poor adherence of trophozoites on plastic and MDCK cells compared to other amoebae isolated from cases of AK. Numerous trophic forms were observed adhered only in limited areas of both substrates, however, in binding sites on MDCK cells, a possible mechanical effect was identified, as deformed cell zones, being more evident between cell junctions where trophozoites were observed emitting cytoplasmic projections allowing monolayer invasion. Phagocytosis was documented during the time of interaction evaluated through phagocytic mouths and by cytoplasmic projections surrounding regions of damaged cells. Trophozoites showed cytopathic effect only in areas of contact with the monolayer, suggesting contact dependent mechanisms similar to those reported in other species of *Acanthamoeba*.

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Or-24

Identification of proteases with mucinolytic activity released by *Naegleria fowleri*

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During nasal infection by pathogens it is known that the production and secretion of mucus are induced. This innate immune response functions as an early mechanism of protection and elimination of pathogens. *Naegleria fowleri* is a free- living amoebae that is the etiological agent of amoebic primary meningoencephalitis. The infection starts with the entrance of the trophozoites by the nasal route, then penetrates the epithelium of the olfactory mucosa, and migrates through the olfactory nerves by crossing the cribiform plate and eventually installing in the central nervous system (CNS). In our laboratory has been studied the early events of infection in the murine model and we observed that mucus protection is insuficient to eliminate *N. fowleri* trophozoites. On the other hand, recently it was reported the precence of mucinolytic activity arround 37 kDa in total crude extracts of *N. fowleri,* but the identification of this protease in secretion products was not detected. This mucinolytic activity could be involved with the ability of *N. fowleri* to evade the immune response. For this purpose, in the present work we analyzed the mucinolityc activity in secretion products. In order to observed mucinolytic activity in conditionated medium (CM), the samples were treated with ethanol (3:1) or with serial precipitations with $(NH_4)_2SO_4$. Zymograms using bovine submaxillar mucin (MSB) as substrate were performed to dectect proteolitic bands. The results revealed mucinolitic activiy in CM, this proteolitic activities were found at 94 and 53 kDa, the activity was induced with 2 mM DTT at 37°C in pH 5 and 7. This study overall provides information about of secretion molecules that could be involved during *N. fowleri* invasion to the CNS. These molecules can be used as drug targets against *N. fowleri*. This work was supported by SEP-CONACYT grant 237523.

Or-25

Highly-pathogenic *Naegleria fowleri* and weakly-pathogenic *Naegleria fowleri* demonstrate differential protein expression

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Naegleria fowleri (*N. fowleri*) is the causative agent of Primary Amebic Meningoencephalitis (PAM). In this disease process, amebae enter the nasal passages, attach to the nasal epithelium, and migrate to the brain where they cause tissue destruction eventually resulting in death of the host. During passage into the brain, amebae are exposed to host-specified cellular and soluble components that putatively trigger the selective induction of proteins that are linked functionally to invasion. The objective of the present study was to apply a proteomic approach to identify amebic proteins that were *de novo* expressed or elevated in expression in association with attainment of the highly-pathogenic state. Weakly-pathogenic amebae were cultured axenically in Oxoid medium. Highly-pathogenic amebae were obtained after serial brain passage at monthly intervals in $(B_6C_3)F_1$ mice. Light microscopic analysis, as well as that using scanning and transmission electron microscopy, revealed no gross morphological differences between axenically cultured and mouse-passaged amebae. Nevertheless, mouse-passaged amebae exhibited a 2 $\log_{10}$ titer decrease in infectious dose 50 (ID_{50}) as compared to the axenically-cultured amebae consistent with their acquisition of a highly-pathogenic state. Whole cell lysates of purified axenically-cultured and mouse-passaged amebae were utilized for assessment of proteomic profiles. Two-dimensional polyacrylamide gel electrophoresis of amebae in concert with silver staining revealed a distinctive protein profile for mouse-passaged versus axenically-cultured *N. fowleri*. Scatter plot analysis and PD Quest software were utilized to identify novel protein species for mouse-passaged amebae. In addition, mouse-passaged ameba proteins exhibiting a greater than two-fold level over that recorded for weakly-pathogenic amebae were identified. These protein-containing spots were excised and subjected to ultra performance liquid chromatography-tandem mass spectrometry and protein identification was accomplished using algorithms to determine homology with known protein species. Homology with proteins involved in downstream signaling of ameba integrins and cytoskeleton components was obtained. The collective results suggest that *N. fowleri*-host cell ligation early in the invasive process serves to trigger differential gene expression that is linked to invasion of the brain. Furthermore, these selectively expressed gene products may serve as targets for therapeutic manipulation of PAM.

ORAL SESSION 6

Chairs

Maryam Niyyati

and

Jacob Lorenzo-Morales

Or-26

The last amoeba standing. Cannibalism as a mechanism for the loss of the ability to encyst in axenic cultures of *Acanthamoeba.*

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The culture of clonal protozoans in axenic medium has many advantages for experimental work but it is also known that this causes changes in the behaviour of these cultured microorganisms. It has been found that the facultative human pathogen *Acanthamoeba* loses its pathogenicity during prolonged axenic culture and it is also reported that the amoebae loses the ability to produce the resistent cyst stage (see other abstract). The cyst stage of this amoeba is an important part of its biology especially in its ability to act as a reservoir for pathogenic bacteria. Here we report that in the very unnatural axenic culture conditions, strong pressure against encystation occurs because stressed amoebae which are the first to encyst tend to be consumed by other amoebae. Post logarithmic culture conditions where resources become limited place increasing selective pressure favouring phagocytic amoebae which are best able to survive by preying on other amoebae, amoebae fragments and developing cysts. One of the attractions of axenically cultured *Acanthamoeba* is that these can be ignored for months and new log growing cultures rapidly obtained by changing the medium, however cultures treated in this manner are likely to change in response to the accidentally altered selection pressure in unpredicatable ways. The cannibalism seen here has been reported in a variety of other amoebal species including the Heterolobosean amoeboflagellate *Heteramoeba clara* [1], *Entamoeba*. In this instance it may be that there is selective pressure to be the last to encyst and to either use the cell constituents expelled by other encysting amoeba or to consume the whole cyst or precyst and use this as a food source. In this manner, aclimatization to axenic media may inevitably lead to a loss of synchronised encystment and perhaps even a loss in this ability altogether since this is no longer an advantage but rather a liability?

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Or-27

Genome Assembly and Annotation of *Balamuthia mandrillaris* using Multi-Platform Data

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The free-living, ubiquitous amoeba *Balamuthia mandrillaris* is an opportunistic causative agent of amoebic encephalitis, a rare but lethal infectious disease of humans and other mammals. Molecular data plays a critical role in unraveling factors governing the metabolic and pathogenic potential of a pathogenic agent and in developing rational and specific therapeutic strategies. The gateway for this concept becomes accessible through the pathogen's genome and transcriptome.

With the help of high-throughput sequencing data, we assembled and annotated the first *de novo* draft genome and transcriptome of *B. mandrillaris*. The integrated data from Illumina, Roche 454, and PacBio sequencing platforms revealed a genome of 56 Mbp in length and predicted 21,623 genes. Representation of 458 core eukaryotic genes was at 94% identifiable as complete ORFs and at 97% when considering also evidence of partial ORFs. Analysis of the k-mer frequency spectrum of Illumina sequencing reads indicated a polyploid genome with a high level of heterozygosity. The mitochondrial genome had a length of 39,892 bp and encoded 48 genes. Comparison of *B. mandrillaris* nuclear genes and mitochondrial structure to that of *Acanthamoeba castellanii* suggested a more distant relationship between these organisms than conventionally assumed.

As a first proof of said concept, we applied the genomic and transcriptomic data to the investigation of genes involved in sterol synthesis to identify fundamental metabolic disparities between this amoeba and its mammalian host with potential for therapeutic intervention.

Or-28

Reviving the ability to encyst in a long established axenic strain of *Acanthamoeba*: Evidence for a common pathway for various encysting stimuli.

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It is known that prolonged cultivation in axenic media alters some properties of *Acanthamoeba* strains, and yet the Neff strain (ATCC 30010, CCAP 1501/1A), which has been in axenic cultivation since 1957, is the standard strain against which others are routinely compared. One of the properties that this strain has lost is the ability to form resistant cysts. Here we test the hypothesis that the ability to produce such cysts can be restored in strains that have been in axenic culture for long periods. We show that this ability can be restored, even in very old axenified cultures by repeated selection cycles, and that the cysts so produced, strongly resemble those originally described for the parent strain in both appearance and at least some of their physical properties. For the selection process, encystation was stimulated by the standard Neff's encystment medium and after 4 days cysts were selected by the addition of SDS. The first selection cycles produced cysts that could survive in small numbers for 10 minutes in 0.1% SDS, however after 10 cycles we could use 0.5% SDS to select cysts. After 16 cycles the cysts survived 2% SDS overnight and were also capable of surviving 3% HCl. The selection process is now in its 47th cycle and although cysts are produced it cannot be said that they do so "synchronously" as approximately half of the trophozoites initially present do not form cysts after 4 days in the encystment media.

After selection it was found that the amoebae could be stimulated to form cysts by a number of different stimuli. These include other salts, gradual desiccation, hyperosmolarity, a variety of drugs, and (weakly), temperature shock. The fact that cells selected to produce cysts by the use of one particular stimuli are able to encyst through other chemical and physical stimuli, provides evidence for a common pathway (or part of a common pathway) in the initiation of this differentiation event. In addition, the re-establishment of an axenic Neff strain capable of encystment/excystment will be of use in further research on this the most widely characterized of *Acanthamoeba* strains.

Or-29

Autophagy in *Naegleria gruberi*

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Naegleria genus is constituted by flagellated protozoans which belong to the group of free-living amoebae. In recent years medical importance has increase because the pathogenic specie *Naegleria fowleri* has been reported in several countries. This microorganism produces primary amoebic meningoencephalitis (PAM), a fulminant infection in the central nervous system. However, biological mechanisms of this amoeba are poorly understood. The non-pathogenic amoeba *Naegleria gruberi* could be an important model in the study of the biological mechanisms such as autophagy, which is involved in the degradation of cytoplasmic components like damage organelles to be recycled by lysosomal pathway, allowing the cell to survive in stressing conditions. In the present work, we analyzed the autophagy process in *N. gruberi* trophozoites induced with Rapamycin, a TOR kinase inhibitor. Our results showed morphological changes in *N. gruberi* trophozoites. We also searched the most representative molecule of autophagy, the ubiquitin like protein Atg8, comparing with the homologue in *S. cerevisiae*. We found a homologue *atg8* in *N. gruberi* genome (*Ngatg8*); with bioinformatics analysis we determinate the identity and the characteristic domains of this protein. We corroborate the presence of the complete gene with specific primers using RT-PCR. In addition, by qPCR we observed changes in the relative expression of *Ngatg8* in trophozoites induced to autophagy with Rapamycin. Finally, we used LysoTracker red to corroborate the acidification of vacuoles present in the cytoplasm of trophozoites. With all these results, we can conclude that *N. gruberi* possess all the necessary machinery to present the classic autophagy phenomenon. The description of autophagy in *Naegleria* genus can give us a better understanding about the cell biology of this microorganism. This work was supported by SEP-CONACYT grant 237523.

ORAL SESSION 7

Chairs

Elizabeth Ramírez

and

Ruqqaiyah Siddiqui

Or-30

PROGRAMMED CELL DEATH IN *ACANTHAMOEBA*

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Members of the genus *Acanthamoeba* are facultative pathogens of humans. They can affect to different tissues, causing a sight threatening keratitis (AK) and the usually fatal granulomatous amoebic encephalitis (GAE). In order to treat those infections properly and due to the different stages of this protozoa which can be found in their life cycle, it is necessary to focus the therapy not only to the trophozoite but also to the cyst. This is because some treatments induced cyst formation but they do not eradicate them, so they can revert to trophozoites after the therapy. Furthermore it may be advantageous to avoid parasite killing by necrosis, which may induce local inflammation, and we must also avoid toxicity of host tissue. Many drugs which target eukaryotes are known to induce programmed cell death (PCD), but this process is poorly characterized in *Acanthamoeba*. Here we study the processes of programmed cell death in *Acanthameoba*, induced by atorvastatin, fluvastatin, simvastatin and voriconazole at the IC50s and IC90s that we have previously established. In order to evaluate this phenomenon when cells are treated with those molecules, we investigated the DNA fragmentation, one of the main characteristics of PCD, with quantitative and qualitative techniques. Also, the changes related to phosphatidylserine exposure on the external cell membrane and cell permeability was studied. Finally, because caspases play a key role in PCD pathways, caspase activity was evaluated in *Acanthamoeba*. Regarding our results, all the assayed drugs in this study induced PCD in *Acanthamoeba*. To the best of our knowledge, this is the first study where PCD induced by drugs is described quantitatively and qualitatively in *Acanthamoeba*.

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Or-31

Apoptotic trends In *Acanthamoeba* spp.

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Objective:
The existence of programmed cell death similar to that of apoptosis has been reported in unicellular eukaryotes in past [1, 2, 3,] We establish the existence of apoptotic pathways and adapter proteins in *Acanthamoeba* spp., and show that this protist pathogen has down regulated the extrinsic pathway receptors and adaptor proteins like caspases, but has a well established intrinsic cascade of apoptosis

Methods:
We establish several biochemical and morphological features of apoptosis in this parasite when exposed to clinically available anticancer drugs like Daunorubicin and drugs targeting cellular receptors and biochemical pathways like, Dicyclomine and Loperamide [4], that have the potential of inducing apoptosis. In vitro apoptotic assays were performed by incubating *A. castellanii* (1 x 10^5 cells/mL/well) with various concentrations of apoptosis inducing drugs in the growth medium in 24-well plates and incubated for 48 h at 30°C.

Results:
A Morphology consistent with apoptotic trophozoite cell shrinkage, DNA fragmentation, Phosphatidyl serine externalization and immunostaining of Cytochrome C and Apoptosis Inducing factor (AIF) have been observed in the protozoan parasite at different time periods. Bioinformatic comparative homologies have revealed several common apoptotic markers identical to metazoan apoptotic phenotype, showing an apoptotic cell death exhibited by *Acanthamoeba Castellanii.* The parasite genome is capable of expression of apoptotic adapters like metacaspase and endonuclease that functions analogous to the mammalian caspases.

Conclusions:
Our study specifies the existence of a caspase independent type of apoptosis in the parasite that gets activated by DNA damage and drugs capable of inducing the intrinsic apoptotic cascade. This protist pathogen in evolution has down regulated the extrinsic cascade of apoptosis to protect itself from incidental contact with extracellular cytokines and drugs that may force it to go into apoptosis.

Significance of Study:
With the limited number of drugs available for treatment of diseases caused by *Acanthamoeba* spp., there is a need for the discovery of newer drugs. Loperamide and Dicyclomine that can induce apoptosis in this protist pathogen, can be evaluated by in-vivo assays to be of chemotherapeutic potential in the treatment of corneal, cutaneous and cerebral infections like granulomatous amoebic encephalitis (GAE) caused by *Acanthamoeba* spp..

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Or-32

Induction of apoptosis by Flavone in *Acanthamoeba*

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Therapeutic methods against several protozoa diseases caused by the genus *Acanthamoeba* strains namely, Granulomatous Amoebic Encephalitis (AGE) and *Acanthamoeba* Keratitis (AK), remains as an issue to be solved due to the existence of a cyst stage which is highly resistant to chemical and physical agents. Recently several researches have been studying the amoebicidal activity of medicinal plants namely the *Origanum*, the *Thymus sipyleus* and the *Olea europea* [1-3]. This activity could be correlated to the presence of several molecules such as the flavonoids. The aim of this study was to evaluate the activity of the flavone on *Acanthamoeba castellanii* Neff. In fact, the flavone or the 2-phenylchoromoneis the flavonoid parent compound [4].

Both form cyst and trophozoïte have been inhibiting by the Flavone with an IC_{50} about 25µg/ml against the vegetative form of the tested parasite. The action mode of this compound was studied by detecting changes in the phosphatidylserine (PS) exposure, the plasma membrane permeability, the mitochondrial membrane potential and the ATP level production in the treated parasites. By using the fluorescent probe SYTOX® Green, the Flavone could affect the membrane permeability without inducing necrosis. In fact, this molecule could stimulate the apoptosis by altering the mitochondrial function: by the membrane depolarization and decreasing the ATP levels to less than 50% in treated parasites with the IC_{90} for 24 h. The tested flavone demonstrated a good activity against *Acanthamoeba castellanii* Neff. Nevertheless, further studies are needed in order to establish the real potential of this molecule against the tested parasitic protozoa.

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ORAL SESSION 8

Chairs

Iva Dyková

and

Patricia Bonilla

Or-33

The genetic structure of amoebae morphospecies

Smirnov Alexey, Nassonova Elena, Glotova Anna, Zlatogurski Vasilij, Kudryavtsev Alexander, Bondarenko Natalya, Mijanovich Olja

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The morphospecies concept, routinely applied for amoebae, was almost satisfactory when most of studies were limited to the ecological, faunistic and taxonomic purposes. Molecular biological tools, applied in order to increase the resolution of species distinction, show that genetic structure of amoebae morphospecies is rather complex. Many of usually applied molecular markers like SSU rDNA, ITS1, ITS2 and even widely applied DNA barcode - Cox I gene show unusually high polymorphism in amoebae [1,2]. Extensive studies of several amoebae species belonging to the genera *Vannella*, *Korotnevella*, *Flamella* and *Cochliopodium* indicated that: (1) every properly defined amoebae morphospecies comprises a number of genetic lineages, but the diversity of lineages is finite and limits to several dozens; (2) The same lineage may be found worldwide, while some lineages appear to be endemics; (3) Every local habitat normally comprises limited number of genetic lineages, some unique for this habitat while some – shared with other (sometimes distant) locations. Our results indicate that for amoebae the morphospecies concept remains applicable. Properly defined amoeba morphospecies possessing remarkable differentiating characters is a genetically distinctive unit. However in many amoebae genera and even families molecular tools seem to be the only way to differentiate genera and species or justify description of new ones. Utilizing of molecular data in taxonomic diagnoses of species and genera nowadays is getting appropriate and even desirable, however these should be molecular characters (e.g. characteristic molecular signatures or unique details of the secondary structure of SSU rDNA gene), but not plain number of nucleotide replacements or position of species in a particular phylogenetic tree.

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Or-34

Occurrence and diversity of free-living protozoa on sprouts

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Despite thorough bio security measurements (cleaning and sanitation) pathogenic bacteria persist on food products, in drinking water and in food related environments. Associations between foodborne pathogenic bacteria and free-living protozoa (FLP) may play a major role in the protection and persistence of these bacteria [1].

While microbiological analysis of food and food related environments has become daily practice for food companies, FLP are usually not incorporated in microbiological surveys as they are considered harmless. As a result, information on FLP on food is scarce and negligible.

Sprouts are often the source responsible for foodborne outbreaks [2][3][4]. As part of a wider investigation into the role of environmental FLP in the contamination of sprouts by foodborne pathogens, an inventory of these unicellular eukaryotes on sprouts was made.

Within batch and between batch variation of the microbial communities on four commonly eaten sprouts (alfalfa, leek, mung bean, cress) and four rarely eaten sprouts (beetroot, red cabbage, green pea, rosabi) were evaluated. Enrichment and cultivation methods were applied to determine the occurrence, enumeration and diversity of FLP on sprouts. In parallel Total Aerobic Bacteria (TAB) and *Escherichia coli* counts on sprouts were obtained.

Flagellates were present in 100% of the examined samples, amoebae in 90% of the samples and ciliates in 79% of the samples. Estimated FLP numbers ranged from 0.03 MPN/g to 1193 MPN/g. In total 72 FLP (30 ciliates, 20 flagellates and 22 amoebae) were identified to species, genus or morphotype level. For the ciliates *Tetrahymena* sp. was an important representative for all sprouted seed types. *Bodo saltans* was a frequently observed flagellate. Amoebal diversity was dominated by *Acanthamoeba* sp. and *Vannella* sp. TAB counts on the sprouts were high, with numbers ranging from 5.85 log cfu/g to 9.33 log cfu/g. *E. coli* counts were in 98% of the examined samples below the limit of quantification (<10 cfu/g).

This study showed that FLP, including some opportunistic pathogens, are a common and diverse microbial group on sprouts and may play a significant role in the contamination of sprouts by foodborne pathogens.

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ORAL SESSION 9

Chairs

Maritza Omaña Molina

and

Sutherland K. Maciver

Or-35

An explanatory model for the colonization success of the pathogenic amoeboflagellate *Naegleria fowleri* in artificial aquatic environments.

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Fifty years after the description of the first cases of PAM in Australia, North America and Europe, *Naegleria fowleri* remains more poorly understood as an environmental protozoan than as a pathogen. Anticipation of its occurrence and the deployment of measures to prevent infections demand a more detailed ecological understanding of this organism. An earlier model, the 'Flagellate-Empty Habitat Hypothesis' [1], recognised the significance of artificial environments but emphasised unduly the role of the flagellate stage: such habitats are colonized rarely or not at all by some other amoeboflagellates (*Naegleria australiensis*, *Willaertia magna*), but readily by *Vermamoeba* species, which do not differentiate to flagellates.

The model presented here explains the distribution and abundance of *N. fowleri* in terms of niche theory. It addresses the comparative questions 'Why does *N. fowleri* occur more widely and at higher densities in artificial than in natural aquatic environments?' and 'Why this is this *not* true of most other *Naegleria* species?' The model is designed to be consistent with the known ecophysiology of *Naegleria* species generally, and with the General Theory of Ecology and its constituent theories. It encompasses the responses of selected *Naegleria* species to important niche dimensions including temperature and food concentration. It also includes some counter-intuitive propositions, e.g. that *N. fowleri* occupies a wider set of conditions (its niche) in artificial than in natural environments, and that this species is favoured over others by relatively low bacterial densities. Some of the propositions require further experimental verification, and the ecological significance of cytological processes that have been little studied (e.g. excystment) is discussed.

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Or-36

Isolation and molecular characterization of *Balamuthia mandrillaris* from soil samples in Iran

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Objectives:
Balamuthia mandrillaris is a free-living amoeba which has been reported as a human pathogen since 1990. B. mandrillaris infections manisfest as encephalitis known as Balamuthia Amoebic Encephalitis (BAE) and could also affect other organs like lungs and skin. Environmental isolation of *B. mandrillaris* is a rare event, however it seems that the most common habitat of *B. mandrillaris* is soil, often associated with elevated amount of nutrients that allow *Balamuthia* to feed on other. In Iran, this pathogen has been previously isolated from a dust sample in the city of Tehran, nevertheless the present study reports the first report on the isolation and molecular identification of *B. mandrillaris* from soil samples in Iran.
Methods:
50 soil samples were selected across the parks of the northwest of Iran from public places and recreational regions in the last three months of 2014. 100 g of soil samples were dissolved in sterile distilled water, which were left for about an hour and filtered with Filter paper. The filters were cut out and placed on 1% Non-nutrient agar (NNA) medium along with *Escherichia coli* as a food source for *Balamuthia and other* amoebae. Identification of the amoeba was carried out morphologically using Page´s key. Furthermore, molecular identification of *Balamuthia* positive samples was performed using the 16S mitochondrial 18S rDNA gene species specific primer pairs and DNA sequencing.
Results
Overall, 5 out of 50 collected samples were positive for *Balamuthia* like amoebae. *Balamuthia* trophozoites prresented irregular shape with fine fingerlike projections resulting in filamentous appearance. Also cysts of *B. mandrillaris* were spherical and appeared multilayered. PCR amplifications resulted in positive bands of 1095 bp and DNA sequencing and homology analyses confirmed *B. mandrillaris* in all the suspected positive samples.
Conclusion
This is the second isolation of *B. mandrillaris* from environmental sources in Iran and the first one from soil samples. Nevertheless, cases of BAE have not been reported so far in this country. The obtained results should raise awareness among the clinicians in Iran, due to the apparent common distribution of *B. mandrillaris* in Iran.

Keywords: Soil samples, *Balamuthia mandrillaris*, Iran

Or-37

Free-living amoebae in Costa Rica: isolation and molecular characterization of potential pathogenic species

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Free-living amoebae (FLA) are ubiquitous protozoa widely distributed in nature, which may behave as parasites under certain conditions. Four genera are considered potential human pathogens *Acanthamoeba, Naegleria, Balamuthia and Sappinia*. In Costa Rica, there are few studies on FLA, and therefore both the distribution as well as their potential impact on public health is unknown. In 2014, our group decided to undertake an investigation in order to isolate and molecularly characterize potentially pathogenic amoebae from different sources related to human activities. Samples were taken from soil, combined emergency units, dental units and air conditioning units. The sampling method varied depending on the type of sample. Each sample was cultured onto 1.5% non-nutrient agar plates, observed microscopically and molecularly characterized using specific PCR methodology, followed by the sequencing of the products obtained. For the determination of potential pathogenicity of the isolates, osmotolerance and thermotolerance assays were performed, as well as protease determination through zymography.

The results obtained indicate that *Acanthamoeba* genotype T4 was the most frequently isolated FLA (75%). Other *Acanthamoeba* genotypes such as T3, T5, T2 and T13, as well as *Naegleria fultoni* and *N. gruberi* were also found. It is important to highlight the isolation of *Balamuthia mandrillaris* from a dust sample, constituting the first finding of this organism in Central America.
Over 90% of the *Acanthamoeba* isolates were determined to be thermo and osmotolerant, and the presence of extracelular serin proteases was determined in all isolates for which an axenic culture was possible. Additionally, by applying the techniques used in our studies, it was possible, for the first time, to isolate of *Naegleria fowleri* in thermal hotsprings in Costa Rica, which was linked to a fatal primary amoebic meningoencephalitis (PAM) acquired in the country.

Funded by: This study was supported by project 803B4050, Vicerrectoría de Investigación, University of Costa Rica. J.L.M. was supported by the Ramón y Cajal Subprogramme of the Spanish Ministry of Economy and Competitivity RYC201108863. MRB was supported by Obra Social La Caixa Fundación Cajacanarias 2014.

Or-38

Free-living amoebae from different water sources in Italy

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Objectives: The present study aimed to detect free-living amoebae (FLA) from different water sources in Italy, and to characterize the isolates at molecular level to better understand the environmental distribution of species/genotypes potentially associated with human disease.

Methods: A total of 160 water samples were collected in Italy. In order to investigate the presence of amoebae, samples were centrifuged and cultured onto NNA-media containing *Escherichia coli*. Molecular characterization was obtained through DNA extraction using the Microkit QIAamp DNA protocol (QIAGEN, Milan, Italy). For *Acanthamoeba* typing, the ASA.S1 region of *18S* rRNA gene was amplified with JDP1 and JDP2 specific primers. Detection of other FLA was performed by the 18S rDNA amplification with primers P-FLA-F and P-FLA-R. Assignment to species/genotypes was obtained by comparing the sequences with those available in GenBankTM through a phylogenetic analysis performed by MEGA version 5.

Results: Forty-six out of 160 (28.7%) water samples resulted to be positive for free-living amoebae. *Acanthamoeba* was detected in 39.1% of samples from all water sources except bottled mineral water. The phylogenetic analysis identifies two well defined clusters corresponding to the potential pathogenic *Acanthamoeba* genotypes T4 (72.2%) and T15 (27.7%). Concerning other FLA, 45.6% of isolates was identified as *V. vermiformis* as evidenced in the Neighbor joining phylogenetic trees. One isolate gave 99% similarity with *Amoebozoa* sp. amMP3 (JX312795) DNA sequences, originated from a mud pond in South Italy. Three isolates evidenced the simultaneous presence of *Acanthamoeba* spp. and *V. vermiformis*.

Discussion and conclusions: The present study has contributed to add new molecular data about the occurrence of *Acanthamoeba* and other free living amoebae in water samples in Italy. The detection of amoebic strains potentially pathogenic suggests that different water source in Italy may act as possible sources of infections for humans.

Or-39

Free-living amoebae (FLA) as reservoirs for *Legionella* spp. and other bacteria – screening of Austrian cooling towers and tap waters

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Free-living amoebae (FLA) are widely spread in the environment and also known to cause rare but often serious infections. Besides this, FLA have an indirect public health significance as they may serve as vehicles of dispersal and replication for bacterial pathogens. In particular, *Legionella pneumophila*, the causative agent of Legionnaires´ disease, replicates within FLA. Intracellular replication in amoebae seems to trigger the ability of the legionellae to infect human alveolar macrophages and besides, intracellular legionellae are protected against disinfection. As in environmental samples intracellular legionellae might not be detected by standard screening methods, the aim of the current study was to evaluate the diversity of FLA in water samples routinely screened for legionellae and to investigate all amoebal isolates for intracellular bacteria. To achieve this, a new screening system for FLA including real-time PCR assays specific for *Acanthamoeba*, Vahlkampfiidae and *Vermamoeba*, was established. Water samples were taken periodically from three cooling towers of public buildings and various tap water facilities from 2013 to 2014 and investigated by culture and molecular methods in parallel. With real-time PCR, an overall of 53/79 samples were positive for *Acanthamoeba* spp. and 38 were positive for Vahlkampfiidae, whereas *Vermamoeba* was detected in five samples. Only half of these samples were also positive for FLA by culture, revealing however also other genera, as e.g. *Cochliopodium* or *Stenamoeba*. Interestingly, a high number of samples (50/79) could not be analyzed for *Legionella* with standard cultivation techniques, due to the composition of this special type of samples (high organic burden), but several of the amoeba isolates revealed intracellular bacteria by fluorescence in situ hybridization (FISH). Of the 29 samples that could be screened by standard techniques, six were positive for *Legionella* spp. and 16 for *Pseudomonas aeruginosa*. Thus, we propose that other methods such as amoeba co-culture or PCR would be more suitable methods for the investigation of cooling towers for legionellae.

ORAL SESSION 10

Chairs

Fernando Lares Villa

and

Yann Hechard

Or-40

Phylogenetic diversity of *Acanthamoeba* revealed by different types of prey bacteria

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Acanthamoeba are traditionally isolated using *E. coli* as the monoxenic source of food. Since there are diverse forms of bacteria in the environment that can be used as food by *Acanthamoeba*, it remains unclear whether the choice of bacteria as source of food can have an effect on the type of *Acanthamoeba* recovered. To address this question, in the current study three different types of bacteria (*E. coli*, *Enterococcus* and *Arcobacter*) were used separately for isolation of *Acanthamoeba.* A total of 102 soil samples were randomly collected from various parts of UK. Each sample was used for isolation of *Acanthamoeba* separately by using either of the three types of bacteria to have *E. coli* (Eco), *Enterococcus* (Ent) and *Arcobacter* (Arc) isolates of *Acanthamoeba*, making a total of 306 sample processing. It was found that the presence of different bacterial types can affect the genotypes and specially subtypes of *Acanthamoeba* recovered. The effect was most prominent in case of Arc isolates which showed greater diversity of 18S rRNA sequences than Eco isolates. T types recovered for Eco isolates included T2(6%), T4(89.2%), T11(2.4%) and T13(2.4%); for Ent isolates included T4(95.1%), T16(3.7%) while 1.2% of isolates were intermediate T13/T16; for Arc isolates included T2(14.3%), T2/6(2.4%), T4(78.6%), T13(2.4%) while 2.4% sequences were intermediate 13/T16. There were also remarkable differences among the T4 types on the basis of subgrouping (T4-A, -B, -C, D, E, F and N) (Fuerst, 2014). Eco isolates had T4-A (54.1%), T4-B (16.2%), T4-C (1.3%), T4-D (8.1%), T4-E (9.5%), T4-N (10.8%); Ent isolates had T4-A (47.0%), T4-B (7.4%), T4-C (11.1%), T4-D (11.1%), T4-E (12.3%), T4-N (11.1%); and Arc isolates had only T4-A (28.8%), T4-B (19.7%), T4-E (34.8%) and T4-N (16.7%). Interestingly, there were also difference in the trends for harbouring bacterial endosymbionts among the Eco, Ent and Arc isolates. The Arc isolates had 15.7% bacterial endosymbionts as compared to 7.8% of Eco and 12.9% of Ent isolates, while T4-B was found to be most susceptible for endosymbionts. Together these results suggest consideration for using different types of bacteria for isolation of *Acanthamoeba* to help surface the masked populations as well.

Key words: *Acanthamoeba*, diversity, bacteria, T types, endosymbionts.

Reference

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Or-41

Food preferences of *Acanthamoeba castellanii*

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Free-living amoebae are protozoa found in the aquatic environment. They feed on microorganisms, such as fungi and bacteria. The purpose of this study was to investigate the potential preference of *Acanthamoeba castellanii* between different bacteria, and if necessary to characterize the molecules involved in this phenomenon.

First, amoebic motility experiments were performed on agar in the presence of various bacteria: *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Stenotrophomonas maltophilia*, *Klebsiella pneumoniae* and *Streptococcus pneumoniae*, to determine a bacterial preference of *A. castellanii* (ATCC 30324). Similar experiments were carried out using cell-free supernatants of bacterial cultures in order to investigate if the attraction could be linked to secreted compounds. Supernatants of interest were then purified by solid phase extraction followed by reverse phase HPLC. Obtained fractions were analyzed by mass spectrometry and tested with anthrone, ninhydrin and primulin to determine biochemical nature of the compounds responsible for the attraction.

The results obtained with amoeba motility experiments varied as a function of the tested bacteria. The amoeba were preferentially attracted by two bacteria: *Klebsiella pneumoniae* (Gram negative) and *Staphylococcus aureus* (Gram positive). We decided to focus on these microorganisms. Similar results were observed with culture supernatants obtained from these two bacteria. The active fractions obtained from HPLC purification appeared positive with ninhydrin and primulin thus suspected to be composed of peptide and lipid moieties. Mass spectrometry was used to determine molecular weights of the active compounds.

In conclusion, according to our results, *A. castellanii* have food preferences and is attracted by molecules secreted by its prey.

Or-42

Acanthamoebae as vectors of Pandoraviruses to humans

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Background: Free-living amoebae (FLA) belonging to the genus *Acanthamoeba* occur ubiquitously in many aquatic habitats and humid soils. In addition to their role as facultative pathogens, Acanthamoebae are known as vehicles for and hosts of various intracellular organisms. They serve as Trojan horses for diverse microorganisms (including bacteria, fungi or viruses) and as training grounds for intracellularly residing or replicating microorganisms. The amoebal passage and the evasion of the digestive mechanisms are possibly important in terms of the development of human pathogenicity.

The host: An *Acanthamoeba* strain LaHel was recently isolated from the contact lens storage cases of a female patient with keratitis. The Acanthamoebae were classified morphologically as *Acanthamoeba* sp. group II, defined as *Acanthamoeba* sp. using real timePCR and were subsequently identified genetically as T4-genotype (sequence type), which is the most common genotype in keratitis associated Acanthamoebiasis cases.

The endocytobiont: These Acanthamoebae harboured endocytobionts, proliferating intracellularly and subsequently leading to the lysis of the *Acanthamoeba* trophozoites. Other Pandoraviruses were found in 2013 within the sediments of rivers. Sequence analysis using the 2013 published primers and proteomic profiles followed by whole genome sequencing proved them as members of the new *Pandoravirus* genus. The spore-like *Pandoravirus* particles, 1,1µm in length, possess a massive electron-dense outer wall.

Conclusion: These Pandoraviruses were initially described as extraordinary microorganisms or endocytobionts without having to make a phylogenetic or taxonomic predefinition at the time of their discovery in 2008. They are the first *Pandoravirus* whose development and morphology were "described" substantially, although it was not possible to classify them correctly at that time. As this is the first documented association of Pandoraviruses with humans as well, we have clearly demonstrated how easily such endocytobionts can be transferred to humans. Although the identification of the Pandoraviruses on the genetic level is partially done, there are a lot of open questions starting with the exact phylogenetic position, the evolutionary significance and the potential extension of the microbial diversity. Nevertheless this case counts as another example of parasites acting as vectors and reservoirs of phylogenetically different microorganisms especially when living sympatric within their biocoenosis of biofilms.

ORAL SESSION 11

Chairs

Donald Munson

and

Patrick Scheid

Or-43

Passive-active resistance of non-tuberculous *Mycobacteria* to amoebal predation: characterization of a survival strategy

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Non-tuberculous *Mycobacteria* (NTM) are acid-fast bacilli frequently found in water systems, sharing the same ecological niches as free-living amoebae (FLA). In a recent study we have shown a strong interaction between *M. llatzerense*, *M. chelonae* and FLA [1] suggesting that these NTM resist amoebal predation.
Using the model *Acanthamoeba castellanii,* the characterization of *M. llatzerense* and *M. chelonae* resistance, the most frequently found NTM within FLA in previous studies, was investigated. Co-culture experiments demonstrated that the two NTM species were highly infectious, 40% of FLA populations were infected after 1 day. This infection was maintained, without cytotoxic effect on the host, for at least 5 days. Treatment of FLA using cytochalasin D, an inhibitor of actin polymerization, clearly inhibited NTM entry, suggesting an actin-dependent internalization. Also, NTM were located inside amoebal phagosomes, as shown by fluorescent staining, confirming internalization by phagocytosis. The stimulation of phagosome maturation in FLA, using rapamycin, triggered a significant drop from 25 to 50% in NTM survival. The inhibition of phagosome acidification, using NH4Cl treatment, did not impact survival of NTM inside FLA. Taken together, these results suggest that the two NTM strains persisted inside FLA by blocking phagosome maturation, in addition to their natural resistance.
In order to understand the genetic factors involved in phagocytosis resistance, the genomes of both *M. chelonae* and *M. llatzerense* were fully sequenced. Both isolates presented the genomic arsenal described in *M. tuberculosis* for phagocytosis resistance. It indicates that genes involved in phagocytosis resistance are conserved within mycobacteria. This finding is in favor with previous studies suggesting a broad resistance towards FLA across the *Mycobacterium* genus [2].
To conclude, our study gives insights into how NTM resist against environmental predation. Our findings suggest that the passive-active resistance model, added to a stable relationship with amoebae, is a very effective strategy for NTM persistence in the environment.

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Or-44

Cysts of free-living protozoa: a potential vector and shelter for foodborne pathogenic bacteria

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The production of resistant, dormant cysts forms an integral part of the life cycle of many free-living protozoa, allowing these organisms to survive adverse environmental conditions. There is increasing evidence that some bacteria escape digestion by bacterivorous protozoa and can survive and even multiply inside active protozoan cells. In some cases, intracystic survival has also been demonstrated, which is especially relevant as cysts may confer high resistance to unfavorable environments. Given the prevalence of free-living protozoa in food-related environments, it has been hypothesized that these organisms play an important yet currently underinvestigated role in the transmission and epidemiology of foodborne pathogenic bacteria.

The present study investigated the survival capacities of foodborne pathogens inside cysts of the model protozoon *Acanthamoeba castellanii*. Invasion assays, encystment monitoring assays and intracystic (stress-)survival assays were performed using the following pathogen strains: *Salmonella enterica* serotypes Typhimurium and Enteritidis, *Listeria monocytogenes* serotypes 1/2a and 4b, enterohaemorrhagic (EHEC) *Escherichia coli* serotypes O:26 and O:157, *Yersinia enterocolitica* bioserotypes 4/O:3 and 2/O:9 and two *Campylobacter jejuni* strains.

Results indicate that important foodborne bacteria (i.e. *Salmonella enterica, Yersinia enterocolitica, Escherichia coli* and *Listeria monocytogenes*) can survive inside cysts of the ubiquitous amoeba *Acanthamoeba castellanii* and resume active growth after excystment, even when they have been exposed to e.g. antibiotic treatment oxidative stress, osmotic stress and highly acidic conditions. Strain- and species-specific differences in survival period were observed, with *Salmonella enterica* surviving up to three weeks inside the amoebal cysts. These differences were not related to variation in trophozoite invasion/uptake efficiency. Transmission electron microscopy revealed that up to 53% of the cysts were infected with pathogenic bacteria, which were located in the cyst cytosol. Apparently intact cells of another common bacterial pathogen, *Campylobacter jejuni,* were observed inside *A. castellanii* cysts, but no cells were observed after excystment.

The present study indicates that long-term survival of foodborne pathogens in protozoan cysts is possible. This has an impact on the ecology and epidemiology of pathogenic bacteria, as cysts may act as a vector and shelter against harsh environmental conditions. Moreover, intracystic bacteria may not be detected by the standardized biochemical protocols used to detected foodborne pathogens in food and food-related environments.

Or-45

Vibrio cholerae maintain fitness with some help from their friend *Acanthamoeba castellanii*

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Vibrio cholerae is a Gram-negative bacterium that occurs naturally in aquatic environment. Only *V. cholerae* O1 and *V. cholerae* O139 produce cholera toxin and cause cholera, other serotypes can cause gastroenteritis, open wound infection, and septicemia. *V. cholerae* O1 and *V. cholerae* O139 grow and survive inside *A. castellanii* [1,2]. Swedish clinical isolates *V. cholerae* O3, *V. cholerae* O4, *V. cholerae* O5, *V. cholerae* O11 and *V. cholerae* O160 have not previously been shown to interact with *A. castellanii*. The interaction between *A. castellanii* and *V. cholerae* strains can be studied by means of amoeba cell counts, viable counts of the bacteria in the absence or presence of amoebae, and of the intracellularly growing bacteria, visualised by electron microscopy. These results show that all *V. cholerae* can grow and survive outside and inside the amoebae, disclosing that *V. cholerae* O3, *V. cholerae* O4, *V. cholerae* O5, *V. cholerae* O11 and *V. cholerae* O160 all can be considered as facultative intracellular bacteria similar to *V. cholerae* O1 and *V. cholerae* O139, and accordingly the intracellularly milieu enhance growth and survival of bacteria. Taken together *A. castellanii* seems to act as a friend to all serotypes of *V. cholera.*

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POSTER COMMUNICATIONS

POSTER SESSION 1

Chairs

Maritza Omaña Molina

and

Jacob Lorenzo-Morales

P-1

Proteomic profiling of the infective trophozoite stage of *Acanthamoeba polyphaga*

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Acanthamoeba polyphaga is a free-living protozoan pathogen, whose infective trophozoite form is capable of causing a blinding keratitis and fatal granulomatous encephalitis in humans [1], [2]. The damage caused by *A. polyphaga* trophozoites in human corneal or brain infections is the result of several different pathogenic mechanisms that have not yet been elucidated at the molecular level. We performed a comprehensive analysis of the proteins expressed by *A. polyphaga* trophozoites, based on complementary 2-DE MS/MS and gel-free LC-MS/MS approaches. Overall, 202 non-redundant proteins were identified. An *A. polyphaga* proteomic map in the pH range 3-10 was produced, with protein identification for 184 of 370 resolved spots, corresponding to 142 proteins. Additionally, 94 proteins were identified by gel-free LC-MS/MS. Functional classification revealed several proteins with potential importance for pathogen survival and infection of mammalian hosts, including surface proteins and proteins related to defense mechanisms. Our study provided the first comprehensive proteomic survey of the trophozoite infective stage of an *Acanthamoeba* species, and established foundations for prospective, comparative and functional studies of proteins involved in mechanisms of survival, development, and pathogenicity in *A. polyphaga* and other pathogenic amoebae.

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P-2

Interactions *Aspergillus fumigatus* / Free Living Amoebae

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Aspergillus fumigatus, a filamentous fungus often involved in infections of immunocompromised patients, can be recovered in water, such as hospital water networks. Free living amoebae (FLA), well known to be able to feed on microogranisms such as bacteria or fungi is also recovered in these aquatic environments. It has been shown that these microorganisms can resist amoebal internalization so that the amoeba acts as a Trojan horse. We decided to investigate the interactions that could exist between *Aspergillus fumigatus* and FLA (*Acanthamoeba castellanii* and *Vermamoeba vermiformis*) in order to know if the amoebae could reinforce *Aspergillus fumigatus* persistence.

In this way, four types of experiments were performed :
1- cocultures of *A. castellanii* or *V. vermiformis* with *A. fumigatus* conidia were performed in PBS with a multiplicity of infection of 0.1 at 27°C up to 96h. Colony forming units (CFU) of *A. fumigatus* were numbered after 48h and 96h of incubation.
2- *A. castellanii* or *V. vermiformis* were incubated in PBS at 27°C during 72h in order to obtain a supernatant in which *A. fumigatus* was then incubated at 27°C. Fungal CFU were numbered at 48h and 96h of incubation.
3- *A. fumigatus* germination was evaluated by microscopic examination of the conidia incubated in amoebae supernatants.
4- Amoebae viability was determined using trypan blue staining after 48h or 96h of contact between amoebae and conidia.

The results showed that
1- the presence of *V. vermiformis* can significantly increase *A. fumigatus* growth after 48h and 96h of contact. No effect was observed when the fungus was incubated with *A. castellanii*.
2- the contents of *V. vermiformis* supernatant also led to an increase in fungal growth
3- amoebae supernatants induce an earlier germination of *A. fumigatus* conidia
4- the amoebae viability was not influenced by the presence of *A. fumigatus* conidia

This work shows how *V. vermiformis* could contribute to *A. fumigatus* development in water networks. The results of this study confirm that the presence of FLA should be taken into consideration in hospital water networks where they may be in contact with *A. fumigatus* and promote fungal growth.

P-3

Proliferation of *Acanthamoeba castellanii* impaired by *Legionella pneumophila*

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Free-living amoebae of *Acanthamoeba* genus are prevalent protozoa found in the environment [1]. They are considered as opportunistic pathogens that may cause amoebic keratitis and granulomatous amoebic encephalitis [1]. Free-living amoebae feed mainly on microorganisms by phagocytosis, while others have acquired resistance and are not digested within phagolysosomes. These organisms are called amoeba-resisting microorganisms [2]. Some of them can grow inside and exit inducing amoebal lysis. This is the case of the bacterium *Legionella pneumophila* that use its natural host *Acanthamoeba castellanii* to persist in the environment [2]. *L. pneumophila* has evolved several mechanisms to modify host physiology to its advantage. However, no study has deciphered consequences of this disturbance on the host proliferation.

Our goal is to study host functions disturbed upon infection by *L. pneumophila* and in particular the impact on the proliferation of *A. castellanii*. These latter were infected for 2 hours at different multiplicity of infection (MOI) (1, 5, 10 and 20) before being incubated in PYG growth medium containing gentamicin. After an incubation period (16h, 24h, 40h and 48h), the multiplication of *A. castellanii* was evaluated by cell counting. We observed that, over the time, the number of *A. castellanii* in infected sample was lower than *A. castellanii* uninfected sample. This effect was dose dependent since this difference was accentuated with higher MOI. To exclude a cell lysis effect, we measured the release of lactate dehydrogenase from infected *A. castellanii*. For all MOI tested, no lysis effect was found. These data were confirmed using a video-microscopy test where we did not observe any figure of division regarding infected amoebae. Our results indicate that *L. pneumophila* might interfere with the amoebal cell division through an original mechanism that is under investigation.

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P-4

The role of *Acanthamoeba species* in soil fertility

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Objective: In this project we investigate the role of *Acanthamoeba* species in soil fertility. **Methods:** Geochemical and microbial data from an urban allotment in Greenock divided into twelve plots, named MP1-MP12 were used to develop a soil fertility index and genetic data was obtained from AmoebaDB [1, 2]. **Results:** We demonstrate that *Acanthamoeba* possess the genes for converting NO_3^- to NH_4^+ and that it can salvage 41% of the NO_3^- available and secrete NH_4^+ to the external milieu. A combination of PCR culture based technique using soil extracts in non-nutrient agar confirmed the presence of *Acanthamoeba spp* in allotments. Phylogenetic analysis showed that the *Acanthamoeba spp* identified belong to 2 out of 18 genotypes. *Acanthamoeba spp* was not present in MP5, which registers a low pH, high aluminum and manganese content. **Significance:** These results suggest that *Acanthamoeba* is a key player in conversion of NO_3^- to NH_4^+ in soil leading to increased soil fertility. The project will seek to determine the ecogenomics of this protist and its microbial community for bioremediation and restoration of soil fertility.

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P-5

Membrane protein profile of the free-living amoeba *Naegleria fowleri*

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The genus *Naegleria* comprised free living amoebae distributed worldwide and it is found in diverse habitats, only *Naegleria fowleri* produce primary amoebic meningoencephalitis (PAM), an acute fulminant rapidly fatal disease that occurs mainly in apparently healthy children and young people with a recent history of swimming. The disease is acquired by the nasal cavity in contaminated water with the microorganism. Diverse virulent factors have been described in *N. fowleri* such as naeglerioporos, cysteine proteases, and a protein capable to bind to fibronectin, among others. Regarding with the plasma membrane and glycocalix, it is reported to be an important component in the cell recognition and adhesion, which permits an intimate contact between both membranes. Only few studies have been reported with carbohydrate residues in the membrane of *N. fowleri*. However, little is known about the molecules that participate in the adhesion process of this amoeba. The objective of the present work was to analyze the differences in the membrane protein profile in the pathogenic *N. fowleri* and the non-pathogenic *N. gruberi*. For this purpose we performed enrichment membrane proteins (Mem-PER PLUS Kit) and SDS-PAGE. Our results showed a different membrane protein pattern between both strains that could be involved in the adhesion process. This study allows us knowing a better and specific drug target against *N. fowleri* because there is no drug to treat the PAM. This is work was supported by SEP-CONACYT 237523 grant.

P-6

Effect of the olfactory bulb neurochemicals on *Naegleria fowleri* trophozoites

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Naegleria fowleri is a protozoan that belongs to the group of free living amoebae. It is the etiologic agent of a fulminant disease, the primary amoebic meningoencephalitis (PAM); which affects mainly children and young people with swimming history. The infection is acquire by the nasal route and migrates to the olfactory neuroepithelium to arrive and establish in the olfactory bulbs (OB); where it has been observed an important increase in the number of trophozoites, suggesting cell division in this organ. The objective of the present work is to establish whether neurotransmitters or growth factors present in the OB could be related with the establishment of the disease and also if they are involved in the proliferation and migration of the amoeba to the central nervous system (CNS). We identified the role of glutamate and taurine co-incubated with *N. fowleri* trophozoites. We realized growth curves in the presence of small fragments of OB. On the other hand, we used purified compound (glutamate and taurine) to elucidate the role of these specific neurotransmitter; we quantified the number of trophozoites using Neubauer´s chamber and cell viability by trypan blue exclusion assay. The study of chemotaxis was made using the Boyden chamber system with the neurochemicals before mentioned. Our results showed an increase in the number of trophozoites evaluated in the growth curves in presence of the OB plus Bactocasitone medium without fetal bovine serum; this was compared with the control growth curves in the presence of complete medium, With these results we conclude that in the OB there are neurochemicals that promote an effective proliferation of *N. fowleri*. In the presence of the neurochemicals purified, we observed an increment in the number of trophozoites compared with the controls. In conclusion we found that in the OB there are some neurotransmitters that could be participating in the proliferation and migration of *N. fowleri* trophozoites in the brain. This is the first study that allows us to know the role of the molecules present in the CNS that participate in the pathogenesis of the PAM. This work was supported by SEP-CONACYT grant 237523.

P-7

Distribution of *Legionella pneumophila* and its host - free living amoebae in water systems of hospitals and public swimming pools in central Slovakia

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Amoebae found in hospital water systems can serve as a reservoir of potential pathogens and thus be indirectly related to healthcare-associated infections.
The present study was aimed at investigating the presence free living amoebae (FLA) and pathogenic *Legionella pneumophila* bacteria in water supply systems of healthcare facilities and pools with water aerosol attractions in Banská Bystrica region. Water samples were collected from July 2013 to October 2013.
Detection and enumeration of *Legionella* spp. in water samples was performed according to STN ISO 11731. After culturing the isolates were confirmed by a standardized latex agglutination assay and immunoassay using monoclonal antibodies have been identified different serotypes of *L. pneumophila*. The part of the isolates of *Legionella* species was confirmed by MALDI-TOF mass spectrometry. Amoebae were isolated by culture in nonnutritive agar medium (NNA) with *Enterobacter* spp. at 20±2 ºC, 36±2 ºC, and 44±2 ºC.

A total 56 water samples (31 from the pool and 25 from medical facilities) was collected. All water samples collected from swimming pools were negative for presence of *Legionella* species. However, the presence of amoebae in 70 % of these samples, may (but may not) indicate false negativity, as a result of interactions of these organisms. The water samples from three medical facilities was taken in two sampling period. A total 15 samples collected in the first sampling period was eight positive for the presence of *Legionella*. Analysis of samples taken in the second sampling period confirmed the presence of *Legionella* and FLA again. One of the three hospitals has not been confirmed presence *Legionella* spp., but the presence of FLA was proven in all collected samples.

This work was developed with the support of grants KEGA 041UK-4/2015 and Operational Programme Research and Development ITMS 26210120024

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P-8

Rhogostoma minus Belar, 1921 diagnosed in gill lesions of farmed rainbow trout *Oncorhynchus mykiss*

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The study based on an outbreak of rainbow trout gill disease in a farm that experienced dramatic changes in the quality of water supply revealed an intense intravital colonisation of fish gills by the testate amoeba *Rhogostoma minus* Belar, 1921 (Cercozoa, Cryomonadida Cavalier-Smith, 1993). This amoeba species was found in gills altered by advanced hyperplasia of epithelium of all fish examined (15/15). In contrast, naked amoebae related to gill lesions were found in minority of these fish (5/15). They belonged to four genera, *Acanthamoeba*, *Naegleria*, *Vannella* and *Vermamoeba*. Results of the study contribute to the mosaic of findings that turn attention to the possibility of heterogeneous, multi-amoeba species and multifactorial aetiology of gill diseases caused by amoebae in freshwater salmonids.

Detail study of ultrastructure of the isolated strain of *R. minus*, first amphizoic strain assigned to this species, revealed its obligate association with an endocytobiotic prokaryote that was characterised by thick cell wall and numerous fimbriae uniformly distributed on its surface. An ultrastructurally identical endocytobiont was also found in an environmental strain of *R. minus* isolated in a far distant locality. The identification of the endocytobiont and assessment of its impact on host amoeba cell is in progress.

P-9

Anti - *Acanthamoeba* activity of selected nanoparticles – preliminary study

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Objectives: Incidences of *Acanthamoeba* keratitis (AK) are increasingly reported worldwide but results of applied therapy are still disappointing [1]. Antibacterial, antifungal and antiviral properties of certain nanoparticles (NPs), especially nanosilver, were recently widely studied and their cytotoxicity was also evaluated [2]. The aim of this study was to investigate *in vitro* activity of two types of nanosilver and one type of gold nanoparticles on two different *Acanthamoeba* strains.
Material and Methods: Clinical isolate *Acanthamoeba* T4 strain and *A.castellanii* Neff strain cultured in BSC medium at 26°C, resuspended in PBS to 0,5-0,7 x 10^5 cells/mL, were exposed to the tested nanoparticles. Hydrocolloid gold NPs, hydrocolloid silver NPs and tannic acid modified silver NPs at effective concentrations 5ppm, 10ppm and 25ppm were examined and compared in term of their antiamoebic activity. Evaluation of *Acanthamoeba* viability was assessed using methylene blue under light microscope in the Bürker hemocytometer after 6h incubation with NPs and compared to the control cultures. All experiments were performed in duplicate; results were analyzed statistically.
Results: Solutions containing both types of silver NPs at concentrations 10 and 25 ppm showed dose dependent 85-100% reduction in the number of viable trophozoites in comparison to the control cultures. 10 and 25ppm concentrations of gold NPs reduced number of living trophozoites by 60-80%. The lowest concentration of all tested NPs was statistically not effective against *Acanthamoeba* Neff strain. Surprisingly 5 ppm concentration of all tested NPs reduced number of viable *Acanthamoeba* clinical strain trophozoites up to 50%.
Conclusions: This preliminary study aimed to assess *in vitro* antiamoebic effect of selected nanoparticles. Amoebicidal activity of both tested types of silver NPs on *Acanthamoeba* trophozoites was confirmed. It is noteworthy that both silver types of NPs were still active against clinical *Acanthamoeba* strain at the concentration which was shown as non-toxic to host cells [3]. Our results may give further arguments for NPs as potential agents that can be used in both prevention and treatment of AK. Further studies of the influence of selected NPs on both adhesion and encystment processes of *Acanthamoeba* are being continued in our laboratory.

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P-10

Free-living protozoa on dishcloths: occurrence, diversity and possible implications for food safety

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Free-living protozoa (FLP) play an important role in the ecology and epidemiology of foodborne bacteria [1][2][3]. At present, no information on the presence of free-living protozoa on dishcloths, and the simultaneous occurrence of foodborne bacterial pathogens is available. Furthermore, to date no standardized protocols for recovering and quantifying FLP from dishcloths are available.
Dishcloths form a potentially important source of cross-contamination with FLP and foodborne pathogens in food-related environments.

Sampling protocols for the recovery and quantification of FLP from dishcloths were developed and evaluated. Two recovery protocols were tested: the centrifugation protocol and the stomacher protocol. For quantification of FLP from dishcloths, the Most Probable Number method (MPN) and a direct counting method were evaluated.
Both recovery methods and an enrichment procedure were applied to assess FLP occurrence and diversity on used dishcloths. In parallel, the presence and concentrations of important foodborne bacterial pathogens in used dishcloths was investigated. Further, the possible impact of various factors on the FLP presence and abundance, and bacterial load was examined.

FLP were found on 89% of the examined dishcloths; 100% of these tested positive for amoebae, 71% for flagellates and 47% for ciliates. The total number of FLP in used dishcloths ranged from 10 to 10^4 MPN/cm^2. Flagellates were the most abundant group, and ciliates the least abundant. Diversity was dominated by amoebae belonging to vahlkampfiids, vannellids, *Acanthamoeba* spp., *Hyperamoeba* sp. and *Vermamoeba vermiformis.* The ciliate genus *Colpoda* was especially abundant on dishcloths while heterotrophic nanoflagellates mainly belonged to the genus *Bodo*, the glissomonads and cercomonads. Detergent use was identified as a prime determinant of FLP concentrations on used dishcloths. Bacterial load on dishcloths was high, with a mean total of aerobic bacteria of 7.47 $\log_{10}$ cfu/cm^2. *Escherichia coli* was detected in 68% of the used dishcloths, with concentrations up to 4 $\log_{10}$ cfu/cm^2. Foodborne pathogens including *Staphylococcus aureus*, *Arcobacter butzleri* and *Salmonella enterica* subsp. *enterica* ser. Halle were also present.

The simultaneous occurrence of FLP and important foodborne bacterial pathogens makes dishcloths a potential risk factor for cross contamination and can have implications for food safety and public health.

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P-11

Acanthamoeba spp. as possible host organism for the pathogen *Burkholderia pseudomallei*

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Burkholderia pseudomallei is a gram negative bacterium and the causative agent of melioidosis. The disease is endemic in Northeast Australia and Southeast Asia and can be associated with different symptoms including severe pneumonia, septicemia as well as bone and joint infections. Moreover, melioidosis can progress seriously in individuals with an altered immune response and underlying diseases e.g. diabetes and tuberculosis. Early detection and adequate treatment can reduce lethality significantly. The disease can be acquired by inoculation of the pathogen through skin lesions, inhalation by bacteria-containing aerosols from contaminated soil and water and also by ingestion. Until recently, melioidosis was mainly known from the aforementioned regions, but more and more cases from Africa have been reported.
Burkholderia pseudomallei are intracellular bacteria and therefore need host cells for replication. *Acanthamoeba* spp. are found in soil, water, dust and even in air samples and as they produce extremely resistant cysts they are known to be reservoirs for numerous bacterial pathogens, e.g. *Legionella pneumophila*. This ability of the amoebae to survive under extreme conditions like high temperatures and desiccation is one of the main reasons why they seem to be the optimal host of *Burkholderia pseudomallei*.
To proof this hypothesis, soil samples from endemic regions will be screened for amoebae and *Burkholderia pseudomallei* in parallel. During this project soil samples will be collected in Burkina Faso, Ivory Coast, Ethiopia as well as Madagascar and further processed using qPCR with the goal to identify the ones positive for *Burkholderia pseudomallei*. Subsequently, pure cultures of all found *Acanthamoeba* spp. are grown on non-nutrient agar plates to the end that the total DNA is gained. Afterwards a PCR reaction of the amoebic 18S rDNA gene is started and the downstream operations as gel electrophoresis and sequencing are used to genotype *Acanthamoeba* spp..
The aim of the current study is to proof the hypothesis that free-living amoebae function as host cells for survival and multiplication of *Burkholderia pseudomallei* in the environment. This project might lead to a better understanding of the global epidemiology of melioidosis and can help undertaking preventive measures.

P-12

Acanthamoeba spp. co-culture for amplification of respiratory tract pathogens from waters of Central Spain

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Free-living amoebae such as *Acanthamoeba* spp. have a high prevalence in water samples from Spain. Due to the fact this protozoa can act as a Trojan horse of a wide variety of pathogens, the aims of this work were to detect the presence of respiratory tract pathogens in waters of the central region of Spain by amplification through *Acanthamoeba* co-culture; also to evaluate the prevalence of these pathogens in patients with pulmonary symptoms.
For environmental analysis, water samples were concentrated using the IDEXX Filta-Max(®) system, heat treated (50ºC/30 min) and co-cultured with *Acanthamoeba* USP-CR5-A35 (genotype T4). After 8 days of incubation at 33 °C, amoebae and bacteria were harvested, and Triton-X 0.5% treated to lyse amoebae and placed onto cetrimide agar plates. Additionally, samples from patients with pulmonary manifestations (sputum, bronchoalveolar fluid and bronchial aspirate) were analyzed by Gram staining and they were inoculated on blood agar, chocolate agar and MacConkey agar plates. All colonies were identified by biochemical tests.
In water samples, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* were isolated, which were also identified in the analyzed patients samples, among other bacteria. Their presence in patients demonstrated the following prevalence: 10.2% (*P. aeruginosa*), 2.9% (*S. maltophilia)* and 0.2% (*A. xylosoxidans*).
These bacteria are usually transmitted through aerosols emanating from man-made water systems and they are involved in cases of nosocomial and community-acquired pneumonia. Therefore, it is important to be aware on the presence of these bacteria on the environmental waters since its presence can indicate contamination and cause opportunistic infections. Also, the amplification of these bacteria on *Acanthamoeba* observed in vitro can suggest the existence of this interaction in the environment, which is a possible way of escaping the routine water disinfection treatments

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P-13

Acanthamoeba griffini (T3) and *Acanthamoeba royreba* (T4), as etiological agents of amoebic keratitis in Mexico

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Amoebic keratitis (AK) is a chronic sight threatening corneal infection, related with the use of soft contact lens and homemade saline contact lens solutions. Currently, *A. polyphaga* and *A. castellanii* are the most common species isolated from corneal scraping. In less proportion *A. rhysodes*, *A. culbertsoni*, *A. hatchetti*, *A. quina* and *A. lugdunensis*, have been identified in ocular infections. We present two cases related with non-common Acanthamoeba species; *Acanthamoeba griffini* and *A. royreba*, isolated from corneal samples of two amoebic keratitis cases in Mexico City. Both strains were identified by morphological criteria and molecular bases: T3 (*A. griffini*), and T4 (*A. royreba*). The amoebic infection related with *A. griffini*, correspond with a chronic case of one month of evolution. Contrarily the bilateral case in which *A. royreba* was implicated, was diagnosed earlier since it was attended after one week of evolution. Both infections were successfully treated with good prognosis.

In the laboratory, primary isolation was performed by using 1.5% non-nutrient agar plates seeded with live *Enterobacter aerogenes*. Selected pieces of agar were transferred to phosphate-biotriptase-serum glucose (PBSGM) and 2% BactoCasitone. To determine the optimal culture medium and growth temperature, amoebae were incubated at 25, 30 and 37 ºC, which was determined at 30 °C in BactoCasitone medium in both strains.

Acanthamoeba keratitis is a re-emerging infection. The optimal care of contact lenses is necessary and prevention of exposures to environmental water during contact lens wear is recommended as preventive strategies. Now It is important to determine the currently incidence of amoebic keratitis in Mexico.

POSTER SESSION 2

Chairs

Thelma Dunnebacke

and

Antonella Mattana

P-14

Comparative proteomic analysis of an *Acanthamoeba polyphaga* attenuated isolate prior and after virulence enhancement by passage in a rat experimental host

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Acanthamoeba spp. are free-living protist pathogen, capable of causing a blinding keratitis and fatal granulomatous encephalitis [1], [2]. The damage caused by trophozoites in human corneal or brain infections is the result of several different pathogenic mechanisms [3]. To identify possible virulence factors of *Acanthamoeba polyphaga*, we started investigating changes in the proteome of a attenuated isolate prior and after virulence enhancement by passage in a rat experimental host. The *A. polyphaga* strain ATCC 30872 proteome was assessed by two-dimensional gel electrophoresis (2DE) in the pH range of 3-10 followed by identification of protein spots by in tandem mass spectrometry (MS/MS). The 2DE profiles prior (ApPI) and after (ApAI) passage in rat experimental hosts were compared. The analysis of each condition revealed an average of 370 spots for the ApPI sample, as previously established proteomic map of *A. polyphaga* [4] and for the ApAI sample, a total of 413 spots were resolved. Among differentially expressed proteins upregulated in ApAI, isoforms of actin, coronin, peroxidase, peptidase, enolase, peroxiredoxin, CBS, LIM, sinapsin, translation elongation factor Tu (EF-Tu), Heat shock protein 90 alpha, hypothetical proteins and others proteins were identified. In order to identify antigenic proteins from *A. polyphaga*, immunoblot assays of 2-DE using the serum of infected rats were performed. The comparison of the immunoblots with the *A. polyphaga* proteomic map (ApAI) allowed the unambiguous identification of antigenic proteins, namely HSP70, chaperonin GroL, hydroxymethylglutarylCoA synthase, GDP dissociation inhibitor, mitochondrial aspartate aminotransferase, RhoGEF domain containing protein, phosphomannomutase, S-adenosyl-L-homocysteine hydrolase, GAMMA CA3 e ubiquinol cytochrome reductase transmembrane region. Our preliminary results indicated changes proteins expressed by *A. polyphaga* trophozoites after passage in rat hosts, during which it acquires competence to cause disease. These alterations involve several biological processes, since upregulated proteins include some involved in stress response, proteolysis, energetic metabolism, phosphorylation, cell cycle control and proliferation.

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P-15

Variability of interactions between *Candida sp* and *Acanthamoeba sp*

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Free living amoebae (FLA) feed on other microorganisms, as bacterial or fungal elements. In water networks, they can be in contact with different yeasts species. In this study, we began to explore relationships between *Candida albicans* or *Candida tropicalis* and two *Acanthamoeba species : A. castellanii* or *A. polyphaga.*

A. castellanii or *A. polyphaga* trophozoites were incubated with *Candida* blastospores. After 24 or 48 h, the cocultures were plated on Sabouraud agar to count fungal colony forming units (CFU). NH_4Cl were then used to evaluate the effect of inhibition of the phagolysosome fusion on the yeasts growth in coculture with amoebae.

Moerover, the attachment of yeasts to amoebae was evaluated in presence of glucose, galactose or mannose.

At last, trophozoites of each strain of amoebae were incubated during 72 h in PBS at 27°C. Amoebae were then pelleted and *Candida* blastospores were incubated at 27°C during 48 h in the resultant supernatant. After this incubation, fungal CFU were numbered on Sabouraud agar.

In coculture experiments, the presence of *A. castellanii* induced an inhibition of *C. albicans* growth after 48h, whereas an increase of *C. tropicalis* growth was observed. The cocultures of the two yeasts with *A. polyphaga* showed an increase of the *Candida* growth.
In our conditions, the presence of glucose, galactose or mannose did not affect the attachement of the yeasts to the amoebae, whereas using NH_4Cl significantly increased *C. albicans* growth in presence of *A. castellanii.*
The incubation of *Candida albicans* or *Candida tropicalis* with amoebae supernatants led to an increase of fungal CFU .

In conclusion, only the presence of *A. castellanii* trophozoites was able to inhibit *C. albicans* growth and the phagolysosome could be involved in the fungal degradation. Moreover, even if *C. albicans* is destroyed by *A. castellanii* trophozoites, its growth can nevertheless be promoted by metabolites released by the amoeba. The coculture experiments have shown that the relationships between *Candida* and *Acanthamoeba* can be quite different depending on the species. It would be interesting to elucidate in further studies which differences between the species are responsible for thes discrepancies in the behaviors.

P-16

Could Artificial Tears prevent *Acanthamoeba*-keratitis?

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The use of CL has increased in the last years as well as the appearance of dry eye due to their use which makes necessary the treatment with artificial tears. Also, their use is common in corneal lesions. Taking into account that *Acanthamoeba* can infect the cornea through these injuries; the present study has been developed in order to know the influence of some artificial tears in the viability of *Acanthamoeba* trophozoites.

Optava FusionTM, *Oculotec*® and *Artelac Splash*® *multidosis* were selected according their formulation. The viability of amoeba trophozoites (*Acanthamoeba* genotype T4) was determined with Trypan Blue stain at different hours, using *Acanthamoeba* maintained in NEFF saline as a viability control.

The results showed a decrease in viability in the case of OptavaTM and Artelac® during the first 4 hours of the study. After then, the amoebae started to divide reaching concentration levels similar to those of the viability control. In the case of Optima®, a complete cell death was observed after 2 hours of incubation.

Optava® includes in its formula Purite® that is a microbicide that can be killing the trophozoites at the beginning of the incubation but loses its strength after 4 hours. Artelac® doesn't have any preservatives in its formula but also produces the decrease in the viability probably due to the change in the osmolality of the medium, nevertheless after 4 hours this effect is lost. It's important to highlight that both tears have sodium hyaluronate in their composition. This salt has been proven as a stimulator of proliferation of the corneal epithelium therefore, can be also stimulating amoebae growth.

In the case of Oculotec®, the preservative/biocide used is Benzalkonium chloride that in this study have been shown as a good amebicide.

The present study shows, for the first time, the amebicide capacity of artificial tears that might be use in the prophylaxis of AK.

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P-17

Interaction between *Pseudomonas Aeruginosa* and *Acanthamoeba Castellanii*

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It was previously shown that *Pseudomonas aeruginosa* can kill populations of *Acanthamoeba castellanii* . In this instance, *P. aeruginosa* PA103, a clinical isolate that produces significant amounts of Exotoxin A, and the type III secretion substrates ExoT and ExoU was investigated.
The aim of this study to investigate whether environmental isolates of *P. aeruginosa* derived from water, from household environment or the clinical environment show the capacity to kill *A. castellanii*. Nine environmental isolates were tested against *A. castellanii* and all of them showed the capacity to kill *A. castellanii* . The environmental isolates killed *A. castellanii* after two or three days. This is a faster killing compared to the clinical isolate *P. aeruginosa* PA103, which required 5 days to completely kill *A. castellanii*. In addition, we could group the environmental *P. aeruginosa* isolates according to the time requirement for killing *A. castellanii*. *P. aeruginosa* isolates which required only two days for killing were members of clones of *P. aeruginosa* recently identified to be widespread in the environment and/or in patients. On the other hand, *P. aeruginosa* isolates, which required three days to kill *A. castellanii*, were not assigned to clonal families.

P-18

Human polymorphonuclears cells, NETs release in response to *Naegleria fowleri* trophozoites

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Naegleria fowleri is a protozoan that infects humans trough the nasal mucosa causing a disease in the central nervous system known as primary amoebic meningoencephalitis that causes death to the host 3 to 7 days post-exposition, during the infection, we have observed that polymorphonuclear cells (PMN's) play an important role in preventing the pathogen adherence, proliferation and infection. PMN's are the first cells of the immune response to arrive at the site of infection, are able to deploy a wide antimicrobial arsenal that includes phagocytosis, degranulation, ectosomes and extracellular traps (NETs). NETs are composed of nuclear or mitochondrial DNA combined with histones and antibacterial proteins that come from the azurophilic granules of the cell. The NETs are released from the cell to focus its antimicrobial attack as well as to reduce tissue damage and the dissemination of the infection. NETs liberation against several protozoans has been observed previously but not against N. fowleri. In the present work we evaluate the capacity of N. fowleri to induce the liberation of NETs by human PMN cells. Our results show that N. fowleri does induce the liberation of NETs by human PMN cells. Myeloperoxidase and elastase show a time-dependent increase, however this is not enough to eliminate the parasites. N. fowleri also presents the capacity to phagocytize PMN cells as shown by the dwindling concentration of PMN cells as the time of the interaction increases. Our studies suggest that it's necessary a previous activation of PMN cells or an adaptive immune response mediated by antibodies to opsonize and eliminate de parasite effectively

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P-19

Detection and molecular characterization of potentially pathogenic free-living amoebae from water sources in Kish Island, southern Iran

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Objectives
Amoebic keratitis a sight threatening corneal infection that mainly affects contact lens wearers with a history of contact of eyes with tap water sources [1]. Tap waters go through filtration and chlorination processes before human use and thus this may be reflect in lower microbial contamination of such waters [2]. However, this fact depends on many factors such as effective filtration and disinfection systems of the mentioned water sources [3, 4]. The present research was conducted in order to identify the occurrence of potentially pathogenic free living amoebae in tap water sources in Kish Island, a touristic region in Iran..

Methods
Amoebae were detected by using a culture enriched method and by PCR/sequencing the Diagnostic Fragment 3 region of the 18S rRNA of *Acanthamoeba* and in the case of other free living amoebae species PCR/ sequencing analysis of the 18S rDNA gene was carried out.

Results
Overall, out of 55 tap water samples collected from five different part of Kish island, 21 (38.2%) isolates were recovered for free living amoebae after three days to one month of incubation. The results of the present study showed the occurrence of *Acanthamoeba* belonging to T3, T4, T5 and T11 genotypes in tap water sources. Additionally, *Vermamoebae vermiformis* was detected in three water samples. Moreover, Two samples positive for *Acanthamoeba* T3 and T4 also contained *Vermamoeba vermiformis*. Other Free-Living Amoebae were detected in four samples (19%) such as *Miniamoebae* and *Thecamoebae* genera but were excluded from our research.

Conclusion
Overall, the occurrence of 38.2% of potentially pathogenic free living amoebae including *Acanthamoeba* T3, T4, T5 and T11 genotypes and *Vermamoebae* in tap water sources in this touristic region in Iran reflects the urgent need to improve water treatment procedures in order to prevent FLA-related infections in this region. This study is the first report of *Acanthamoeba* genotypes T3, T4, T5 and T11 and *Vermamoeba vermiformis* species in tap water sources in a touristic region in Iran.

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P-20

Acanthamoeba strains isolated from the effluent of a textile factory in Mexico.

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Free-living amoebae present a phase of resistance, which allows them to withstand adverse environmental conditions. However, textile industrial effluents generated mainly in the process of finishing and dyeing of the fabrics, are typically characterized by the presence of residues from colorants and chemicals used in these processes. The objective of the study was to determine the presence of free-living amoebae in the effluent of a textile factory. Samples were taken from the effluent of a textile factory, an aliquot of 50 mL of samples was taken and centrifuged at 1200 g for 15 minutes, and the sediment was seeded on non-nutritive agar with *Enterobacter aerogenes* (NNE). The plates were incubated at 30, 37 and 42°C. At first the amoebae were identified by taking into account the morphological characteristics of the trophozoite and the cyst. For the classification of the isolates at the genotype level, the diagnostic fragment 3 of the 18S rDNA gene was amplified. The obtained products were purified and sequenced and the DNA sequences were compared to the ones available in the Genbank database. There were isolated two strains belonging to *Acanthamoeba* genus. Phylogenetic analysis revealed that isolate AIII-A37 was 95% homologous to other strains belonging to genotype T5, *Acanthamoeba lenticulata*, and that strain AII-A30 was grouped in the cluster of genotype T4, being the higher homologous sequences related to *Acanthamoeba polyphaga*. Amoebae growth at 37 and 42 °C, but were not pathogenic when they were inoculated in mice. The presence of *Acanthamoeba* in the effluent of the textile factory, which contains a variety of chemical residues, confirms the strength of the genus to extreme environmental conditions.

P-21

Eukaryotic cell encystation and cancer cell dormancy: is a greater devil veiled in the details of a lesser evil?

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Objective:
The encysted state of a pathogenic eukaryote and the dormant state of cancer not only have a remarkable conceptual resemblance, but as both are hibernating states of eukaryotic cells, molecular similarities may also exist in signalling pathways, receptors and cytokines [1] that are involved. With the rationale for studying a similar, if not identical process of encystation and excystation in primitive eukaryotes that could possibly help our understanding of the metabolism and protein expression in dormant cancer cells. We intended to extend the knowledge gained by identifying any similarities, to categorize potential therapeutic targets, as was done in the past [2], that is, either to wake and kill or maintain this state of a dormant form of cancer cells for therapeutic gains.

Methods:
We subjected the healthy cancer cell of the prostate gland and healthy Acanthamoeba trophozoites to well known encystation provoking stimuli. The biochemical and epigenetic changes that occurred until a morphological transition to a hibernating (dormant and encysted) stage was documented. The cells were kept in the dormant and encysted state for prolonged periods of time, under constant temperature, osmolarity, pH, and nutritive status. The provoking stimulus was then removed to revert the active cancer cell state of the dormant cells, and trophozoite forms of encysted amoeba.

Results:
Mitochondria appeared to play a key role in nuclear wall development, as these organelles assumed a centripetal layout [3] helping to delimit membrane border, resembling the walling effect seen in cysts. Vital cell surface receptors, enzymes, and minimally needed metabolic pathways involved were found to be similar if not identical in the cells studied for encystation and dormancy. Calmodulin, nutrition sensing glucose receptors (GLUT-1-4), PI3PK, AKT, ERK, MAPK, Na-K ATPase, NHE-Pumps and T3-T-4 cytoplasmic receptors, in particular, were the homologies detected while going into and coming out of the hibernation states of these two eukaryotic cells.

Conclusion:
Drugs targeting the above proteins and enzymes were able to induce, maintain and reverse the cancer cell dormancy. Collectively, these in-vitro studies demonstrate for the first time to our knowledge the homology that exists between cancer cell dormancy and encysted states of pathogenic eukaryotic hibernation states

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P-22

Acanthamoeba sp: fresh isolate from a severe case of keratitis possess electron-dense granules that are lost after long-term cultivation

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Different species of *Acanthamoeba* are capable to produce amoebic keratitis (AK) especially in persons wearing contact lenses. Incorrectly treated, amoebic keratitis may result in permanent visual impairment or blindness. In addition, these amoebas are causative of granulomatous amoebic encephalitis.

The *Acanthamoeba* pathogenicity includes contact dependent (formation of acanthopodia and endocytic cups, phagocytosis of target cells) and contact independent mechanisms that involve parasite-mediated cytolysis and degradation of extracellular matrix components, also secretion of several proteases with cytolytic activity have been reported.

Here we present a comparative transmission electron microscopy study on the presence of EDG in trophozoites of an *Acanthamoeba* strain morphologically identified as *A. culbertsoni* but with genotype T4, in the fresh isolate and after long-term cultivation.

Trophozoites of *A. culbertsoni* recently obtained from a severe human case of keratitis were grown at 36.5 °C and maintained in axenic culture in 2% Bactocasitone (DIFCO, Spaks, MD) supplemented with 10% fetal bovine serum (Equitech-bio, Kerville, TX), and 1% (w/v) antibiotics (10,000 U/lg/ml penicillin-streptomycin). Long term culture of these trophozoites was carried out by more of six months.
Our study reveals abundant EDG in *A. culbertsoni* recently obtained from a human keratitis case. These granules were not present after long term cultivation. This finding suggests that EDG may be produced during contact of the amoeba with the host and they are lost during *in vitro* growing.

P-23

Transmission electron microscopy sample preparation protocols for the ultrastructural study of cysts of free-living protozoa

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Detection and localization of intracystic bacteria and examination of the en- and excystment dynamics is a major challenge, as no suitable protocols for ultrastructural analysis of cysts are available yet. While Transmission Electron Microscopy (TEM) is ideally suited for these analyses, conventional TEM protocols tend to result in low cyst yield and images of poor quality, making them not optimal for further cell biological analysis.

Cysts of free-living Protozoa (FLP) have received increasing attention as they can act as a vector and shelter for bacteria. This identifies a hitherto little known role of FLP cysts in the ecology and epidemiology of pathogenic bacteria. As such, there is a need for a suitable TEM sample protocol for protozoan cysts.

In this study, different protocols for TEM sample preparation of cysts were designed and tested. Two protocols, one based on chemical fixation in coated well plates and one on High Pressure freezing with Automatic Freeze substitution, were selected as most effective for TEM-based ultrastructural studies of cysts. These protocols will allow a better analysis of the cyst structures and a better understanding of bacterial survival mechanisms in cysts. We suggest that the proposed TEM protocols can also be used for weakly adherent cells and fragile cells.

P-24

High prevalence of IgG antibodies to *Naegleria fowleri* in residents of southern Sonora, Mexico

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The presence of free-living amoebae of the genera *Naegleria* and *Acanthamoeba*, which contain pathogenic species for humans and animals, has been demonstrated in different natural aquatic environments in southern Sonora, Mexico. In the particular case of *Naegleria fowleri*, causal agent of primary amoebic meningoencephalitis, isolation has been achieved in some cases in the Yaqui Valley but not in the Mayo Valley. In order to determine indirectly whether populations of Valle del Yaqui and Mayo have been in contact with *N. fowleri*, a comparative study of the immune response was performed in sera from residents of both valleys, through the presence of IgG anti-*N. fowleri*. 500 serum samples from healthy male adults, who attended to donate blood to the Mexican Social Security Institute hospitals at Navojoa (Valle del Mayo), and Ciudad Obregon, Sonora (Valle del Yaqui), were assayed for antibodies against *N. fowleri*. Study participants authorized the use of the serum sample through a letter of informed consent. Humoral IgG response *N. fowleri* was analyzed in duplicate to titles 1: 100 and 1: 500 by ELISA, whereas the characterization of the specificity of the antibody was detected using Western blot. The Mann Whitney U test was used to estimate differences between groups. The results showed a high prevalence of IgG antibodies to *N. fowleri* in residents of southern Sonora, however, the observed data show a significant difference in antibody titer. Western blot analysis revealed the identification of heterogeneous proteins with molecular mass of 37-70 kDa for lysate *N. fowleri*. Also, among the participants' donors' residents from both valleys, the presence of IgG antibodies to *N. fowleri* showed more reactivity in the Mayo Valley, where there are no reports of cases of primary amoebic meningoencephalitis. On the other hand, sera did not recognize the same antigen proteins under study, despite the proximity of the two valleys. We conclude that the titers against *N. fowleri* indicate that exposure to the ameba is common and the differences in proteins recognition could be due to other *Naegleria* sp. that is antigenically similar to *N. fowleri*.

P-25

Free Living Amoebic Infections in Colombia: epidemiological data from 2009-2013

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Introduction: Although *Acanthamoeba* and *Naegleria* were described in 1930 and 1965, respectively, few studies and reports have characterized incidences of their main clinical infections (granulomatous amebic encephalitis and primary amebic meningoencephalitis). Then epidemiological studies are required.

Methods: Current observational, retrospective study aimed to estimate incidences of acanthamoebiasis and naegleriasis in Colombia for the years 2009-2013 based on data extracted from the so-called personal health records system (*Registro Individual de Prestación de Servicios*, RIPS), using the ICD-10 codes B60.1 and B60.2 respectively. Using official population estimates of National Department of Statistics, crude (national and territories) and adjusted incidence (age, sex) rates were estimated (cases/100,000pop).

Results: During the period, 107 cases were reported (median 22/year), 88 due to *Acanthamoeba spp* and 19 due to *Naegleria fowleri*, for a crude national rate of 1.89 and 0.39 cases/1,000,000pop, respectively; 50.5% corresponded to male (for naegleriasis, 73.7% were female [OR=3.52 {95%CI 1.17-10.61}]); 34.6% were 0-9.999 year-old. From the total, for acanthamoebiasis, 25.3% were from Caldas department (9.18 cases/1,000,000pop 2011), followed by Bogota with 16.1% (1.38 in 2009) and Santander with 10.3% (1.97 in 2012). For naegleriasis, 5 cases were from Bogota and 4 from Magdalena.

Discussion: FLA are neglected in many countries in Latin-America and the Caribbean, including Colombia (none articles on Medline of *Naegleria* in the country). Despite the limitations of this study, this is the first attempt to provide estimates of FLA infection incidence in the country, with consistent findings regard affected age groups and geographical distribution. More studies are expected and deserved.

P-26

Spatially distribution of free-living amoeba in industrial water retention

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Free-living amoeba (FLA) are highly diverse and ubiquitous protozoa of freshwater and soil [1]. FLA have been isolated from natural environments (rivers, lakes, springs) and man-made water systems (drinking water networks, poorly chlorinated swimming pools, cooling waters) [2-5]. In their habitats, FLA is a crucial part of microbial communities because they feed on bacteria in biofilm [6] and can also serve as reservoirs of bacteria. Some FLA species might be pathogenic [7] and/or bear pathogenic bacteria [8-9]. Within the FLA population, *Naegleria fowleri* is a thermotolerant FLA of health interest, because it is the causative agent of a primary amoebic meningoencephalitis (PAM) [10]. *N. fowleri* could be found in naturally or artificially heated freshwaters, such as industrial cooling waters [11-12]. Then, to reduce health risk, particularly during recreational activities, treatments are implemented in cooling water systems of French power stations as their cooling waters are released into rivers [13].

The aim of our study was to evaluate the occurrence of FLA, *Naegleria* spp and *N. fowleri* in a industrial water retention where a part of make-up water for a cooling system of a power plant is withdrawn and a part of the blowdown collected.

Water samples of five stations were collected four times a year during two years. Samples were taken at a shallow water, - 3 m and at a maximum depth. Water samples were transferred onto non-nutrient agar plates overlaid with E. coli and maintained at 30°C (NTA) and 43°C (TA). The FLA cells were counted by the most probable number approach [14]. *N. fowleri* were identified using an enzyme-linked immunosorbent assay [15].

No *N. fowleri* has been detected in water samples. The density of FLA cultured at 30°C and 43°C ranges from 10^2 to 10^4 NTA/L and 10 to 10^3 TA/L, respectively, and less than 10^2 *Naegleria*/L have been counted everywhere into the lake. In conclusion, similar horizontal and vertical distribution of FLA and *Naegleria* spp. has been shown into the water column of the retention. Further studies about physicochemical and microbiological parameters are currently performed to better understand ecology of FLA in heated water retention.

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