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Keynote Lecture (Kn)-01

Free-living amebae and infection of the central nervous system

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Infection of the central nervous system by free-living amebae *Naegleria fowleri*, several species of *Acanthamoeba,* and *Balamuthia mandrillaris,* is almost always fatal. The term amphizoic has been applied to these amebae since they are free-living but can cause disease in humans and animals. For the most part, the mechanisms of pathogenicity and the characterization of virulence factors contributive to the pathogenic process remain to be defined. *Acanthamoeba* cause Granulomatous Amebic Encephalitis (GAE), a chronic disease that is more common in debilitated and immune suppressed individuals. A number of *Acanthamoeba* isolated from clinical cases of acanthamoebiasis have been shown to harbor endosymbionts and pathogenic bacteria, indicating a potential for increased pathogenesis. Recent results suggest that proinflammatory cytokines produced in response to *Acanthamoeba* and direct destruction of host cells by amebae contribute to neuropathology, in addition to proteases and phospholipases that the amebae secrete. *Balamuthia mandrillaris* causes encephalitis that also is a chronic progressive infection of the CNS. *Balamuthia* destroy mammalian cells in culture and induce proinflammatory cytokines from microglia that may contribute to neuropathology. Recent studies suggest that these amebae access the brain by attaching to the extracellular matrix protein, laminin. *Naegleria fowleri* causes Primary Amebic Meningoencephalitis (PAM), a rapidly fatal infection of the CNS. *N. fowleri* has "developed" strategies to evade the host immune response; it is resistant to complement-mediated lysis and destroys antibodies. Induction of proinflammatory cytokines and lytic pore-forming proteins appear to be major factors in the pathogenesis of infection. In summary, there is an increasing awareness that free-living amebae have the potential to serve as human pathogens. This potential may be heightened in view of the increasing number of individuals whose immune systems are compromised due to immune suppressive therapy, infection with the human immunodeficiency virus, or use of illicit drugs.
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Kn-02

From *Hartmannella* to the genome project: Past and future of *Acanthamoeba* research

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Found in a wide variety of ecological niches, the ubiquitous isolates of the free-living genus *Acanthamoeba* are one of the most extensively studied protists in science. Since its discovery and initial placement in the genus *Hartmannella* in 1930, until the recent inclusion of *Acanthamoeba* in a genome-sequencing project determining the primary nucleotide sequence of the estimated 33Mb genome of *Acanthamoeba*, this genus has been extensively studied. *Acanthamoeba* has provided a model system of fundamental aspects of cell biology, for studies of cellular movement, of cytoskeletal structures, of mechanisms and variability of pathogenicity, for phylogenetic and evolutionary studies investigating eukaryotic evolution and protistan relationships, and recently in studies investigating mechanisms and evolution of symbiotic events. Numerous bacterial species have been shown to be temporary or long-term symbionts of *Acanthamoeba*. This relationship may offer protection and enhance the pathogenic potential of these bacteria. The understanding of these host/symbiont relationships is an active area of current investigations. In addition, the existence of gene transfer from symbionts to *Acanthamoeba* is also under investigation. The genome project is being performed on *Acanthamoeba castellanii* Neff, a widely studied isolate whose mitochondrial genome has been sequenced previously. Classification of *Acanthamoeba* species and iso-

lates has been enhanced in recent years by molecular sequencing methods, resulting in a more quantitative, robust, and repeatable identification of isolates. Sequencing of the *A. castellanii* Neff genome will allow researchers to expand numerous aspects of *Acanthamoeba* research and provide insights when genome-wide comparisons to other protists are completed. However, significant sequence variation has been observed between different *Acanthamoeba* species, and the sequencing of the Neff genome alone will not completely answers questions regarding the differences between *Acanthamoeba* species. Nonetheless, the excitement provided by the current genome project cannot be overstated and its significance to the current status of *Acanthamoeba* research will be discussed

Oral (Or)-01

Environmental behavior of FLA released by artificially heated water

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Free-living amoebae (FLA), such as the pathogenic *Naegleria fowleri*, can proliferate in water cooling circuits due to the increase of temperature. Presence of *N. fowleri* is detected in the cooling circuits of nuclear power plants. In order to reduce the quantity of *N. fowleri* released in the aquatic environment, water treatments (UV or monochloramine) are performed on cooling circuits. These treatments reduce the amoebal concentration in the circuit waters in order to respect the authorized values in downstream rivers. However there is still some small concentration of *N. fowleri* released in the river and nothing is yet known about the long term effect of this pathogen release on the FLA community either in the water column and/or the sediment river.

The FLA could either survived and be transported in the water column and/or fall in the sediment where they could stay alive and eventually be released in the water column under certain environmental conditions. All theses hypothesis were studied by sampling water and sediment in plant downstream rivers (Vienne River, Loire River, Garonne River). Several campaigns were performed between 2004 and 2006 upstream and downstream of the plants. The identification and quantification of thermophilic and non thermophilic amoebae, *Naegleria* species and *N. fowleri* were performed using traditional methods of culture (at 30°C and 43°C). A flagellation test and a ELISA assay were performed respectively to confirm the identification of *Naegleria* genus and *N. fowleri* species.

The results of the campaigns showed that some *N. fowleri* were detected in the river, closest to the plants but the concentration of *N. fowleri* measured was always more or less equal to the detection limit. Moreover, as the distance from the plant increased, the percentage of positive samples decreased. Finally, no accumulation of *N. fowleri* was detected in sediments and no sign of *N. fowleri* transfer from sediment to water was observed.

Or-02

Selecting treatments to control pathogenic amoebae *Naegleria fowleri* in cooling systems: An integrated approach for power plants

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Closed loop water-cooling circuits of nuclear power plants are likely to face pathogenic free-living amobae (*Naegleria fowleri*) proliferations. Therefore EDF has implemented monochloramine as an effective cooling water treatment since 1999. However, to reduce the chemical releases in the environment, alternatives are explored. Compared to other cooling circuits, EDF nuclear power plants circuits are characterized by high water flows (several thousands m^3/s), high exchange areas (several millions m^2) and a river water supply without any pretreatment. Thus, the choice of the appropriate disinfection treatment is always a tricky issue. Four major criteria have to be attained: effectiveness, absence of environmental and public health impact, preservation of equipment and thermal exchange performances as well as cost-acceptability. For these purposes, EDF R&D has developed a methodology in three phases:
First phase: static laboratory experiments assessing:

> the effectiveness of the treatment: e.g. determination of the couples biocide concentration/application time with regard to pathogens reduction.
> the behaviour of the biocide with regard to water supply quality: characterization of biocide consumption and identification of its by-products.
These trials lead to select biocides for the second phase.
Second phase: experiments conducted with two industrial pilots simulating nuclear power plants cooling circuits (one of which being used as a reference without any treatment) to:
> test the treatment in dynamic operating conditions,
> identify the optimal treatment conditions with regard to effectiveness in water as well as in biofilms, biocide consumption by surfaces, nature and quantity of by-products...
These trials lead to a first sizing on full scale and assess the nature and quantity of products released by the treatment.
Third phase: When first and second phases results are promising, complementary studies can be conducted to evaluate the ecotoxicity of the discharge.

Different treatments have been tested using this approach: chlorine dioxide, peracetic acid, hydrogen peroxide, combination of hydrogen peroxide and UV, organic filming agent. Results are presented and compared with monochloramine as a reference.

Or-03

Ecology of free-amoebae in real in-house water networks - *Amoeba/Legionella* interactions: disinfection elements –

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Free amoebae are protozoa largely encountered in environmental and hydric niches. They have the ability to multiply without the help of any host. A few species are direct human pathogens (e.g. *Naegleria fowleri*) and a lot of them can be reservoirs for pathogenic bacteria like *Legionella pneumophila*.

To evaluate the occurrence and the characteristics of free living amoebae in in-house water networks, an analytical campaign was conducted in real distribution systems (cold and hot water). Moreover, another objective was to study the impact of curative treatments on amoebae and intra-amoebae *Legionella*. The applied treatments were those usually used and accepted by French authorities on real networks to manage *Legionella* risk.

Methods: Analysis campaign and Amoeba identification: 36 sites were tested for the presence of amoebae (five sampling points for hot and cold water). Total amoebae were quantified by the method (MPN) described by Pernin et al. Identification was made by microscopic morphology determination.
Treatment assays: Different disinfection or cleaning treatments were studied *in vitro* on an amoeba model (*Acanthamoeba castellanii*) which was infected or not by *Legionella pneumophila*. The treatments were:
> *Thermal shock : : 70°C, 1h*
> *Chlorine shock: 50 mg/l, 1 h*
> *Chlorine dioxide: 1; 5; 10 mg/l, 1h*
> *Basic shock: pH 12 , 1 h*

Results & Conclusions: In French in-house networks, the most representative amoebal genus is *Hartmannella*. For several samples, *Acanthamoeba* were isolated.
Among installation network parameters (temperature, pipe material, presence of loops, type of hot water production), temperature (up to 60°C) seems to be the preponderant parameter controlling the presence of amoebae.
Disinfection assays show that
> Chlorine treatment permits a 2.5 Log decrease of amoebal trophozoites but does not lead to encystations while thermal and basic treatments promote encystation.
> Our results confirm the protective effect of the amoebae for *Legionella* against disinfection based on oxidative molecules.
These results may have an impact on the way to manage in-house water networks.

Or-04

Legionella species in amoebae isolated from saline environments

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Amoeba cultures were enriched from sediments from four sites in the New England estuarine system of Mt Hope Bay, and from sediments from Farmington Bay on the east side of Antelope Island in the Great Salt Lake. Sampling sites in Mt Hope Bay included the thermal plume region of a power plant, a secondary sewage outfall, a brackish environment and a coastal marine region considered impacted by normal bay conditions. The sites in the Great Salt Lake included a canal used by sewage treatment plants to release water into Farmington Bay, areas near the opening in the causeway that separates the Farmington Bay from Gilbert Bay, the bathing beach in Bridger Bay (in Gilbert Bay) and in Bear River Bay. Cultures were enriched using both minimal and non-nutrient agar plates, made with fresh water, brackish water or saltwater. Recovered amoeba cultures were assayed for the presence of *Legionella* species using nested PCR and primers specific for the genus, and positive samples were then screened with nested primers specific for *L. pneumophila*. Fifty-two of 90 isolated amoeba cultures were positive for the presence of *Legionella* species. *L. pneumophila* was detected by PCR in ten of the amoeba cultures growing on marine media. Our results show that amoebae capable of growing in saline environments harbor not only a diverse collection of *Legionella* species, but also species pathogenic to humans.

Or-05

N-chlorotaurine (NCT) reveales highly cysticidal activities against *Acanthamoeba* spp.

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Objectives: *Acanthamoeba* spp. are the causative agents of *Acanthamoeba* keratitis (AK) occurring mainly in contact lens wearers, and of skin lesions, granulomatous amoebic encephalitis (GAE) and other disseminating infections in the immunocompromised host. Problems of therapy are the high toxicity of the drugs and the resistance of the cysts against them. Therefore, new anti-*Acanthamoeba* drugs are needed. We investigated the anti-*Acanthamoeba* activity of N-chlorotaurine (NCT), an endogenous mild antiseptic. Tolerability of 55 mM (1%) NCT on the human skin and up to 0.1 M (3%) in the human eye was demonstrated before.

Methods: Trophozoites and cysts were cultured according to their needs and were treated with NCT and cotreated with NCT and NH_4Cl and β-alanine, respectively in different culture media. Effectiveness was observed after 1, 6 and 24 h by microscopy and trypan blue staining.

Results: It was shown that NCT has amoebicidal and cysticidal qualities, both in phosphate buffered saline (PBS) and in specific amoebic culture medium. Already after 6 hours treatment with 10 mM NCT, trophozoites of all strains investigated showed at least a 2 $\log_{10}$ reduction. A delay of excystation was observed when cysts were treated with 55 mM (1%) NCT, whereas a complete failure of excystment was achieved by cotreatment with 1% NCT and 1% NH_4Cl. Efficacy of NCT was generally higher in PBS, than in culture medium.

Conclusion: Altogether, NCT clearly demonstrated amoebicidal activity at concentrations well tolerated by human tissues and might be useful as a topic drug in *Acanthamoeba* infections. Addition of ammonium chloride can be considered to enhance the activity.

Or-06

Anti-*Acanthamoeba* efficacy and toxicity of miltefosine in an organotypic skin model

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Acanthamoebae are the causative agents of the so called *Acanthamoeba* keratitis (AK) on the one hand and of disseminating infections such as skin lesions, pneumonitis and the granulomatous amoebic encephalitis occurring mainly in immunocompromised individuals on the other hand. The treatment of *Acanthamoeba* infections is still problematic, due to the lack of sufficiently effective and also easily manageable drugs. In a previous study we have shown that out of eight different alkylphosphocholines tested miltefosine (hexadecylphosphocholine) had the highest activity against *Acanthamoeba* spp.

In the current study the suitability of miltefosine for the topical treatment of *Acanthamoeba* eye and skin infections was evaluated in vitro. The storage life and the time dependency of the efficacy of different concentrations of miltefosine against trophozoites and cysts of various *Acanthamoeba* strains were assessed in a microtiter plate system. Moreover, the susceptibility of other important eye pathogens to miltefosine and synergistic and adverse effects of combinations of miltefosine with other anti-*Acanthamoeba* substances were evaluated. An organotypic skin model was adapted for investigating the penetration of acanthamoebae into the epidermis and the human tissue tolerability against anti-*Acanthamoeba* effective concentrations of miltefosine. It was shown that miltefosine can be stored as a 2 mM stock solution but also as 50 µM dilution over a period of 6 months at 4°C without any loss of activity. Moreover, acanthamoebae were shown to penetrate the skin within 48 h, while treatment with miltefosine prevented this penetration. The toxicity of anti-*Acanthamoeba* effective concentrations of miltefosine lies considerably below those of substances used for AK treatment.

Miltefosine has been successfully used for the oral and topical treatment of leishmaniasis and may therefore also be a promising new candidate for the topical treatment of *Acanthamoeba* infections.

Or-07

Effect of therapeutic chemical agents *in vitro* and in experimental meningoencephalitis due to *Naegleria fowleri*

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Objective: Inhalation of fresh water containing the free-living ameba, *Naegleria fowleri,* leads to a potentially fatal infection known as primary amebic meningoencephalitis (PAME). Amphotericin B is an only agent with clinical efficacy in the treatment of PAME in humans, but therapy with this drug is often associated with adverse effects on the kidney and other organs.

Methods: In an attempt to select other useful therapeutic agents for treating PAME, the amoebicidal activities of the drugs, such as antibacterial (clarithromycin, erythromycin, hygromycin B, neomycin, rokitamycin, roxithromycin, and zeocin), antifungal (amphotericin B), antiprotozoan (miltefosine), and anti-brain disease antipsychotic agent (chlorpromazine) were examined in this study. Then, the *in vivo* efficacy of the drugs showed the effective amoebicidal activity was investigated in a mouse model of experimental meningoencephalitis due to *N. fowleri*.

Results: In results, the growth of amoeba was inhibited effectively in treatment of hygromycin B, rokitamycin, roxithromycin, amphotericin B, miltefosine, chlorpromazine, respectively. Particularly, when the *N. fowleri* was treated with rokitamycin and amphotericin B, the minimal inhibitory concentrations (MIC) of the drug were 6.25 µg/ml and 0.39 µg/ml on 2 day. Also, when the amoeba was treated with hygromycin B, rokitamycin, and amphotericin B on 6 days, each drug showed 100% of amoebicidal activities against *N. fowleri*. In experimental meningoencephalitis, survival rates of *N. fowleri* infected mice treated with roxithromycin, amphotericin B, miltefosine, chlorpromazine, and rokitamycin showed 25%, 40%, 55%, 75% and 80% during one month, respectively. Their average of mean time to death was 17.1 days, in comparison with 11.2 days in control. In all cases, effect of drug was the best in 3 times administrations following 3, 7, and 11 days at dose of 20 mg/kg except amphotericin B (10 mg/kg).

Conclusion: Finally, rokitamycin and chlorpromazine have both *in vitro* and *in vivo* activities against *N. fowleri*

and may be useful therapeutic agents for treatment of PAME, instead of amphotericin B.
Key Words: *Naegleria fowleri*, amphotericin B, rokitamycin, chlorpromazine, PAME

Or-08

Structure-activity relationship and selectivity of amoebocidal activity

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At the XIth International Meeting on the Pathogenic Free-Living Amoebae, we had explained a screening method for amoebocidal compounds from microbial metabolites; a diffusion method with impregnated paper disc on agar plated for inhibition of growth of *Naegleria*. The active compound, which was later identified as 1-mono-O-linoleoyl-grycerol, had inhibited the growth of *N. lovaniensis* trophozoites at MIC of 31.2µg/ml by the diffusion method and induced cell destruction at IC_{50} of 8.0µg/ml in a hole-slide glass.

In the present experiment, we have examined the quantitative structure- activity relationship analysis of amoebocidal activity of this compound and its selective activity on amoeba species. Using a hole-slide glass with 10 wells with a diameter of 6mm and 0.1mm in depth, each of the wells was filled with 20µl of different compound solutions at a final concentration of 100µg/ml in ethanol-Tween 80, and was air-dried. Then, 20µl of the amoeba suspension (around 100cells/well) supplemented with 0.25% Trypan Blue was transferred into the wells, and the whole-slide glass was attached firmly with a cover glass with double-sided sticky tape to make wet chambers. Observation was made under light microscope for up to 120 min to determine the number of cell death.

It was demonstrated that around 90% of the *N. lovaniensis* trophozoites underwent destruction within first 10 min of incubation when they were transferred into a well precoated with 1-linoleoyl-glycerol, while those in a well precoated with 1-stearoul-glycerol remained intact. The trophozoites transferred in a well precoated with 1-oleoyl-glycerol gradually underwent destruction in 80 or more minutes of incubation. Interestingly, the trophozoites were not affected by the compounds in the form of free acids, namely linoleic or oleic acid, or in the form of alcohols, namely linoleyl or oleyl alcohol.

Possible amoebocidal activity of the compounds was also tested against other amoeba species such as *Hartmannella* sp. H1-1 and *Acanthamoeba* sp. AC-1 isolates both of which were originally isolated from the soil in our university campus and have been maintained by serial passages *in vitro* in our laboratory. *Hartmannella* sp. HT-1 was slightly sensitive to 1-Linoleoyl-glycerol and 1-oleoyl-glycerol, and 20% to 40% of the trophozoites underwent destruction within 120 min of incubation. Neither of these compounds exhibited any amoebocidal activity against *Acanthamoeba* species.

Or-09

Amoebic gill disease in farmed Atlantic salmon in Tasmania: Research progress

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Amoebic gill disease (AGD) has impacted on the marine culture phase of Atlantic salmon (*Salmo salar* L.) in Tasmania since the early 1980's and remains a serious health problem in Tasmania. Some fundamental aims of the research are to unequivocally identify the aetiological agent of the disease, refine the disease challenge model and to test the efficacy of vaccine candidates. It has been known for some time that the causative agent(s) of AGD belonged to the genus *Neoparamoeba,* a marine Gymnamoeba, but identification to species level was problematic with 2 species, *N. pemaquidensis* and *N. branchiphila* both being isolated and cultured from AGD-affected salmon. However, using PCR amplification and specific oligonucleotide probes on AGD-affected gill tissue significant progress has been made and the aetiological agent has now been identified (Young et al., 2007). However, this new species remains to be cultured. Two AGD challenge models have been validated and used to monitor attachment of the amoeba to the gills and the onset of pa-

thology and to test the efficacy of prospective vaccines. The first is a short term model with potential to test the ability of treatments to block amoeba attachment to the gills and the second a traditional challenge to a pre-determined level of morbidity in challenged salmon. This paper will present an update of the most recent developments.

Young, N.D., Crosbie, P.B.B., Adams, M.B., Nowak, B.F. and Morrison, R.N., 2007. *Neoparamoeba perurans* n. sp., an agent of amoebic gill disease of Atlantic salmon (*Salmo salar*) International Journal of Parasitology (in press)

Or-10

Another real-time PCR assay detecting *Balamuthia mandrillaris* DNA

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A TaqMan real-time PCR assay was established specifically targeting the RNase P gene of *B. mandrillaris* amoebae. The assay detected down to 0.3 amoebae-equivalents in dilutions of DNA extracted from axenic *B. mandrillaris* cultures. The assay did not detect DNA from closely related *Acanthamoeba* species (*A. hatchetti* 2HH, *A. lenticulata* 72/2, *A. castellanii* 1BU), nor DNA extracted from a murine macrophage-like cell line (RAW 264.7), murine tissues (brain, lung), a human neuroblastoma cell line (Kelly), or human tissue (brain). The assay efficiently detected *B. mandrillaris* DNA in spiked cell cultures, spiked murine organ homogenates, infected mice, and DNA preparations from 2 patients with proven balamuthiasis. This real-time PCR assay showed similar sensitivity as the one published by Qvarnstrom et al. (2006 J Clin Microbiol 44:3589) which targets *B. mandrillaris* 18S ribosomal DNA and it could be combined with the latter in a duplex assay for enhanced specificity and reliability.

Or-11

Challenges in the diagnosis of balamuthiasis

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Balamuthia mandrillaris is a free-living ameba and is known to cause a devastating central nervous system infection, granulomatous amebic meningoencephalitis (GAE), in humans and other animals. In some cases the disease may disseminate to other organs including lungs, prostate, kidneys etc. Although GAE manifests as CNS infection in many cases the infection may occur initially as a skin ulcer especially on the extremities. In South America, especially Peru, lesions can occur on the face and torso as well, before invading the CNS. Balamuthiasis cases have been recorded from all over the world and the outcome, almost always, is death. There are about 150 reported cases of balamuthiasis worldwide. The infection is usually identified at autopsy but in several cases it was identified premortem in brain biopsy sections. In three instances, because of early detection, the patients have survived the infection with minimal or no neurological deficits after treatment with multiple drugs. Identification of *Balamuthia*, especially in the biopsied brain sections can be challenging. We discuss here the difficulties involved in arriving at a diagnosis in two recent cases. Of the two cases, one was a young girl of Hispanic ethnicity from Colorado, and the other a 35-year-old male also of Hispanic ethnicity from California. In both cases initial examination of H&E-stained sections did not reveal the identity of the infecting organism, even though the serum antibody titer was $\geq$ 64. Additionally, in the California case, a PCR of the fresh brain was positive for *Balamuthia* but the immunofluorescence staining was negative. Further examination of multiple stained and unstained sections together with immunofluorescence and PCR finally led to the correct identification of the infecting agent. It is believed that both patients are under treatment for balamuthiasis and may survive this infection.

Or-12

AFLP fingerprinting reveals intraspecific genetic diversity in *Naegleria fowleri*

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Genetic diversity among different *N. fowleri* strains, as well as other ecological relevant amoebae species was evaluated with the AFLP method. Discriminatory and reproducible DNA profiles were found for all different reference species. The *N. fowleri* strains clustered into two main groups, containing the same strains as the two groups obtained after rDNA sequencing studies. One subcluster of *N. fowleri* containing discriminatory and reproducible DNA fingerprints differentiated into three intraspecific genotypes. The results indicate that the AFLP technique is a valuable tool for (intra)specific genotyping of *Naegleria* sp. This molecular fingerprinting method could be useful in tracking the source of (possible) *N. fowleri* contamination in aquatic habitats or even tracking the source of human or animal infection with this pathogen.
Keywords: *N. fowleri*, AFLP, intraspecific genotypes

Or-13

The phylogenetic structure of *Acanthamoeba* as seen through the window of the small subunit ribosomal RNA gene: Genotypes and "species" do not reflect the same images

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The genus *Acanthamoeba* contains more than 20 described species, based on morphological or cytological characteristics. Phylogenetic analysis of the nuclear small subunit ribosomal RNA gene *(Rns)* suggests that species do not correspond to phylogenetic units. Previous studies identified at least 12 sequence types within *Acanthamoeba* (>5% pairwise divergence between *Rns* sequences). Many sequence types also contain significant phylogenetic subgroups, with divergence between 1-5%, especially in type T4, the most common sequence type seen in environmental or clinical samples. Phylogenetic analysis suggests at least six significant subtypes within T4. Data from "almost complete" *Rns* sequences (sequences > 2000 bases) are available for over 185 *Acanthamoeba* isolates, with 133 genotype T4 isolates. Examination of correspondence between sequence type and species classification indicates that sequence types contain multiple nominal species, while particular nominal species occur in multiple sequence types. Twenty different nominal species are represented, constituting 71 sequences, while the term *Acanthamoeba* sp. applies to the remaining sequences. Twelve of twenty named taxa are represented by multiple isolates, but only three taxa have all isolates placed within a single sequence type or T4 subtype (12 *A. lenticulata* isolates in type T5; 2 *A. giffini* isolates in T3; 2 *A. lugdenensis* isolates in the same T4 subtype). *A. castellani* is associated with T1 and four subtypes of T4, while *A. polyphaga* isolates are identified as T2, T3 or four different subtypes of T4. The disconnection between species and genotype is even more evident when examining the distribution of nominal species within sequence types. Seven sequence types (T2, T3, T5, T6, T9,T11 and five subtypes of T4) contain multiple nominal species. Only sequence type T5 contains a single species (*A. lenticulata*), while other sequence types contain 2-6 nominal species each. Clearly, species nomenclature within *Acanthamoeba* has little relationship to phylogenetic classification. (Supported by grants from NIH)

Or-14

Acanthamoeba spp. in tap water of houses of contact lens wearers

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Several species of Acanthamoeba can produce Acanthamoeba keratitis (Ak) and Granulomatous Amebic Encephalitis (GAE) in human and animals. A survey was carried out in the metropolitan area of Mexico city to determine the presence of Acanthamoeba in tap water of houses of contact lens wearers and its relation with the quality of the water. One liter samples of tap water of 27 houses were taken by duplicate and the saline solution contained in the contact lens boxes were collected. A total of 108 samples were analyzed. Samples were filtered and put onto NNE media; plates were incubated at 25 ºC and 37 ºC respectively. The isolates were identified on the basis of their morphological features. Total (TC) and faecal coliforms (FC), water temperature, pH, dissolved oxygen (O.D.) and residual free-chlorine were measured. Forty-nine isolates of Acanthamoeba (45.3%) from the 108 water samples were obtained. The greater number was obtained from the cistern (49%) followed by the roof tanks (27%), the municipal network intakes (12%) and tap water of wash basin of the bathroom (12%). No amoeba was isolated from the saline solutions of the contact lens boxes. FC were detected in 37% of the water samples, and TC in 44.4%. The higher number of amoebae was isolated from the places where the water is stored, which favour the evaporation of residual free-chlorine and thus stimulate the proliferation of amoebae, bacteria and other microorganisms are stimulated. Besides, the containers are not always are cleaned regularly; therefore the possibility of contamination is higher. Most of the isolates were associated with the presence of O.D. (1.2 - 5.4 mg L^{-1}), low levels of residual free-chlorine (below 0.2 mg L^{-1}) and bacterial contamination. Most of isolates were non pathogenic, however the presence of Acanthamoeba in domestic tap water is a potential hazard since some species of these genera are able to cause Acanthamoeba keratitis and GAE. These results show that domestic tap water is an important source of these organisms.
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Or-15

Characterization and identification of various *Acanthamoeba* isolates from the Philippines

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Six *Acanthamoeba* spp were obtained from different sources. Two were from hot springs, two from human nasal cavity, and two from keratitis patients. Isolates were cloned and each clone was grown either in non-nutrient agar plates with heat-killed *E. coli* and/or in proteose-peptone-yeast-glucose medium after axenization. Characterization was based on (a) trophozoite and cyst morphology using phase contrast and electron microscopy, (b) temperature tolerance, (c) growth rates in agar culture (d) growth patterns, (e) ability to migrate in an "under-agarose system, (f) osmotolerance and (g) cytopathic effects on Vero cells.

Based on cyst morphology 5 clones belong to Group II, while one clone from nasal cavity isolates is classified as Group III. From the DNA sequence of the 18s rRNA gene, 2 isolates were identified as identical to *A. castellanii*, and 1 each were identical to *A. lenticulata* and *A. triangularis*. Remaining two of the *Acanthamoeba* isolates are yet to be identified.

Or-16

Occurrence of *Acanthamoeba* spp. in the nasal cavity of selected human populations in Metro Manila, Philippines

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Free-living potentially pathogenic and opportunistic amoebae, especially those belonging to the genus *Acan-*

thamoeba are ubiquitous unicellular organisms. They have been isolated from soil, dust, air, sewers, eyewash stations, air-conditioning units, contact lenses, nasal epithelium and many other common areas. These organisms have two life forms, an amoeboid trophozoite from and a dormant cyst form. Some *Acanthamoeba* species cause fatal granulomatous amebic encephalitis (GAE), amoebic keratitis as well as cutaneous lesions and sinusitis in humans. They may also harbor pathogenic bacteria in their cytoplasm. In this study, nasal swabs were obtained from volunteers of different sample populations in Metro Manila, Philippines to associate presence of ubiquitous free-living amoebae with occupational risk. The populations consisted of fifty high-rise office workers, fifty factory workers and fifty public utility jeepney (PUJ) drivers. An additional fifty-seven unemployed volunteers were included in the study. Organisms from the nasal swabs were allowed to grow in agar plates containing heat-killed *E. coli*. Plates inoculated with nasal swabs from office and factory workers as well as from the unemployed volunteers were negative for growth of amoebae. However, cultures of nasal swab samples from 2 PUJ drivers showed trophozoites and cysts. The amoebae were cloned and labeled as Clone D-26 and Clone D-38. Morphological characterization showed that the cysts and trophozoites are from the genus *Acanthamoeba*. Based on Pussard and Pons classification, Clone D-26 belongs to Group III (GIII) while Clone D-38 belongs to Group II (GII). Results of 18srDNA sequence typing/T-typing revealed that Clone D-26 is identical to isolates of *A. lenticulata* which is grouped as GIII and belongs to T5. Clone D-38 is identical to isolates which include *A. castellanii* which is grouped as GII and belongs to T4. This is the first report on the detection and isolation of *Acanthamoeba spp.* from nasal cavities among Filipinos.

Or-17

Recent interesting identifications of free-living amoebae from the Brussels laboratory

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Since the last conference two years ago I have obtained the following interesting identifications by sequencing PCR amplified rDNA from new isolates.

Eight new *Naegleria* spp. from Arctic and sub-Antarctic locations constitute two new clusters in the phylogenetic tree of the genus. The total number of *Naegleria* spp. is now 47.

Identification of *N. byersi* and *N. indonesiensis* in samples from Mexico (collaboration with ITSON, Cd. Obregon, Mexico). Both species were previously only known from the opposite side of the world. Identification of *N. angularis* in water samples from Benin in Africa (collaboration with Institute of Tropical Medicine, Antwerp, Belgium). This species had been detected only in Peru and is one of the few *Naegleria* spp. with a particular cyst morphology.

Identification of two new *Tetramitus* spp. isolated in Arizona (USA), *T. anasazii* and *T. hohokami*, the latter also found later in samples from Sinoloa and Sonora (Mexico).

Identification of *Vahlkampfia ciguana* in Chihuahua (Mexico), species previously found only in Spain (Isle of Tenerife).

Identification of *Acanthamoeba* genotype T4 in a fatal encephalitis in a patient treated for cryoglobulinemia (collaboration with Gasthuisberg University Hospital, Leuven, Belgium). Identification of genotype T5 (*A. lenticulata*) in a fatal disseminated *Acanthamoeba* infection in a hart transplant patient (collaboration with Hôpital Pitié-Salpêtrière, Paris, France).

50 % of environmental *Acanthamoeba* isolates from Arizona (USA) were identified as genotype T5 (*A. lenticulata*), the other 50% were genotype T4. The majority of *Acanthamoeba* isolates in samples from Chihuahua (Mexico) and Mongolia were identified as genotype T4, but in Mexico also genotypes T1 and T15 (*A. jacobsi*) were identified.

Or-18

The feeding of *Balamuthia mandrillaris* on cultured cells

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Microscopic observations of live cultures of *Balamuthia mandrillaris* on a sheet of mammalian cells showed that individual amebas could be distinguished and followed before the occurrence of cell sheet disruption and/or the development of lesions, areas cleared of cells bordered by a dense rim of feeding amebas. An early indication of ameba-cell contact was the presence of small, clear vesicles (cvs) that looked like “holes” in the cytoplasm of the cells although such cells excluded vital dyes. The cvs occurred only in cultures with amebas; they may represent a deposit of an amebic enzymes, or the removal of cellular material. Both the number of cells with cvs, the number of cvs per cell and the numbers of ameba increased near expanding lesions. Timed photographs showed an ameba extend a pseudopodium until the tip reached into the cytoplasm of a cell where it moved about leaving visible change in the cell when it was retracted; amebas were seen with their pseudopodia spread in the cytoplasm of several cells at the same time; an ameba within the cytoplasm of two adjacent cells was seen to move and to exit the cells. In rounded cells near the rim of a lesion, whole amebas were seen to enter, and move about within the cytoplasm before leaving the cell. The cytoplasm was consumed first, then the denuded nuclei that no longer excluded vital dyes; mammalian chromosomes in the form of a mitotic plate were not consumed. Cellular invasion of monkey kidney cells or CHO cells by the pseudopodia and/or the whole ameba was seen for *B. mandrillaris* isolates from two humans and from two soil samples. There was no indication of cellular cytopathology prior to the invasion by amebic pseudopodia. *Balamuthia mandrillaris* amebas feed by the invading mammalian cells.

Or-19

Morphological characterization of inflammation during experimental primary amoebic meningoencephalitis (PAM)

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Naegleria fowleri is a free-living amoeba that produces an acute and rapidly fatal infection of the central nervous system. Trophozoites reach the brain by penetrating the olfactory epithelium, passing thru the cribriform plaque, until reaching the olfactory bulbs. Experimental studies have shown that an intense inflammatory reaction is produced in the brain, and it is always associated with extensive tissue damage. Detailed studies of the early inflammation stages, as well as their role in pathogenesis of the PAM have not been addressed yet. We analyzed the morphological changes of this inflammatory process during experimental PAM production, using both optical and electron microscopy. After three to four days post-inoculation, several *N. fowleri* trophozoites were observed in the olfactory bulbs. At 3 days, no inflammation or apparent tissue damage was observed. The number of amoebae increased rapidly in the next 24 h. Soon after, eosinophils and neutrophils were seen surrounding the parasites. Ultrastructural analysis showed features suggesting leukocytes attempt to eliminate the amoebae. The inflammatory reaction was increased rapidly, with leukocytes showing important signs of damage and evident necrosis of parenchyma. We suggest that lysis of the host polymorphonuclear cells, added to other parasite factors, may contribute in the tissue damage. To corroborate the former hypothesis, we also used knockout (KO) CD38 mice, which have deficiencies in leukocytes chemotaxis. Their mortality rate was compared with the parental strain C57BL/6, after a lethal challenge with live parasites. Results showed that KO mice had a statistically significant higher survival. The histological analysis of brain tissue of KO mice showed a delayed appearance of inflammation. This may explain the slight, but significant increase in their survival rate. We conclude that the inflammatory reaction observed during experimental PAM may contribute importantly in the tissue damage and eventual death of mice.

Or-20

Experimental murine granulomatous amoebic encephalitis: Immunohistochemichal characterization of early stages of the infection

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Early stages of experimental murine granulomatous amoebic encephalitis with *Acanthamoeba castellanii* were immunohistochemically characterized after 48 and 72 h post intranasal instillation of 1×10^6/20µl trophozoites. At the end of time point interactions, mice were sacrificed; lungs, kidneys, spleens, liver and heads were fixed and processed for their inclusion in paraffin, sectioned to obtain 5 µm-thick slices and then treated accordingly with immunohistochemical techniques, using IgG of rabbit anti-*Acanthamoeba* and goat anti IgG of rabbit conjugated to peroxidase, finally revealed with H_2O_2-diaminobenzidine and counterstained with Harris hematoxylin.

The immunohistochemical analysis of mouse tissue during the initial stages of *Acanthamoeba castellanii* encephalitis, revealed the invasion of trophozoites through all tissues in study. The immunolocalization of amoebae after 48 and 72h of interaction was very similar. By light microscopy the analysis of the head's slides showed trophozoites in contact with the surface of the mucus layer of the olfactory epithelium. In some zones, trophozoites moved through the mucus with disruption of a number of areas. Invasion of the olfactory epithelium was observed. Several cyst forms were observed in the brain too.

The analysis of lung, spleen, kidney and liver showed trophozoites as well as cysts in all organs. In some cases disorganization but not inflammation of the tissues was evident. Amoebae were located near vessels, frequently migrating through the different tissues.

The present work describes the sequence of histopathological events following *Acanthamoeba* infection during the initial penetration into nasal mucosa and other tissues. It was believed that *Acanthamoeba* spp. reached the brain by hematogenous dissemination but we have proven that it is able to adhere, migrate and penetrate through the olfactory epithelium, probably by mechanical and enzymatic processes. In contrast to *Naegleria fowleri*, *A. castellanii* migrates and penetrates slowly explaining the chronic course of the infection.

Immunohistochemistry is a useful technique to identify free-living amoebae which are difficult to observe with conventional staining techniques.

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Or-21

Balamuthia mandrillaris traversal of the blood-brain barrier

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Balamuthia mandrillaris granulomatous encephalitis is a serious human disease that almost always results in death. However, the pathogenesis and pathophysiology of *Balamuthia* encephalitis remains incompletely understood. An important step in *Balamuthia* encephalitis is amoebae invasion of the bloodstream followed by their haematogenous spread. *Balamuthia mandrillaris* entry into the central nervous system most likely occurs at the blood-brain barrier sites. However, it is not clear how circulating amoebae cross the blood-brain barrier. Here we studied *B. mandrillaris* interactions with the human brain microvascular endothelial cells (HBMEC), which constitute the blood-brain barrier. Adhesion assays revealed that *B. mandrillaris* exhibit more than 90% binding to HBMEC in a galactose-inhibitable manner. Affinity chromatography using galactose-sepharose column, a galactose-binding protein (GBP) was isolated from detergent extracts of unlabeled amoebae. The isolation of GBP from cell surface biotin-labeled amoebae suggested its membrane-association. One dimensional SDS-PAGE confirmed the proteinaceous nature of GBP and determined its molecular mass in the range of 100 kDa. Using Western blotting assays, we showed that *B. mandrillaris* inhibits HBMEC cell cycle by modulating protein retinoblastoma phosphorylations. Furthermore, it was shown that *B. mandrillaris* target zonula-occludens-1 (ZO-1) and occludin. Both ZO-1 and occludin are involved in the formation of tight junctions, suggesting that *B. mandrillaris* disrupt tight junctions to induce HBMEC permeability and/or HBMEC monolayer disruptions, leading to blood-brain barrier perturbations. Subsequently, *B. mandrillaris* produced HBMEC cytotoxicity. The ability of *B. mandrillaris*

to produce HBMEC death may also be dependent on extracellular molecules released by *B. mandrillaris*. To this end, we demonstrated that *B. mandrillaris* ecto-ATPases hydrolyse ATP to release ADP, which may exert toxic effects on HBMEC. In support, suramin, i.e., P2 receptor antagonist, inhibited *B. mandrillaris*-mediated HBMEC death. Future work will continue to study *Balamuthia encephalitis* pathogenesis which should help identify potential targets for therapeutic interventions.

Or-22

A novel model for the study *in vivo* of central nervous system infections due to free-living amoebae

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A whole-organism approach to the study of disease is recognised as essential to gaining a full understanding of the inter-relationships between infectious agents and their hosts. While vertebrate model systems are seen as immediately more relevant, it is proposed here that the use of an invertebrate model at an early stage can offer several advantages in terms of speed, cost, technical convenience, and ethical acceptance. It is shown for the first time that the African migratory locust can be used as a model to study *Acanthamoeba* pathogenesis. Mature adult locusts were allocated randomly in groups of ten to different treatments. In the experimental groups, each locust was injected intra-abdominally with 10 µl of a suspension of 1 x 10^6 *A. castellanii* (a clinical isolate belonging to T4 genotype) in PYG medium. In control groups, locusts were injected with the same volume of PGY alone. Mortality was recorded every 24 h, when any dead locusts were removed to minimise cannabalism. The injection of amoebae killed almost 100% of the locusts within 14 days. To determine whether parasites disseminate via haematogenous spread, samples of haemolymph together with various tissues were dissected and cultured on non-nutrient agar plates containing bacterial lawns. Amoebae were recovered from locust haemolymph, muscles, fat bodies, but not faeces. To determine *A. castellanii* association with the central nervous system, locust brains were isolated. Briefly, the injected locusts were killed and the left side of the head was removed by a saggital cut through the base of the left antenna using a sharp scalpel. The brain was dissected out from the right-hand side of the head using fine sterile forceps. After several washes with PBS, the brains were incubated with pentamidine (40 µM; final concentration) at 37°C for 60 min to kill extracellular amoebae. The brains were then washed a further three times, homogenised and cultured for the presence of amoebae. Brain lysates from amoebae-infected locusts were positive for *A. castellanii*, whereas brain lysates from PYG-infected locusts showed no organisms. Overall, these results suggest that locusts can be used as a model to study *Acanthamoeba* pathogenesis *in vivo*. Because insects rely for their protection against infection on an entirely innate immune system, the use of an insect model is particularly relevant in the study of human *Acanthamoeba* encephalitis, the control of which has significant dependency on the innate immune system.

Or-23

Encystment in *Balamuthia mandrillaris*: A property that enhances resistance and/or recurrence of infection

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Balamuthia mandrillaris is an emerging protozoan pathogen that is known to infect the central nervous system to produce fatal granulomatous encephalitis. Early diagnosis, followed by aggressive treatment using a combination of drugs is a prerequisite in successful treatment but even then, prognosis remains very poor (~98% mortal-

ity). In addition, amoebae can transform into dormant cyst forms, which may provide resistance and/or reactivate following drug therapy. Here, we tested *B. mandrillaris* resistance to physical, chemical and radiological conditions. Following these treatments, viability was determined by culturing amoebae on human brain microvascular endothelial cells for up to 12 days. *B. mandrillaris* cysts were resistant to repeated freeze-thawing (5X), temperatures of up to 70°C, 0.5% SDS, 25ppm chlorine, 10μg/ml pentamidine and 200mJ/cm^2 of ultraviolet irradiation. When amoebae were cultured with primary human brain microvascular endothelial cells (HBMEC) which constitute the blood-brain barrier, cycloheximide, ketoconazole or pre-exposure of organisms to cytochalasin-D prevented amoeba-associated cytotoxicity. In an assay for inhibition of cyst production in the absence of feeder cells, these 3 agents prevented the production of cysts. These findings indicate that the biosynthesis of proteins and ergosterol and the polymerization of actin are important in cytotoxicity and encystment. Of the three targets, ketoconazole and other azoles are of most interest for further investigation, because ergosterol is not produced by mammalian cells.

Or-24

Characterization of membrane carbohydrates in pathogenic *Naegleria fowleri* and non-pathogenic *Naegleria gruberi*

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Naegleria fowleri is a free-living ameboflagellate and the etiologic agent of the primary amoebic meningoencephalitis (PAM). This is a fulminant and rapidly fatal infectious disease of the central nervous system. Knowledge of the parasite surface molecules including glycoproteins may be an important factor in understanding the parasite mechanisms of pathogenesis. Presently, we characterized the carbohydrate residues by using different lectins, which interact specifically with glycoconjugates by binding to a specific carbohydrate. We analyzed the differences in the membrane glycoprotein composition between *N. fowleri* (pathogenic) and *N. gruberi* (non-pathogenic) strains, and their possible role in cell adhesion. The following biotinilated lectins were used: *Canavalia ensiformis* (Con A), *Tetragonolobus purpureas* agglutinin (TPA), *Helix pomatia* agglutinin (HPA), *Pisum sativum* agglutinin (PSA) and *Galanthus nivalis* lectin (GNL). Trophozoites of both strains were fixed and incubated with the specific biotinilated lectins. After washing, amoebae were incubated with streptavidin-fluorescein and analyzed by FACS (Fluorescent-activated cell sorting). Results showed that *N. fowleri* expressed with a higher intensity than *N. gruberi,* the glycoproteins that are detected with Con A (which recognizes α-D-Glucosyl and α-D-mannosyl residues) and TPA (which recognizes α-L-fucosyl residues). This recognition was 2.7 fold higher in *N. fowleri* using both lectins. The role of these glycolconjugates in amoebic adhesion to epithelial cells was also evaluated. Trophozoites of both strains were interacted with MDCK cells for 1 h in the presence of different concentrations of specific sugars. D-mannose and L -fucose inhibited 70% and 66% of *N. fowleri* cell adhesion, respectively. On the other hand, the same carbohydrates inhibited 40.3% and 43% of *N. gruberi* adhesion to MDCK cells. The differences observed suggest that parasite membrane glycoproteins with mannose, glucose and fucose residues may be involved in amoebic adhesion to host cells, prior to tissue invasion.

Or-25

Acanthamoeba castellanii differentiation: A two-faced drama

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Acanthamoeba castellanii of the T4 genotype is an environmentally ubiquitous protozoan pathogen that can cause a rare, but fatal, encephalitis and a blinding keratitis. One of the distressing aspects in combating *Acanthamoeba* infections is the prolonged and problematic treatment. This is due to the ability of *Acanthamoeba* to rapidly adapt to harsh conditions and switch phenotypes into a resistant cyst form, resulting in both disease resur-

gence and longevity. One possibility of improving the treatment of *Acanthamoeba* infections is to inhibit the ability of these parasites to switch into the cyst form. In keeping with other protozoans, *Acanthamoeba* elaborate proteases, considered to be key determinants of pathogenicity and cytolysis of host cells. However, there is now growing evidence that their localised incitement and amplification may equally be synonymous with morphological changes. Previous research has established that *Acanthamoeba* secretes serine proteases. Using a number of protease inhibitors and corroboratory evidence from short interfering RNA primers (siRNA), we have found that serine proteases play an important role in *A. castellanii* differentiation. Inhibition of serine proteases attenuates *A. castellanii* metamorphosis, as demonstrated by arrest of both encystment and excystment of *Acanthamoeba*. Further, as serine and threonine residues are important biological regulatory sites, the foregoing has led to an initial investigation into the contribution that phosphorylative signalling cascades play in determining *Acanthamoeba* transposition.

Or-26

RNA interference (RNAi) as a tool for the silencing of differentiation process in *Acanthamoeba*

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The genus *Acanthamoeba* undergoes encystation under unfavourable environmental conditions. Cellulose is a major component of the inner cyst wall in this organism. The process of cellulose synthesis is not clearly understood yet, however in order to synthesise cellulose, these amoebae must have storage of glucose for its synthesis regarding unfavourable conditions. It has been suggested that the glycogen, which amount is decreasing during encystation, is the main source of free glucose for the synthesis of cellulose in *Acanthamoeba*. The glycogen phosphorylase is supposed to be the main tool that these amoebae use in order to maintain the free glucose levels during the encystation process.

In this study the glycogen phosphorylase of four strains, with different pathogenic levels, of *Acanthamoeba* was silenced using RNA interference (RNAi) and the effects on the differentiation process in RNAi-treated and non-treated cells was evaluated at different stages using inverted and electron microscopy and by measuring the levels of glycogen phosphorylase using northern and western blots.

Our results demonstrated that the free glucose during encystation was exclusively delivered via glycogen phosphorylase activity, which was increasing due to higher levels of mRNAs codifying this enzyme. Additionally, this study highlights the potential of RNAi technology as an attractive candidate for future development of new agents for the treatment of *Acanthamoeba* infections.

Or-27

Losses of the encystment potential of *Acanthamoeba* spp. involve epigenetic regulation

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The extremely high abundance of *Acanthamoeba* in a broad range of habitats is mostly due to their ability to evade unfavourable environmental conditions by encystment. Apart from their high tolerance against desiccation and prolonged starvation, *Acanthamoeba* cysts show high resistance against therapeutic agents, and thus represent a challenge in therapy of *Acanthamoeba* infections, because remaining cysts have been reported to lead to recurrent infections. In the laboratory, encystment of *Acanthamoeba* trophozoites can be induced by replacing the

nutrient medium by a defined salt medium. However, prolonged culture of microorganisms under laboratory conditions is known to change their physiological properties. Accordingly, loss of virulence and the decrease in cellular enzyme activities have been reported for long-term cultured *Acanthamoeba.*

In this study, the encystment potentials of four *Acanthamoeba* strains of genotype T4, previously cultured axenically for different periods of time, were compared in three previously described encystment media and a correlation of strain age and loss of encystment potential could be demonstrated. However, it was shown that decreases in the encystment potential were partly reversible by growth in vero-cell monolayers or by treatment with 5-azacytidine and trichostatin A, suggesting that epigenetic mechanisms might be involved. Our long-term aim will be the evaluation of these mechanisms on the protein level.

Adaptations to long-term culture represent a common problem and the identification of underlying mechanisms and approaches to overcome this phenomenon could be beneficial for studies on pathogenicity or drug susceptibility assays of microorganisms.

Or-28

Naegleria fowleri excystation process: An ultrastructural study

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Naegleria fowleri is an amphizoic amoeba causative of human primary amoebic meningoencephalitis (PAM). The life cycle of *N. fowleri* present three morphologically different phases: the trophozoite, from which may originate the flagellated form, and the cyst, which represents the resistance phase. The cyst is not considered infective, but its importance lies in its capacity to assure the persistence of the parasite, and is therefore of epidemiological importance for this microorganism. Moreover, it has been reported that the amoeba may excyst in a relatively short time, thus giving rise to the invading trophozoite.

We have studied the structural events that occur during the *Naegleria* sp. excystation process by transmission electron microscopy (TEM). The amoeba used in the present experiments was the isolate recently isolated from a hot spring water source and associated with a PAM case in a child. Cysts were collected from an asynchronic population of the amoeba grown on non-nutritive agar plates pre-coated with *Enterobacter aerogenes* at 37°C. Cysts were induced to excyst by plating them on the fresh culture plates for 12 or 24 h to 37°C. By fluorescence microscopy mature cysts presented two to three ostioles, each of one sealed by an operculum. By TEM the operculum shows two areas different in the compaction grade of their fibrillar structure. When the excystation process was induced, small electron dense granules were associated both to the parasite plasma membrane and to free fibrillar material present in the peritrophic space. Images suggesting the removal of the operculum by the amoeba were also found. After removal of the operculum, the presence of pseudopodia through the ostiole was frequently seen. The cytoplasm of the pseudopodia contained vacuoles with a fibrillar material. This fibrillar content may be derived from the incorporation of the operculum into the cytoplasm. Finally, the parasite emerges from the cyst wall. These observations suggest that in *Naegleria* sp. lytic products, endocytic and pseudopodic activities may be involved in the process of excystation.

Or-29

Protein expression profile during encystment of *Acanthamoeba castellanii*

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Most amoebae are switching between two different forms: trophozoite and cyst. Trophozoite is the vegetative

form that could multiply, feed and move. Cyst is the dormant form, which appears in case of adverse conditions such as nutrient limitation. Of particular importance is the study of cyst, as this form is highly resistant to biocides and would be responsible for amoebae persistence in the environment. In addition, *Acanthamoeba* cysts have been shown to protect pathogenic bacteria such as *Legionella pneumophila*. Little is known about the encystment process in free-living amoeba like *Acanthamoeba*. Our work is aimed to decipher, at the molecular level, the mechanism of encystment in *Acanthamoeba castellanii.*

We first compared protein expression of *A. castellanii* cells in the process of encystment at different times (0h, 6h, 24h and 8 days) by 2D gel electrophoresis. Several protein spots were induced or repressed during the encystment. These spots were analysed by mass spectrometry and among them six proteins were identified. We are currently measuring, by real-time RT-PCR, expression of genes encoding these proteins. The level of RNA expression will be then compared to that of proteins. Identification of proteins related to amoebae encystment might help to understand this process and, later on, to struggle against encystment.

Or-30

Characterization of encystation-mediating factor, serine proteinase of *Acanthamoeba*

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The genus *Acanthamoeba* is an opportunistic protozoan parasites causing granulomatous encephalitis (GAE) or amebic keratitis. Proteinases are reported to play roles in various biologic actions of *Acanthamoeba* including host tissue destruction, pathogenesis and digestion. Serine protease of *Acanthamoeba* is known to role in parasite physiology and in host-parasite interactions. In the present study, we purified and characterized a serine proteinase mediating the encystation of *Acanthamoeba*. By the Differential Expressed Gene analysis, we identified 16 genes expressed higher in cyst than in trophozoite. One of those, a serine proteinase was confirmed the highly expressed mRNA level during encystation by real time PCR analysis. Encystation of *Acanthamoeba* was inhibited by serine proteinase inhibitor, PMSF. Chemically synthesized small interfering RNA (siRNA) of the encystation mediating serine proteinase (EMSP) was significantly reduced the encystation efficiency in *Acanthamoeba*. Western blot analysis detected the serine proteinase of 33 KDa mature form in encysting amoebae but not in trophozoite. A EMSP-EGFP fusion protein localized at the vesicle-like structure in the amoeba. Using Lyso Tracker analysis, these vesicular structures were confirmed to be lysosomes rather than secretory vesicles. These data show that EMSP may play an important role in the differentiation of *Acanthamoeba* by being partly responsible for autolysis.

Or-31

Encystment in *Acanthamoeba* spp.: Application of two-dimensional gel electrophoresis for the elucidation of stage-specific proteins involved in the developmental process

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The capability to encyst is *Acanthamoeba*'s most effective measure to withstand adverse conditions and thus has medical as well as environmental implications. The cyst stage of *Acanthamoeba* is remarkably resistant to chemotherapeutic agents, which often leads to recurrence of disease in patients who were supposedly treated successfully against *Acanthamoeba* keratitis. Moreover, *Acanthamoeba* cysts often harbour pathogenic bacteria, including legionellae, mycobacteria, *Listeria monocytogenes* and others, thereby rendering them a potential risk to public health.

It is, therefore, important to gain a better understanding of the encystment process in *Acanthamoeba* spp., its underlying mechanisms and its interdependence with endocytobiotic bacteria. We applied two-dimensional gel electrophoresis (2D PAGE) in order to monitor and identify the differentially expressed proteins in the encysting cell. Samples of encysting *Acanthamoeba* spp. cultures were taken at several time-points until the mature cyst stage was attained. The protein expression patterns of encysting cells were found to differ dramatically from the trophozoite stage, already at a remarkably early stage of development. A large number of protein spots was isolated from 2D gels and analysed by mass spectrometry.

In the future, the identified proteins will be studied at the genetic level in order to identify regulatory DNA sequences that are involved in directing encystment and in order to isolate and to define encystment specific transcriptional regulators of *Acanthamoeba*. The ultimate goal of our study will be the establishment of a molecular model of encystment in *Acanthamoeba* spp., which enables to draw comparisons to other encysting protozoa and to assess the impact of endosymbiotic bacteria on encystment by RNA profiling and proteomics.

Poster (Po)-01

An update on the *Acanthamoeba* DNA sequence database at the Ohio State University: A resource for the study of clinical and environmental isolates

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The genus *Acanthamoeba* has a worldwide distribution, inhabiting a wide range of environmental niches. It has been isolated from soil, fresh and saltwater, humans, domestic and feral animals. The genus includes opportunistic pathogens responsible for the sight threatening disease *Acanthamoeba* keratitis (AK) in otherwise healthy individuals. *Acanthamoeba* is also responsible for life threatening infections in immunocompromised patients. Variation in the pathogenicity of *Acanthamoeba* strains has been observed, but the relevance of these results to human disease is unclear. Lack of a reliable subgeneric classification system has complicated analysis. *Acanthamoeba* traditionally has been classified using morphological markers such as cyst morphology and trophozoite size and shape. Questions regarding the reliability of morphological methods led our laboratory to examine molecular methods to determine whether more definitive subgeneric classifications could be accomplished. Our initial studies focused on the nuclear small subunit ribosomal RNA gene (*Rns*). Subsequent studies added mitochondrial loci as additional markers and as a test of the consistency of conclusions drawn from the *Rns* studies. The data collected over the previous decade now allow us to quickly examine a clinical or environmental sample using molecular methods and determine and classify the sequence genotype for an unknown specimen. Twelve *Rns* sequence types were originally identified and designated T1-T12. Several more may exist. The database currently includes ~190 “nearly complete” sequences (defined as sequences of 2000 or more nucleotides). Of these, 133 (70%) are classified as sequence type T4. In addition, more than 200 isolates have been examined for which partial *Rns* sequences have been reported, including 135 isolates with sequences of 400-1000 nucleotides and 20 isolates with sequences of 1000-2000 nucleotides. While mitochondrial *rns* sequences have been less extensively analyzed, there are over 80 sequences that have been reported, spanning almost all nuclear sequence types. (Supported by grants from NIH)

Po-02

Construction of EST database for comparative gene studies of *Acanthamoeba*

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The genus *Acanthamoeba* is the causative agent of granulomatous amoebic encephalitis and amebic keratitis.

But little information has been reported on the genome of *Acanthamoeba* up to now and moreover, specific database for *Acanthamoeba* gene has yet to be constructed. Three kinds of cDNA library with *Acanthamoeba* of different virulences or different developmental stages were constructed. A total of 2,864 ESTs from 3,115 random selected clones from the cDNA libraries was generated and subjected for BLASTx homology search. Among the ESTs that have significant matches, 1,607 ESTs were found to be identified as unique genes, and 1,574 genes were found in *Acanthamoeba* for the first time. Each ESTs data was classified by KOG analysis, predicting function. Comparing EST data by KOG analysis between the two species, actin related proteins, subtilisin-like serine proteinase in *A. healyi* ESTs show higher percentage than *A. castellanii* ESTs. Comparing EST data by KOG analysis between the two developmental stages of *A. castellanii*, cullin4, heat shock protein, calreticulin, ubiquitin conjugation enzyme, proteasome, and serine type endopeptidase was found in only cyst ESTs. Being based on result of *Acanthamoeba* ESTs data with download data related with *Acanthamoeba* from NCBI, database of *Acanthamoeba* called 'Acanthamoeba EST DB' was constructed. The sequence data of 'Acanthamoeba EST DB' is consisted of 36,227 nucleotide and 285 amino acid data. The contents of 'Acanthamoeba EST DB' are 'Home', 'BLAST_NT', 'BLAST_AA', 'BLAST_results', '2-sequences', and 'statistics'. In 'BLAST_results', each gene has links for the significant information including sequence data, gene orthology annotations, relevant references and a BLAST result. Using various blast program, nucleotide analysis in 'BLAST_NT', protein analysis in 'BLAST_AA', and alignment of two sequence in '2-Sequence' are possible. As first attempt in the *Acanthamoeba*, 'Acanthamoeba EST DB' would make the gene study easy, providing many new opportunities for the scientific community.

Po-03

Genotyping of *Acanthamoeba* strains based on mitochondrial DNA RFLPs and 18S rDNA sequences

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The genus *Acanthamoeba* is distributing in a variety of environment, including soil, fresh and saltwater, house dust, and contact-lens solution and others. Recent molecular studies of this genus have revealed that DNA analyses, such as mitochondrial (mt)DNA RFLP typing or 18SrDNA sequence typing, are more reliable than morphology of the cyst or the trophozoites for the identification of the species. Interestingly, most of the keratitis isolates can be assigned to major 7 mtDNA RFLP types or have an 18SrDNA sequence of T4 type in different studies. Since little is known about the relationships between mtDNA RFLP types and 18SrDNA sequence types, we extensively studied to know whether they are closely related or not. Ninety-nine isolates of keratitis isolates and some of the authentic strains were analyzed both with mtDNA RFLPs and 18SrDNA sequences. Mitochondrial DNA RFLPs were obtained by BglII and EcoRI digestion of whole mtDNA extracted from the cultures. Sequences of 18SrDNA were obtained according to the published methods (Booton GC et al, 2005, JCM). Reference sequences of T-types based on the 18SrDNA sequencing in Genbank were involved for phylogenetic analysis. Major 7 mtDNA RFLP types which encompass most of the human isolates had T4 sequence. More precisely, however, these major mtDNA RFLP types except for JAC/E4 distributed in different sub-clusters within T4 clade, namely most of the 18SrDNA sub-clusters consist of multiple mtRFLP types.

Po-04

Keratitis by *Acanthamoeba* spp, a great threat to users of contact lenses

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Background: Most of the information described on kinds of free living amoebas (FLA) pathogenic to humans refers to *Naegleria fowleri*, *Acanthamoeba* spp, *Balamuthia mandrillaris* and *Sappinia diploidea*. The first published case of keratitis caused by *Acanthamoeba* was in 1973, but its association with users of contact lenses was recognized only in 1984. In Chile, the first isolation of FLA from environment was carried out in 1981 and it cor-

responded to *Acanthamoeba palestinensis*; later in 1993 the first isolation from a patient was carried out and it corresponded to *Acanthamoeba polyphaga*. It came from a sample of corneal scraping of a patient with severe bilateral keratitis. Both species were typed at the Center for Disease Control, Atlanta, United States (G. Visvesvara).

Objective: To report the isolations of *Acanthamoeba* spp. from samples of patients with keratitis.

Material: Sixty-eight samples of corneal scraping or corneal biopsy; the patients included 48 females and 20 males with ages between 20 and 60 years.

Methods: The samples were cultivated in non-nutrient agar covered with an emulsion of *Escherichia coli*, and were incubated at 37ºC and at room temperature. Observation of the culture was done every 24 hours for 7 days.

Results: Positive samples showed filamentous trophozoites and cysts of smooth walls with polygonal enclosure. Eighteen out of 68 patients (26 %) were positive, 16/43 from female patients and 2/20 from male patients. Ten out of 18 patients were users of contact lenses, 1 did not use contact lenses and 7 patients the information.

Conclusions: With the results obtained, it is recommended to detect the presence of this protozoan parasite in keratitis patients who do not respond to the usual treatment procedures and in those from which no other pathogenic agents have been isolated.

Po-05

Evaluation of potential keratopathogenicity of *Acanthamoeba* isolated from contact lens storage cases and domestic tap water

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In a previous study, we reported on the contamination rate of free living amoeba, including *Acanthamoeba,* isolated from contact lens storage cases (CLSC) and domestic tap water in Korea. In an effort to evaluate the potential kerato-pathogenicity of 5 isolates from CLSC and 17 isolates from domestic tap water, we have conducted an investigation into the morphological features, mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) phenotypes, 18S rDNA sequences, and drug sensitivities of these isolates, and have compared the results with those of 20 amoebic keratitis (AK) isolates from Korea, as well as 14 reference strains. Cysts from 22 isolates obtained from CLSC and domestic tap water showed typical characteristics of morphological group 2. A total of three and five mtDNA RFLP patterns generated by *Eco* RI were found in 5 of the isolates from CLSC and 17 of the isolates from domestic tap water respectively. The mtDNA RFLP patterns of four of the five isolates from the CLSC were found to be identical to those of the isolates from domestic tap water of students who had contaminated CLSC. The majority had mtDNA RFLP patterns identical to those of AK isolates in Korea. The results of 18S rDNA sequencing analysis were also shown to coincide with the results of mtDNA RFLP analysis. KA/WP12 was determined to be profoundly sensitive to chlorhexidine (MCC; 6.25 μg/ml), and KAWP2 was the most sensitive strain to polyhexamethylene biguanide (PHMB) (MCC; 4.69 μg/ml). Some difference in the cytopathic effects of isolates against human corneal epithelial cells was observed according to their mtDNA genotypes. In conclusion, domestic tap water may constitute a source of *Acanthamoeba* contamination of CLSC, and most isolates from CLSC and domestic tap water appear to be potentially keratopathogenic.

Po-06

Granulomatous amoebic encephalitis due to Acanthamoeba *sp*. Case report

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A 41-year-old men resident of Villa Santiago, N. L., Mexico initiated the sickness with clinical history of five months of partial convulsive crisis. Signs and symptoms most important were: consciousness deteriorated, somnolence, severe headache, vomiting and convulsive crisis. Nuclear magnetic resonance imaging showed a right frontal -parietal tumor of the brain. Neurosurgery was practiced and showed a right frontal lesion with neoplastic aspect. Histopatology analysis showed subacute cerebral abscess with granulation tissues and necrosis. Amoebic trophozoites were observed and morphologically identified as *Acanthamoeba sp*. The patient was treated with rifampin, trimethoprim-sulphamethoxazole, fluconazole and metronidazole. The neoplasic process was not demonstrated. Because of patient´s neurological status continued declining and convulsive crisis persisted, a second surgery was performed. Samples of cerebral tissue were inoculated in NNE media with *Enterobacter sp.* and incubated at 25 °C for 7 days. The culture was negative for free-living amoebae. Besides, representative samples of the brain were stained with H & E and Gomori trichromic tincion. Amoebae seen in tissue sections from the patient were identified as *Acanthamoeba* sp., and Amphotericin B was added. Samples were fixed and processed for their inclusion in paraffin and sectioned to obtain 5 μ m-thinck slices and then processed for immunohistochemistry using polyclonal serum of rabbit anti-*Acanthamoeba* and goat anti-IgG of rabbit conjugated to peroxidase and revealed with H_2O_2 diaminobenzidine. Samples were counterstained with H & E.

Two months after, the patient showed signs of increased intracranial pressure and new surgery was performed. Findings were compatible with *Glioblastoma multiforme* and cerebral abscess with numerous trophozoites of *Acanthamoeba* were observed. The patient died after 3 months after diagnosis of EAG.

Po-07

Possible role of immunoglobulin IgA in the protection against experimental *N. fowleri* meningoencephalitis

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We have reported that intranasal administration of Cry1Ac protoxin alone or with amoebal lysates increases protection against *Naegleria fowleri* meningoencephalitis in mice. Our data indicated that IgA responses elicited in the nasal mucosa seem to be related with protection and suggest they may participate in the initial defense against this parasite. In the present work we performed an immunohistochemical analysis of *N. fowleri* and IgA at early times after an intranasal lethal challenge with *N. fowleri* in mice, that previously had been immunized by the intranasal route with Cry1Ac alone or with amoebal lysates and in control mice. Our results showed that intranasal immunization provoke changes in the olfactory epithelia of mice immunized with any of the three distinct treatments tested, and was clearly appreciated an increase in areas with metaplasia towards respiratory characteristics. Moreover we found a markedly increased localization of IgA in the epithelia with metaplasia of immunized mice, as well as IgA interacting with trophozoites. These data suggest that the increased IgA induced in the nasal mucosa by intranasal immunization, probably impedes amebic adhesion and subsequent invasion of the nasal epithelia. In support to this notion in immunized mice, trophozoites were mainly detected in the nasal lumen while in unimmunized mice they were detected in the lumen as well as invading the nasal mucosa. Present results suggest that immunization provokes cellular changes in the olfactory epithelia which may allow a major secretion of IgA, and may contribute to the higher protection against *N. fowleri* observed in mice intranasally immunized and challenged with this parasite.

Po-08

Role of antibody responses in the protection against experimental *N. fowleri* meningoencephalitis in STAT6-deficient mice

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We have reported that intranasal administration of Cry1Ac protoxin alone or with amoebal lysates increases protection against *Naegleria fowleri* meningoencephalitis in mice. In that study we proposed that adaptive immune mechanisms such as antibodies (IgG and IgA) appear to play an important role in the protection against the infection. The aim of the present study was to investigate whether antibody responses were essential for resistance to *N. fowleri.* STAT6-deficient mice (STAT6-/-) were used and compared with similarly treated wild-type (STAT6+/+) mice. The mice were immunized by the intranasal route with a combination of *N. fowleri* lysates and Cry1Ac, and subsequently challenged with lethal doses of trophozoites of *N. fowleri*. STAT6+/+ mice had a 100% protection, whereas no protection was observed in STAT6-/- mice. Moreover, a significantly higher level of titers of Th2-associated immunoglobulin G1 (IgG1) as well as interleukin-4 (IL-4) were found in STAT6+/+ mice, whereas in STAT6-/- mice significantly more IL-12 and IFN-γ as well as significantly higher levels of titers of Th1-associated immunoglobulin G1 (IgG1) were observed. These findings suggest that the humoral response is critical for defense against *N. fowleri* infection because STAT-6+/+ mice showed a 100% protection and a Th2- response, while STAT6-/- mice showed a cellular response that apparently does not participate in the protection against *N. fowleri.*

Po-09

Molecular identification of *Naegleria* spp. isolated in Mexico

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In the genus *Naegleria* 47 species have been described which can be identified by rDNA sequencing. Only four of these had been identified in Mexico until now: the human pathogen *N. fowleri* and the closely related nonpathogenic *N. lovaniensis*, the mouse pathogenic *N. australiensis*, and *N. mexicana*, which is known to occur only in Mexico until now.

In the present study we isolated in 2004-2005 *Naegleria* strains from different states of Mexico in an attempt to identify as much different species as possible. The isolates were identified by sequencing the PCR amplified ITS1-5.8S-ITS2 and comparing the sequences to those of established species. Nine isolates were identified as *N. lovaniensis,* five as *N. tihangensis*, two as *N. pagei*, and one each as *N. clarki*, *N. indonesiensis*, *N. byersi* and *N. americana*. Of these, *N. lovaniensis* is the only one that had been identified previously in Mexico. One of the new *N. lovaniensis* isolates is unique as it is the first time a one bp deletion is observed in the ITS2 of this species. *N. tihangensis* is closely related to the pathogenic *N. australiensis*, differing from each other only by an indel of 2 bp in the ITS2. The isolation of *N. indonesiensis* and *N. byersi* is important because they had been reported previously as having a restricted geographic dispersal.

Po-10

Molecular identification of *Acanthamoeba* sp. Isolates in an *Acanthamoeba* keratitis outbreak

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Objectives: An increase in *Acanthamoeba* keratitis (AK) cases has been documented in the Chicago, Ill., USA area. Epidemiological analysis indicates this is a significant increase in cases compared to historical numbers. It was hypothesized that the increased infections may be due to changes in water treatment. Alternatively, a more pathogenic strain of *Acanthamoeba* may be responsible for the increase in AK cases. Here we use genotypic data to test the hypothesis that a new, or more pathogenic known genotype of *Acanthamoeba,* is the cause of the AK surge.

Methods: Previous sequence analysis of the 18S ribosomal RNA gene (18S rDNA) of *Acanthamoeba* isolates resulted in the placement of *Acanthamoeba* strains into 15 different genotypic classes. Most cases (~97%) of AK are associated with a single genotype (T4) of *Acanthamoeba*. Rarely, AK cases are associated with other genotypes. In this study we determine the genotypes of 23 *Acanthamoeba* sp. isolates from the Chicago AK outbreak by sequencing a highly informative region of the 18S rDNA.

Results: DNA sequencing shows these isolates are predominantly genotype T4 (91%), whereas the remaining isolates were genotype T3 (9%). Both genotypes have previously been observed in AK cases. In addition, DNA sequences are overwhelmingly similar to previously sequenced isolates.

Conclusion: There is no support for the hypothesis that cases of AK in this outbreak are the result of infection by a new *Acanthamoeba* genotype. High sequence similarity between these isolates and the 18S rDNA database does not support the hypothesis that these isolates represent more pathogenic *Acanthamoeba* of known genotypes. Lastly, results lend support to the hypothesis that increased AK cases are due to changes in water treatment, permitting increased bacterial colonization of the water, increased *Acanthamoeba* grazing, and ultimately an increase in AK cases due to an increased abundance of *Acanthamoeba* in the water supply.

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Po-11

Selective grazing of free-living naked amoeba on methane-oxidizing bacteria

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Biological methane oxidation at the oxic-anoxic interface in wetland soils and sediments is a key process in methane cycle, preventing large amounts of this greenhouse gas from escaping into the atmosphere. Methane oxidizing bacteria (MOB) is the only group of aerobic bacteria that oxidize methane and assimilate methane-C. However, the fate of MOB-biomass is largely unknown. Here we demonstrate that a microbial food web can be driven by methane. Soil microcosms, in which a thin layer of water-saturated rice field soil was incubated under opposing gradients of oxygen and ^{13}C-labelled methane, revealed ^{13}C-enriched "heavy" RNA which could be affiliated to MOB, to the scavenging prokaryotic Myxobacteria, and to protistan grazers including Lobosea and Heterolobosea. Amoeba-derived RNA demonstrated preferential enrichment with ^{13}C among the group. Different protistan communities developed depending on methane concentrations. Amoeba isolated from the soil demonstrated selective grazing on MOB. These results indicated that amoeba may shape the community structure of MOB and vice versa. Recent work using MOB as a model has shown that microbial community structure matters for ecosystem functioning. Our study suggests that not only microbial, but also protistan diversity and microbe-protist interactions have to be considered to understand and control methane oxidation in wetland soils.

Po-12

A mycophagous amoeba-flagellate isolated from a *Phytophthora ramorum*-infected lesion of California bay laurel

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An amoeba-flagellate, termed strain ANN04, was isolated from hyphae of *Phytophthora ramorum* originating from an infected California bay (*Umbellaria californica*) leaf lesion. *P. ramorum*, an oomycete, is the causative agent of "Sudden Oak Death", a disease syndrome currently afflicting forests along the coasts of Central and Northern California. ANN04 was separated from *P. ramorum* by culturing on heat-killed bacteria and the co-culture reestablished on a separate *P. ramorum* strain. Trophozoites (~10 µm in length) show putative feeding behavior on hyphae and sporangia of *P. ramorum*. Higher densities of trophozoites and cysts could be observed by co-culturing with an ascomycete, termed strain F1, which was also isolated from the same infected bay leaf lesion as ANN04. Further studies to determine the feeding range of strain ANN04 are underway. Characteristic of *Naegleria* spp., suspension of ANN04 trophozoites into dilute medium induced a temporary flagellated stage. However, to our knowledge, there are no reports of mycophagy by *Naegleria*. To better establish the taxonomic identity of strain ANN04 we will be conducting rDNA and actin gene sequence analysis. The potential application of ANN04 as a biocontrol agent will be discussed in the context of studies by Old and Chakraborty [1] on the apparent role of mycophagous amoebae in suppression of soil-borne *Phytophthora* diseases.

[1] Old, K.M. and Chakraborty, S. 1986. Mycophagous soil amoebae: Their biology and significance in the ecology of soil-borne plant pathogens. In: Patterson, D.J., & Corliss, J.O. eds, *Progress in Protistology* 1:163-194.

Po-13

Presence of free-living amoebae in two habitats with different conditions of oxygen

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Free-living amoebae (FLA) are aerobic organism and as such, it is supposed cannot exist as the trophozoite stage in environments with low oxygen content; however, cysts of *Acanthamoeba* have been isolated from anaerobic material such as faeces. The aim of the investigation was to compare the population of free-living amoebae of two habitats with different conditions of oxygen. Samples of wastewater and sludge were taken from an anaerobic digester tank used as treatment of domestic wastewater. Samples were seeded on non nutritive agar with *Enterobacter aerogenes* and the cultures were incubated at 30ºC. Dissolved oxygen concentration of wastewater was in an average of 0.6 mg/L and the sludge was anoxic. Seventeen species of free-living amoebae of 6 genera were isolated from sludge and wastewater. Eleven species of amoebae were isolated from sludge; while 16 species were isolated from wastewater. Both habitats shared 10 species of amoebae: *Acanthamoeba astronyxis, Acanthamoeba tubiashi, Acanthamoeba quina, Acanthamoeba triangularis, Acanthamoeba royreba, Acanthamoeba lenticulata, Hartmannella vermiformis, Vahlkampfia avara, Vahlkampfia aberdonica, Dactylamoeba stella* and *Mayorella microeruca*; and 6 species were found only in wastewater: *A. mauritenesis, A. polyphaga, A. lugdunensis, Vannella lata, Vannella platypodia* y *Vannella simplex*. Higher number of FLA was found in sludge (16,800 NMP/100 mL) than in wastewater (2,028 NMP/100 mL).

In anoxic condition (sludge) the richness of species was low, but the number of amoebae was higher; while in low condition of dissolved oxygen (wastewater), the richness of species was higher, but the number of amoebae was low.

Cyst forming amoebae like *Acanthamoeba*, *Hartmannella* and *Valhkampfia* were more frequent; however some cyst not-forming amoebae like *Dactylamoeba*, *Mayorella* y *Vannella* were also present. This finding indicates that these amoebae can support hostile condition of oxygen in vegetative form. This would be especially significant to their survival and distribution in the environment.

Po-14

Prevalence of *Acanthamoeba* in Chicago area tap water

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Objectives: A significant recent increase in *Acanthamoeba* keratitis (AK) has been documented in the greater Chicago, Ill. area. It was hypothesized that changes in water treatments may have led to an increase in *Acanthamoeba* in the water supply. This study was done to identify the prevalence of *Acanthamoeba* in tap water in the Chicago area.

Methods: Over the course of 12 months (June 2006-June 2007), water samples were collected from sites in the greater Chicago area. Water was sampled in all cases using sterile swabs from the inside surface film of the lavatory cistern reservoir tank. Further, 50mL of tank water serving the lavatory was also sampled. In all cases, the ultimate source of water was cold municipal mains water. The presence of amoebae in samples was assayed using an enrichment cultivation method appropriate for *Acanthamoeba*. Amoebae were identified based on diagnostic features discernable by light microscopy and select samples were genotyped.

Results: Over 130 households' samples were processed and amoebae were noted in over half of these. *Acanthamoeba* were found in a higher percentage of homes than in previous US studies. Amoebae (regardless of genus) were also present in a higher percentage of samples than found in previous US studies.

Conclusions: Tap water in the home has been associated with corneal infection in contact lens wearers who have exposed their lenses to tap water. The recent outbreak of AK in the Chicago area prompted a study of the tapwater in that area. Initial findings reported here indicate a much higher incidence of *Acanthamoeba* in the tap water of Chicago than was observed in S. Florida, possibly a link to the higher incidence of AK. These initial findings support the hypothesis that the new water treatments may be permitting increased biofilms which result in an increase of *Acanthamoeba*.

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Po-15

Identification of endosymbiont in *Acanthamoeba* isolated from domestic tap water

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The genus *Acanthamoeba* is an amphizoic amoeba which is a causative agent of amoebic keratitis, pneumonitis and granulomatous amoebic encephalitis (GAE) in immunocompromised host. Also the important feature of the genus is that it is well-known to function as a carrier or vehicle for some pathogenic bacteria. In this study we investigated presence of endosymbiont from 16 *Acanthamoeba* isolates from domestic tap water samples. We identified endosymbiont from 5 (31%) isolates (KA/PW3, KA/PW4, KA/PW8, KA/PW9, and KA/PW12) using orcein stain methods and assumed their species using 16s rDNA sequencing analysis. To determine two or more different endosymbionts live in one *Acanthamoeba* isolates, we analyzed 16S rDNA PCR products using denaturing gradient gel electrophoresis method. Ultra-structure analysis of endosymbiont achieved using transmission electron microscopy. The 16s rDNA sequence from endosymbionts of KA/PW3, KA/PW4 were similar to *Candidatus* Amoebophilus asiaticus, their similarity were 96.7% and 95.4% respectively. The endosymbionts of KA/PW8, KA/PW12 have 95.4% and 95.3% 16s rDNA sequence similarity to *Candidatus* Odyssella thessalonicensis, respectively. In this study, uncommon endosymbiont which is very similar to *Methylophilus* sp. was found from KA/PW9 isolates. This is firstly report that *Methylophilus* isolated from *Acanthamoeba*. The morphologic characteristics of endosymbionts in KA/PW3, KA/PW4, KA/PW8, and KA/PW12 were rod shape (1.40 - 1.80 μm × 0.20 - 0.30 μm) and they were located sporadically in cytoplasm. But endosymbionts of KA/ PW9 were amorphous (1.50 μm × 0.20 μm) and distributed with groups each other in the vesicles of cytoplasm.

In the near future, we will evaluate the pathogenicity of endosymbiont, include *Methylphilus* which is first reported as endosymbiont in this study.

Po-16

Detection of four adenovirus serotypes within water isolated strains of *Acanthamoeba* in the Canary Islands, Spain

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We surveyed 236 potentially pathogenic *Acanthamoeba* strains, isolated from water sources in the Canary Islands, for the presence of human adenoviruses (HAdV) using a PCR based typing assay. A total of 34 of these strains were found to be positive for adenovirus belonging to four different HAdV serotypes (HAdV-1, 2, 8, and 37). We found that HAdV-2 was the most frequency encountered serotype amongst the *Acanthamoeba* strains and their identification was confirmed by a nested PCR specific for this serotype. We showed that *Acanthamoeba* genotype T4 was highly associated with serotype HAdV-2, while *Acanthamoeba* genotype T3 was most often associated with adenovirus serotypes related to ocular diseases. Based on these data, we suggest that *Acanthamoeba* should be considered as a potential reservoir and perhaps even a transmitter of adenoviruses to human and other secondary hosts.

Po-17

Novel culture medium for *Naegleria fowleri*

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Background: For axenic cultivation of *Naegleria fowleri*, some culture media were reported up to now. Most of these culture media are excellent for growth of *Naegleria fowleri*, but they have a few disadvantages. Therefore we developed a novel culture medium for *Naegleria fowleri* axenic cultivation.

Method: In this study, *Naegleria fowleri* YT9611 strain, originally isolated from a patient with a fatal case of primary amoebic meningoencephalitis, was used. Commercially available basal media for mammalian and insect cells with or without fetal bovine serum were tested at 37°C and 40°C.

Result: Results from short-term trial at 37°C indicated that the IPL-41, insect cell culture medium, supplemented with fetal bovine serum is suitable for *Naegleria fowleri* cultivation. Interestingly, growth rate of *Naegleria fowleri* in case of diluted IPL-41 condition was faster than in undiluted IPL-41. As a result of examination in a series of mixed ratio, the best ratio of IPL-41 : fetal bovine serum : water was 1:1:8 (named IFW-118) on growth rate of *Naegleria fowleri*. Under axenic cultivation with IFW-118 at 37°C and 40°C, cell shape and motility of trophozoites were normal. Part of trophozoites transiently transformed into flagellates and cysts without medium change. At present, we are continuing long-term cultivation of *Naegleria fowleri* using IFW-118 at 40°C over one year. Further information will be presented.

Po-18

Application of flow cytometry for detection and quantification of *Naegleria fowleri* amoebae in cooling water systems

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Since 1978, the water used for cooling in French power plants has been tested for the presence of pathogenic amoebae and especially *Naegleria fowleri* (*Nf*), the causative agent of Primary Amoebic Meningoencephalitis

(PAM). The replacement of brass condensers by stainless steel condensers, to reduce corrosion and copper discharges, have resulted in increase of *Nf* developments. In some cases, during summertime, densities could reach up to 10000 *Nf*.l^{-1} in power plants cooling water with stainless steel condensers. Consequently, to reduce the release of amoebae in the rivers, continuous chlorinations of the closed-loop cooling systems were implemented in these power plants.

Traditional methods for detection and quantification of *Nf* in water samples are growth- based and results require several days to obtain the result. A new method based on the ChemScan scanning cytometer was evaluated. It is based on the fluorescent labelling of *Nf* initially concentrated on a filter membrane and automatically detected and enumerated by a laser-scanning instrument This rapid method (results in the daytime) was evaluated for the testing of water used for cooling French power plants.

During 2 years, more than 950 water samples were tested by both methods. Results on river water samples showed that, for concentrations of *Naegleria* superior to 200 *Nf*.l^{-1}, ChemScan method appeared to give the same trends of *Nf* developments as traditional standard plate count method (up to 60% of results were strongly correlated). For concentrations under 200 *Nf*.l^{-1}, ChemScan and count plate method are not so well correlated due to low volumes filtered and scanned (10 to 25 mL) with the ChemScan. In this case, a pre-filtration method should be adapted to increase analysed volumes and detection limit.

In conclusion, ChemScan method, by giving significant results in daytime, is a very useful tool for evaluation of a treatment efficiency and risk assessment.

Po-19

Quality assessment of the molecular tools used for the diagnosis of *Acanthamoeba*

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Aims: Early and sensitive diagnosis of *Acanthamoeba* infections may reduce the risks for the clinical condition from worsening. We studied the yields of *Acanthamoeba* DNA that can be obtained by means of different procedures after introduction of a labile DNA tag (internal control) in the specimens.

Methods: *Acanthamoeba* cysts mixed with a tag virus - (seal herpes virus, DNA extraction-yield marker and PCR inhibition detector) - were processed according to different DNA preparation methods: heat, Proteinase K (ProtK), alkali lysis, QIAmp kitR, MagNA Pure (DNA Mini kit, MagNA PureR Nucleic Acid isolation kit), ProtK + QIAmp and ProtK + MagNA Pure. In each extract the parasite-DNA loads were assessed by real-time PCR.

Results: Heat and NaOH did partially hydrolyze the nucleic acids in the samples and therefore, did not allow the DNA extraction yield assessment. Treatment with Prot K was not able to eliminate the PCR inhibitors. Both, the QIAmp and the MagNA Pure partially improved the levels of detection and eliminated the inhibitors. Significant increase in positive results (more than 10X) was obtained just by adding a ProtK pre-treatment before the commercial extraction kits.

Conclusion: The *Acanthamoeba* cysts do not release DNA for amplification if samples are processed with methods used for other cells and viruses. To minimize false negative *Acanthamoeba* diagnosis the cysts should be efficiently lysed without affecting the DNA structure and inhibitors should be eliminated. Significant increase in the detection rates were obtained by adding a ProtK treatment (10min at 56°C) before conducting the commercial procedures. ProtK + MagNA Pure produced in 30 min the highest level of positive results followed by ProtK + QIAmp (150 min).

Po-20

Aquaporin is responsible for high water permeability of the contractile vacuole membrane in *Amoeba proteus*

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To control the cell volume, unicellular organisms living in fresh water have evolved an organelle, the contractile vacuole (CV) complex. *Amoeba proteus* has a large CV that moves with the cytoplasmic streaming. Understanding of mechanism of water uptake is still poor. The aim of the present study is to elucidate the mechanism of water uptake by CV in *A. proteus*.

To evaluate water permeability of CV membrane, CV was isolated from *A. proteus*. When the medium containing cells between the slide glass and the cover glass was soaked up with a piece of filter paper, cells were pressed between two glasses. They became flattened and finally CVs were released into the bathing medium. When the isolated CV was incubated in a hypertonic medium, its volume quickly decreased within 10 sec. This suggested that the CV membrane is semi-permeable and that water should be collected along the osmotic gradient *in vivo*. From the rate of osmotic volume change, the water permeability of the CV membrane was calculated to be 0.94 μm/ sec. This high value suggested the presence of water channel in the CV membrane.

We cloned an aquaporin gene *ApAQP* from *A. proteus*. *ApAQP* encodes a protein consist of 295 amino acids, which shows six putative transmembrane domain and two NPA motifs. Using *Xenopus* oocytes, we demonstrated that the *ApAQP* functions as a water channel. Immunofluorescence microscopy with an anti-ApAQP antibody revealed that ApAQP was localized on the CV membrane and the vesicles around CV. This is the first success in explaining high water permeability of the CV membrane by aquaporin. On the other hand, we found that V-ATPase was highly concentrated on the vesicle membranes around CV. These findings suggest that vesicles are involved in generating the osmotic gradient via the activity of V-ATPase and that water moves into the vesicles along the osmotic gradient through aquaporin.

Po-21

The heat shock protein 70 of pathogenic *Naegleria fowleri* is necessary for rophozoite growth and cytotoxicity

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OBJECTIVES: To evaluate the role of heat shock 70 protein (Hsp70) in the pathogenicity of *N. fowleri,* we cloned, characterized a constitutive and inducible heat shock 70 gene from pathogenic *N. fowleri,* and named as *Nf-cHSP70.* To investigate role of *Nf-cHSP70,* we inhibited the Nf-cHsp70 protein with benzylidene lactam compound, KNK437, which has effect on Hsp70 synthesis and knock-downed *Nf-cHsp70* gene with antisense oligimers which has designed with a start region specific antisense oligonucleotides (24 mers) and modified with phosphorothioate.

METHODS: 1. Amoeba : Trophozoites of *N. fowleri* (Carter NF69; ATCC No. 30215) were axenically cultured in PYG medium at 37˚C.

2. Proliferation inhibition assay: To evaluate the effect of KNK437 (Hsp70 inhibitor) on proliferation of N. fowleri, the trophozoites of N. fowleri were treated with various concentration of KNK437 and incubated at 37˚C for 24hrs. Proliferated cells were counted by hemocytometer after trypan blue stain.

3. Phosphorothioated Oligomers: The target sequence for antisense oligomer (24mer) corresponded to the start region of *Nf-cHsp70.*

4. AS-Oligomer transfection: To evaluate the gene specific inhibition effect of AS-Oligomers, the trophozoites of *N. fowleri* were transfected with various oligomers modified with phosphorothioated and complexed with PEI (polyethylenimine) as transfection reagent.

RESULTS: KNK437 inhibited the induction of *N. fowleri* HSP70 in a dose-dependant manner. After treatment with 300μM of KNK437, the proliferation of *N. fowleri* was reduced to 79.4% compared with untreated control (100%). *Hsp70*-knock downed *N. fowleri* with antisense oligomer showed reducing proliferation to 70% compared with untreated control. Hsp70 inhibited *N. fowleri* showed reducing cytotoxicity against CHO target cells as to 50%.

CONCLUSION: AS Oligomers directed against *Nf-cHsp70* caused a dramatic and sequence specific decrease of target protein level, resulting in the inhibition of growth and cytotoxicity on target cells of pathogenic *N. fowleri*. Our study suggests that *Nf-cHsp70* is essential proliferation and pathogenicity of *N. fowleri*.

Po-22

In vitro activity of perifosine against the trophozoite stage of *Acanthamoeba*

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Perifosine is a novel alkyl-phospholipid that has displayed significant antiproliferative activity *in vitro* and *in vivo* in several human tumor model systems and has recently entered phase II clinical trials. This drug was previously used against *Leishmania* promastigotes. In this study, a modification of a previous colorimetric assay for the assessment of antiamoebic drugs was developed and also the activity of perifosine was tested, against the trophozoite stage of *Acanthamoeba castellanii* Neff, a type strain, and a clinical isolate of *Acanthamoeba* previously identified as *A. polyphaga*.

A previously developed colorimetric 96-well microtiter plate assay, based on the oxido-reduction of Alamar Blue® Assay, was optimized for the determination of drug efficacy against the trophozoites of *Acanthamoeba* spp. The drug used for the optimization of the protocol was the alkyl-phospholipid perifosine.

The obtained results demonstrated that the newly optimized assay is more comparable to a manual counting than the previously developed one and also the activity of perifosine was demonstrated against *Acanthamoeba* spp, using the developed protocol.

The activity of perifosine is demonstrated for the fist time against the trophozoite stage of *Acanthamoeba* spp. The newly optimized assay offers a number of advantages such as being less labour-intensive, faster and more reliable than those previously described, as well the results can be read in a non-subjective manner.

Po-23

Acanthamoeba encystation versus „toxic reaction“: differences in morphology, cellulose synthesis and resistance

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Under unfavourable environmental conditions *Acanthamoeba* spp. undergoes encystation. The mature cyst is characterized by the presence of double-layered wall – exo and endocyst; both of them are containing cellulose. Encystation is a relatively slow process continuing for 48 hrs, when the first mature cysts are observed in-vitro. In contrast to encystation, usage of different solvents leads to „toxic reaction“, where acanthamoebae form single-layered pseudocysts with minor content of polysaccharide with beta linkages. Cellulose synthesis during encystation and toxic reaction was detected by calcofluor staining. The formation of exocyst takes 24 hrs. During the next 24 hrs endocyst is formed and first mature cysts are observed. Toxic reaction is a rapid process where trophozoites detach and round up within 30 min of exposure and patchy cellulose remnants on the cell surface appear 1 hr after induction. Single-layered pseudocyst is formed within 2 hrs. To test the sensitivity of cysts, pseudocysts and trophozoites, acanthamoebae were exposed 0.5% SDS as well as different range of pH (3-11) and temperature (40-65°C). In contrast to mature cysts, trophozoites and single-layered pseudocysts are not resistant to the SDS treatment. Exposure to different pH revealed that in contrast to mature cysts, pseudocysts were sensitive to acidic pH (3-6) and within 24 hrs of exposure most cells underwent lysis. Mature cysts were shown to be resistant to 5 min incubation in 65°C in contrast to pseudocysts (resistant to 55°C for 10 min) and trophozoites (resistant to 45°C for

10 min). „Toxic reaction“ is a rapid reaction of acanthamoebae to the agents, which might act as amoebicidal. However, even if the speed of the response is suitable, single-layered wall of pseudocysts provides only relative protection against different unfavourable conditions and mature cysts are the most important resistant forms of acanthamoebae.

Po-24

Gene profile of encysting *Acanthamoeba castellanii*

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The members of the genus *Acanthamoeba* are the causative agent of GAE and amebic keratitis. The trophozoite, the vegetative state of *Acanthamoeba* transforms to the cyst, the resistant form under harmful environments such as starvation, cold and by which poor responses in medical treatment are resulted in. To investigate the factors mediating encystations, expressed sequence tag (EST) of encystations-induced *A. castellanii* was analyzed and compared to that of trophozoite. Total 1,021 and 905 ESTs were generated from the cyst and trophozoite cyst cDNA libraries of *A. castellanii* Castellani. We compared the predicted proteins from the ESTs of the cyst and the trophozoite by reciprocal BLAST analysis, KOG (euKaryotic Orthologous Groups) assignment, and gene annotation. Various genes were identified in the cyst ESTs. In addition to previously reported cyst specific genes such as cyst specific protein 21, protein kinase C, enolase, proteasome and heat shock protein, we identified several genes like as cullin 4, caleticulin, autophage protein 8 and ubiquitin-conjugating enzymes seems to be related to encystation. Five kinds of proteinases including subtilisin-like serine proteinase, cysteine proteinase, peptidase M28, serine-type endopeptidase and aspartyl aminopeptidase were detected in cyst ESTs. The information on the encystation mediating factors identified by ESTs opens the way for further study on differentiation and resistance of cyst-forming pathogenic protozoa.

Po-25

Variabilities in protein profiles and immunoreactivities among *Acanthamoeba* genotypes

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Acanthamoeba is a free-living protozoan found in a wide variety of habitats. *Acanthamoeba* has also been isolated from humans and animals. Several representatives of the genus *Acanthamoeba* are known as causative agents of *Acanthamoeba* keratitis (AK), a sight-threatening disease of the eye, and granulomatous amoebic encephalitis (GAE), an infection predominantly occurring in the immunocompromised host. Recently, fifteen genotypes (T1-T15) of *Acanthamoeba* have been established. Most clinical isolates group into genotype T4 but also genotype T3, T5, T6, T11, T12 have each caused individual cases. Previous studies have indicated physiological differences between pathogenic and non-pathogenic *Acanthamoeba* strains. However, there is little information on differences between genotypes.

The aim of this study was to compare protein profiles and immunoreactivities between *Acanthamoba* strains of different genotypes, and between pathogenic and non-pathogenic stains within genotype T4, the genotype most of all linked with disease. Altogether, thirteen strains, both pathogenic and non-pathogenic, from seven genotypes (T3, T4, T5, T6, T9, T11, T12) of *Acanthamoeba* were studied for their protein profiles and immunoreactivities with IgA, IgG, IgM. It was shown that protein profiles and immunoreactivities are notably different among genotypes. Moreover, differences were also observed between pathogenic and non-pathogenic strains within genotype T4.

Key words: *Acanthamoeba*, genotypes, protein profiles, immunoreactivities

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2D-page protein profiles of pathogenic and non-pathogenic *Naegleria* species (II)

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Naegleria fowleri is a thermophilic free-living amoeba that causes primary amoebic encephalitis in humans, while *N. lovaniensis,* the morphologically identical species, is not. A two-dimensional (2-D) gel analysis was employed to compare total proteins in *N. fowleri* to those of *N. lovaniensis* in order to identify proteins that may link to its pathogenesis. Protein was extracted from log-phase growing amoebae and recovered by TCA precipitation. The results of 2-D PAGE image analysis showed that 228 protein spots were common in *N. fowleri* strains and so were 246 protein spots in *N. lovaniensis* strains. A comparison of the average gel showed that 88 protein spots were common between two species. The number of species-specific protein is 102 proteins in 5 strains of *N. fowleri*. Among the species-specific spots of *N. fowleri*, 63 spots were analyzed N-terminal and/or internal amino acid sequences along either with Cleveland peptide mapping or with EST-IT tandem MS followed by similarity search for known proteins against NCBInr database using Mascot search engine. In addition, some of the MS/MS spectra have also been de novo sequenced by means of PEAKS Studio v2.4, revealing many peptide candidates from each spot. The latter method showed an ultimate advantage for the identification of the proteins from the internal sequences of protein spots especially those whose N-terminal is blocked like many of those of *N. fowleri.*

According to amino acid sequences of about 20 residues, 14 spots were identified with high confidence as cyclophilin, nucleoside diphosphate kinase, NADP-isocitrate dehydrogenase, and MP2CL5, *N. fowleri* HSP70, albumin, actin, and others. We partially cloned the genes of 2 protein spots, tentatively designated as #15 (24.1kDa, pI6.5) and #35 (50.9 kDa, pI 16.7), that are specific to *N. fowleri* by means of degenerate PCR. The respective PCR products had approximately 250 bp and 400 bp in sizes. From the amino acid sequence predicted from the amplified DNA, #15 was identified with high confidence as Thioredoxin peroxidase (22.3 kDa, Q6DV14) with 80% (54/67) homology. Similarly the predicted amino acid sequence of #35 showed 55% (67/120) homology to that of glutamate dehydrogenase (55.0kDa, Q54KB7). The 2D-PAGE database will be updated spot by spot without delay for public access.

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Observations on *Acanthamoeba* isolates from the Philippines

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Acanthamoeba sp., a free living amoeba that is distributed worldwide, is considered to be one of the most prevalent protozoa distributed in the environment. The organism can be found almost everywhere, from natural or treated water, seawater, swimming pools, air-conditioning units, soil and contact lenses. Being an opportunistic pathogen, it can also be isolated from humans. The organism exists as an actively dividing trophozoite and as a dormant cyst. In our study we isolated *Acanthamoeba* sp. from various sources. Four were human isolates and two were from hot springs. Two of the four human isolates were isolated from corneal specimens, and two came from nasal swabs. The isolates were cultured on non-nutrient agar and incubated for 36 hours at 37°C. The trophozoites vary in size ranging from 25–40µm and feed on bacteria and yeast in the environment. There are several ways the trophozoites acquire food either through pseudopod formation and phagocytosis or by food cup formation. The amoeba moves in a sluggish movement with the formation of a hyaline pseudopodium. The cysts measure 15–20µm in diameter and are doubled-walled and usually polygonal and spherical in shape.

In higher magnification, trophozoites of acanthamoeba possess a trilaminar plasma membrane that surrounds the cytoplasm. The trophozoite serves as the feeding stage of the amoeba. It also comprises of a nucleus, vacuoles, mitochondria, ribosomes and endoplasmic reticulum. The amoeba has a double-walled wrinkled cyst that is com-

posed of an ectocyst and an endocyst. The cyst is formed during adverse conditions like food deprivation, change in pH and temperature. Cysts are resistant to chlorination, and are able to survive at low temperatures ranging from 0–2˚C. Excystation and encystations of the amoeba occur when conditions are favorable. This paper describes both the trophozoite and cyst stages of the organism.