SUPPLEMENT

XI[th] International Meeting on the Biology and Pathogenicity of Free-Living Amoebae České Budějovice, September 5–9, 2005

NON-REFEREED ABSTRACTS

In 1958, Culbertson and collaborators were the first to observe that small free-living amoebae of the limax group were able to infect mammals and turn into serious pathogens. Their discovery initiated a gradually snowballing fruitful research. These organisms, also termed amphizoic amoebae, are today well known as often lethal human parasites and disease agents of other animals, especially fish. They enjoy a gradually increasing attention, reflected by a series of international meetings devoted to presentation of results in this field of research. We publish the abstracts of papers presented at the last, XI[th] International Meeting on the Biology and Pathogenicity of Free-Living Amoebae, held in České Budějovice on 5–9 September 2005.

These abstracts have not been refereed. Apart from correcting a number of obvious misspellings they have not been edited by the journal and the authors with Meeting organizers are responsible for the accuracy and contents of their abstracts. These abstracts are not considered regular papers and should not be cited as papers by authors of future publications and database administrators.

Molecular taxonomy of the genus *Naegleria* and other vahlkampfiids

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Naegleria spp. have been described using morphology, pathogenicity, serology and allozymes. The ribosomal DNA (rDNA) sequences have confirmed and refined these species identifications. Analysis of the small subunit (SSU) rDNA has been superseded by that of the internal transcribed spacers (ITS) including the 5.8S rDNA sequence. Due to this precise molecular identification of a species it is now possible to study in detail the ecology of these amoeboflagellates. Since I demonstrated 30 years ago that *N. fowleri* proliferates in water thermally polluted by industries, many new species, the total being 36 *Naegleria* spp. by now, were isolated from warm waters. Hence, most known *Naegleria* spp. have high maximum temperature ranges. When sampling from cold environments, like the Arctic and Antarctic region, it appears that another genetic pool of *Naegleria* is present in these sites. It will be interesting to see any genetic changes in these populations with global climate change. While it had almost become a dogma that in *Naegleria* spp. with a group I intron, or twintron, in the SSU rDNA, all strains carry it, I have detected in two of these species one strain each that lacks the intron. Several new species have also been described in the other vahlkampfiid genera using molecular typing. The rDNA is on a circular plasmid in all vahlkampfiids. Because plasmids could be exchanged between organisms there is a possibility that it is the rDNA phylogeny which is actually obtained, instead of the amoeba phylogeny. Therefore, a protein is investigated to see whether the same phylogeny would be obtained with a chromosomal gene. The sequences of the largest subunit of RNA polymerase II (RPB1) indeed confirm the taxonomy of the genus *Naegleria*. This RPB1 gene also detects two types in *N. fowleri*. Although species delineation remains something artificial in protists without sex, species names are necessary in order to make international information exchanges on the diversity and occurrence of these amoebae possible.

A quantitative study of thermophilic *Naegleria* during summer season, in natural waters used for recreational purposes in Sonora, Mexico

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In an attempt to quantify *Naegleria fowleri* in our region, where a case of primary amoebic meningoencephalitis occurred recently, a natural reservoir and running water were sampled during the hottest months of the year. The concentration of *Naegleria* was determined by the Most Probably Number (MPN) method. Water samples were seeded on non nutritive agar plates, covered with *E. coli*, and incubated at 42°C for 5 days. All isolates were tested for enflagellation and temperature tolerance. Axenic cultures were used for pathogenicity testing in mice, and identification with PCR using ITS and *N. fowleri* primers, followed by sequencing. Maximun abundance reached 1724 amoebae L^{-1} and minimum abundance was 16 amoebae L^{-1}. Although the concentration of total thermophilic amoebae changed according to the temperature of water, concentrations of *Naegleria* did not follow the same pattern and never reached 100 amoebae L^{-1}. Preliminary results of 39 *Naegleria* strains recovered from plates, seeded during June to October, shows that 17 strains are not pathogenic for mice after intranasal instillation and also negative with the *N. fowleri* primers in PCR. All the strains sequenced

with the ITS primers were identified as *N. lovaniensis*. This is the first attempt in Mexico to estimate concentrations of *N. fowleri* in the natural environment and data will be useful to recommend a legal standard for natural freshwater used for recreational purposes.

Reliable detection and quantification of *Naegleria fowleri* in water samples using a real-time PCR assay

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The free-living amoebae *Naegleria fowleri*, the causative agent of primary amoebic meningoencephalitis, are frequently found in environmental and man-made aquatic environments. Therefore, monitoring the presence of *N. fowleri* in these sites is crucial. The classical culture methods used for the quantification of amoebae are time-consuming and not very accurate. For this reason, new culture-independent molecular methods are being developed. It has already been proven that real-time PCR is suitable for fast and accurate enumeration of microorganisms in water and soil. In this study, primers and probes were designed to amplify and quantify *N. fowleri* DNA in a single duplex reaction. Each real-time PCR assay also contained an internal positive control to check for PCR inhibition, which is a common problem in the analyses of e.g. cooling water samples. The PCR assay was found to be 100% specific for *N. fowleri* and was capable of detecting as little as 4 *N. fowleri* cells in a single PCR reaction. Afterwards the *N. fowleri* real-time PCR assay was used in the analyses of several cooling water samples. Results showed that the test is well suited for the rapid and quantitative detection of this human pathogen in water samples.

Restriction fragment length polymorphism (RFLP) profiles of five *Naegleria* sp. isolated from various water sources in the Philippines

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Naegleria are free-living protozoans whose life cycle consists of 3 morphological stages: trophozoite, flagellate and cyst. Some members of the genus are opportunistic pathogens, the most notable of which is *Naegleria fowleri*, a causative agent of the primary amoebic meningoencephalitis (PAM). In this study, five *Naegleria* isolates were obtained from different water sources: a river, swimming pool, man-made pond, stream, and factory effluent. DNA was extracted from each isolate and then subjected to polymerase chain reaction (PCR) to amplify the internal transcribed spacer (ITS) regions and 5.8S rDNA gene, using appropriate primers. After agarose gel electrophoresis, results showed that the isolates had amplicon sizes of 500, 406, 450, 472 and 461 base pairs, respectively. This suggests that the isolates differed from each other. Differences in band patterns in polyacrylamide gel were observed among the isolates when the amplicons were digested with restriction enzymes, Mse I and Nla IV. With the latter enzyme, a 70-bp fragment was common in four of the isolates and in the reference strain *N. philippinensis* H15K416. Based on the restriction patterns observed, the isolates were neither *N. fowleri* nor *N. australiensis*, since the fragments obtained did not have the markers for these known species.

Cell-surface interactions of *Naegleria gruberi* amoebae with substrata

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Complex ligand-receptor interactions with substrata occur in metazoan cells for example via integrin associations. In these cells the internal extracellular milieu is finely regulated. In contrast free-living amoebae can operate within an extracellular fluid displaying a wide variety of chemical components and physical physical parameters. Our studies on *Naegleria gruberi* amoebae demonstrated modulation of cell adhesion, motility and cell-substratum separation distance following alteration of ionic strength (cation component), pH, temperature. Surprisingly alteration of the physical nature of the substratum (glass, fluorocarbon oil, air-water interface) made no observable difference to amoeboid motility rates. Hence alteration of ambient levels of physicochemical components in the extracellular medium can determine cell behaviour. This can be demonstrated using a variety of experimental techniques including cell adhesion measurement, videomicroscopy, interference reflexion and fluorescence microscopy. It will also be considered to what extent these laboratory studies relate to natural aqueous environments.

However chemical specificity could be demonstrated. Amoebae bound strongly to a glass surface treated with Concanavalin A thus suggesting carbohydrate residues present at the amoeba surface bound to the substrate associated lectin. It is of interest to note several glycan-binding proteins of viral/bacterial origin can bind to eukaryote carbohydrate arrays in a specific manner. Parallel experimental studies were also carried on *Acanthamoeba castellanii* although the the profile of physicochemical interactions was different. Also chemical interactions identifed glycan residues at the amoeba surface possibly as a prelude to phagocytosis of bacteria in a specific manner.

Organotypic slice cultures from rat brain: a new approach for *Naegleria fowleri* infection in vitro

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The free-living amoeba *Naegleria fowleri* is the etiological agent of primary amoebic meningoencephalitis (PAM), a disease with fatal outcome in the vast majority of cases. In patients suffering from PAM and in corresponding animal models, the brain undergoes a massive inflammatory response, followed by hemorrhage and tissue necrosis. Both, in vivo and in vitro models are currently being used to study PAM infection. However, animal models may pose ethical issues, are dependent upon availability of specific infrastructural facilities, and are time-consuming and costly. Conversely, cell cultures lack the complexity of organ-specific morphology found in vivo, and thus, findings obtained in vitro do not

necessarily reflect the situation in vivo. In the present study, we report on infection of organotypic slice cultures from rat brain with *N. fowleri*, comparatively assessed with in vivo infection in a rat model of PAM. We found that brain morphology as present in vivo is well retained in organotypic slice cultures, and that infection time course including tissue damage goes in line with observations in vivo in the rat. Therefore, organotypic slice cultures from rat brain offer a new in vitro approach to study *N. fowleri* infection in the context of PAM.

Screening of amoebocidal compounds using paper disk method

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Our focus to date has been to screen chemical compounds with amoebocidal/static activity against *Naegleria* species. To screen amoebocidal substances, we have designed an impregnated paper disk method using *Naegleria lovaniensis* as a target microbe with slight modification of the method originally developed to evaluate antibacterial effectiveness of new compounds on inhibiting growth of pathogenic bacteria. Briefly, an aliquot of 0.5 ml of a mixed suspension of *N. lovaniensis* (1.8×10^6) and heat-inactivated *E. coli* (1.3×10^9) was poured onto non-nutritive agar plates in 90 mm in diameter petri dish, and spread evenly over entire surface of the plates. The plates were kept in a safety cabinet for several min with the cover open to evaporate off excess amount of water. Paper disks with known concentration of compounds to be tested were placed on inoculated agar surface of prepared petri dish. After 18 h of cultivation at 37°C for 18–24 h, diameter of each zone of growth inhibition was measured.

This method is useful to screen the effectiveness of both crude extract and purified material on inhibiting growth of *Naegleria*, and found crude extract from a fungal culture (F1557) with marked amoebocidal effect on *N. lovaniensis*. Sequential purification was made to extract amoebocidal fraction(s) by means of silica-gel column chromatography, preparative silica gel TLC and gel column chromatography on Sephadex LH20. As a result, 9 mg of colorless oily syrup was purified from 2.5 g of crude extract. The purified fraction induced zone of inhibition, and the calculated MIC was 31.2 μg/ml. Under microscopic observations, the fraction exhibited lytic activity against *N. lovaniensis* cells. The fraction was then identified as 1-mono-O-linoleoyl-grycerol mainly based on NMR spectral analysis, and demonstrated that the authentic compound consistently induces the amoebocidal activity to the same extent with the extracted fraction.

A multiplex real-time PCR assay for simultaneous detection of *Acanthamoeba* spp., *Balamuthia mandrillaris* and *Naegleria fowleri*

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Acanthamoeba spp., *Balamuthia mandrillaris* and *Naegleria fowleri* are pathogenic free-living amebas found worldwide in the environment. *Acanthamoeba* spp. and *B. mandrillaris* are capable of causing granulomatous amebic encephalitis (GAE) in individuals with compromised immune systems. *N. fowleri* produces an acute and usually lethal central nervous system infection called primary amebic meningoencephalitis (PAM). PAM is commonly diagnosed by finding *Naegleria* trophozoites in fresh CSF samples. Diagnosis of GAE is more difficult since trophozoites are rarely seen in CSF and culturing is very time consuming. The use of polymerase chain reaction (PCR) is an attractive alternative for laboratory diagnosis of infections caused by these free-living amebas. Different PCR protocols have been published, but none of these are designed to simultaneously detect all three free-living amebas. This work presents a multiplex real-time PCR assay for simultaneous detection of *Acanthamoeba* spp., *B. mandrillaris*, and *N. fowleri* in CSF specimens. The assay is based on TaqMan probes that target three regions of the chromosomal small subunit ribosomal gene where the amebas differ from each other. The ability of this assay to distinguish between the different amebas was evaluated with cultured trophozoites: six strains of *Acanthamoeba*, including representatives of the three clinically relevant genotypes T4, T7 and T10; four strains of *B. mandrillaris*; and seven strains of *N. fowleri*. The limit of detection was determined with cultured trophozoites serially diluted in parasite-free CSF. Using this rationale, we were able to detect one single organism of each free-living amebas in spiked CSF with no cross reactions, representing a detection limit of 20 trophozoites per ml. These preliminary results suggest that this multiplex real-time PCR assay is a fast and efficient method to detect free-living amebas in spiked CSF. Further studies are necessary to evaluate the usefulness of this assay in clinical samples from cases of infection.

Identification of free-living amoebae in environmental and clinical samples using polymerase chain reaction

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Four genera of free-living amoebae (FLA) including *Sappinia diploidea*, *Naegleria fowleri*, *Balamuthia mandrillaris*, and *Acanthamoeba* spp., have been associated with central nervous system infections in humans. The number of reported cases of amoebic encephalitis and of emerging pathogens has increased in recent years. In addition, the number of immune compromised individuals is rising. Thus, the availability of specific and rapid tools for distinguishing between genera is important. Diagnosing clinical infections is important as treatment differs between amoebae. However, current approaches to diagnosis of infections are characterized by variety of confounds as well as time constraints. Polymerase chain reaction (PCR) is a rapid and sensitive tool that can be used to identify different genera of amoebae in environmental and clinical samples. We have employed PCR analysis to identify free-living amoeba from different genera. Results demonstrate that *Naegleria*

fowleri is detectable in domestic water sources using a nested PCR assay. PCR also was used to distinguish *Acanthamoeba* spp. from *Balamuthia mandrillaris* in paraffin-embedded brain tissue from a 41 year old HIV$^+$ individual with brain, skin, bone, and liver lesions. Histological examination confirmed the presence of amoebae in brain tissue. PCR results indicated that *Acanthamoeba* was the causative agent of amebic encephalitis in this patient. The use of PCR in clinical samples may provide a more rapid and highly sensitive approach to diagnosis and treatment. (This study was supported in part by NIH/NIDA 5P50DA05274.)

Epidemiologic, serologic and molecular identification of *Acanthamoeba* and *Balamuthia* amebas isolated from brain, lungs, sinus and skin tissues of 14 humans and brain tissue of two dogs and an Orangutan

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During the past two years we have received fresh or Formalin-fixed and paraffin-embedded brain, lungs, sinus and skin tissue samples from 14 humans, two dogs, and an Orangutan with central nervous system disease (CNS) of granulomatous type. Of the 14 humans, 10 were males and 4 were females and ranged in age from 3 to 70 years. At least five of the patients were HIV positive. The human cases originated from three different countries, USA, Australia and Japan. The USA cases came from eight States including Florida, California, Utah, Colorado, Massachusetts, Texas, Pennsylvania, and Arizona. One of the two dogs came from Arizona and the other from Australia. The Orangutan was from Florida. Fresh tissue specimens were processed for culture by inoculating into mammalian cell cultures and on to agar plates seeded with *Escherichia coli*. The paraffin-embedded tissue samples were sectioned, deparaffinized and indirect immunofluorescence test performed using either anti-*Acanthamoeba* or anti-*Balamuthia* serum. We have also successfully extracted DNA from unfixed brain tissues received from two humans and the Orangutan cases and performed PCR using specific primer sets for the amplification of *Acanthamoeba* or *Balamuthia* 16S ribosomal DNA (rDNA) gene sequences. Based on these investigations we have identified *Acanthamoeba* in six humans and a dog and *Balamuthia* in four humans, one dog and the Orangutan indicating that PCR can be performed on fresh brain tissue to identify infections caused by *Acanthamoeba* and/or *Balamuthia*. *Acanthamoeba* organisms were also established in culture from four cases. Two of the four isolates of *Acanthamoeba* were typed as belonging to genotype T4 and the other two as T1. The two amebas with T4 genotype were isolated from the skin whereas the two amebas with the T1 genotypes were isolated from the brain tissue. These studies indicate that these two opportunistic amebas are an important cause of granulomatous amebic encephalitis in humans and other animals worldwide.

Demonstration of *Balamuthia* and *Acanthamoeba* DNA by PCR in clinical specimens and archival materials

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Since 1999 the California Encephalitis Project (CEP) has screened >300 serum samples by immunofluorescent antibody staining (IFA) to detect cases of *Balamuthia mandrillaris* and *Acanthamoeba* spp. encephalitis. During this time period, IFA and/or immunostaining of brain tissue identified 7 cases of balamuthiasis. Cerebrospinal fluid (CSF) and brain and other tissue samples were also received, though not for all cases.
In addition to IFA testing, we have employed the polymerase chain reaction (PCR): 1) as a means of further confirmation of *Balamuthia* DNA in CSF and brain tissues and, 2) as a potential diagnostic tool for detection of *Balamuthia* in encephalitis patients. Three out of 4 of the CSF samples that were submitted tested were positive for *Balamuthia* DNA; the fourth was negative. *Balamuthia* DNA, however, was present in all brain tissue samples tested.
No case of acanthamebiasis was detected in any of the serum samples submitted to the CEP. Two *Acanthamoeba*-suspect cases were examined independent of the CEP. One case was from Tennessee (U.S.) and the second from India. Immunostaining and PCR of brain tissue sections for *Acanthamoeba* DNA confirmed the presence of the etiologic agent.
The PCR technique was extended to include slide material from the CDC archive (positive for *Naegleria fowleri*, *Acanthamoeba* spp., or *B. mandrillaris*) for the presence of *Balamuthia* or *Acanthamoeba* DNA. Formalin-fixed sections of brain tissue on slides from 25 different cases were deparaffinized, scraped off the slides, and their DNA extracted. There was agreement between immunostaining and PCR results for 12, disagreement for 9, and indeterminate results for 4 of the slides tested. *Balamuthia* DNA was detected in none of the *Acanthamoeba*-positive slides, and the reverse was also true. Potential variables in these results are probably the length of time tissues were fixed/stored in formalin, and the age of the brain tissue sections.

Retrospective studies on human amoebic encephalitis in Japan

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Six human cases of amoebic encephalitis have been diagnosed in Japan since the first case was reported in 1976. In 1996, one PAM case due to *Naegleria fowleri* infection was diagnosed, and the pathogenic amoeba was isolated successfully from the cerebrospinal fluid of the patient. The remaining 5 encephalitis cases were suggested to be either due to *Acanthamoeba* or due to *Balamuthia* infection at postmortem examinations. In the present experiment, 6 cases were re-examined by means of indirect enzyme-labeled antibody stain method using combination of rabbit anti-*Acanthamoeba*, anti-*Naegleria* and anti-*Balamuthia* antibodies given by the courtesy of Dr. Visvesvara of US CDC. Three amoeba species can be differentiated by their stainability to the antibodies; *Naegleria* and *Acanthamoeba* are stained with respective specific antibodies, and

Balamuthia is stained both with anti-*Balamuthia* and faintly with anti-*Acanthamoeba* antibodies.
Amoebae in the brain sections of the PAM case were confirmed to be *N. fowleri* by its specific stainability to anti-*Naegleria* antibody. Amoebae in the second encephalitis case showed strong positive reaction with anti-*Acanthamoeba* antibody, but no positive reaction was obtained with two other antibodies. In the remaining 4 cases, *B. mandrillaris* were identified in the brain sections by their stainability to anti-*Balamuthia* antibody and faintly with anti-*Acanthamoeba* antibody. Histopathological findings of these specimens together with those obtained by the electron microscopy were comparable to those specific to *Balamuthia*: (a) relatively large trophozoites (mean size; 12.9–16.6 μm) tending to cluster in perivascular space; (b) uninucleated with a large, densely staining nucleolus with two or three nucleoli in some trophozoites; and (c) cysts can be visualized in brain specimens. Further detailed immunological and histopathological results of these 6 amoebic menigoencephalitis cases will be discussed in the presentation.

Balamuthia mandrillaris: clinical diagnosis and pathogenesis

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Balamuthia mandrillaris is an increasingly important pathogen that can cause fatal central nervous system infections. Recently, we have isolated *B. mandrillaris* from the brain and the cerebrospinal fluid of a 33-year old male suffering from *Balamuthia* granulomatous encephalitis (BGE). The patient had no history of HIV infection or of being immunocompromised and his CD4 count was only slightly depressed (621 cell/μl, normal range 775–1385 cell/μl). We successfully employed 1) indirect immunofluorescence assays for the clinical identification of BGE and 2) developed PCR-based assays for the sensitive detection of *B. mandrillaris*.
Several lines of evidence suggest that haematogenous spread is a prerequisite in BGE but it is not clear how circulating amoebae cross the blood-brain barrier to gain entry into the central nervous system to produce disease. Using human brain microvascular endothelial cells, which constitute the blood brain barrier, our aims are to identify host as well as pathogen factors that contribute to parasite traversal of the blood-brain barrier. For example, we have shown that *B. mandrillaris* stimulates interleukin-6 (IL-6) release from HBMEC in a phosphatidylinositol 3-kinase (PI3K)-dependent manner. This is shown using LY294002, a specific PI3K inhibitor, as well as by using HBMEC expressing dominant negative PI3K. IL-6 is a pleiotropic cytokine responsible for initiating an early inflammatory response, which may play a role in blood-brain barrier perturbations. In addition, for the first time, zymography assays revealed that *B. mandrillaris* exhibit protease activities. We observed several major protease bands of approximate molecular weights in the region of 40–50 kDa on SDS-PAGE gels using gelatin as substrate. The protease bands were inhibited with 1,10-phenanthroline suggesting metallotype proteases and exhibited degradation of extracellular matrix (ECM) proteins. The metalloproteases exhibited activity over a pH range of 5–11 with optimum activity at neutral pH. Among variable temperatures, the optimum activity was observed at 37–42°C, indicating their physiological relevance. Future studies will continue to identify both host and parasite factors that are involved in the pathogenesis and pathophysiology of *B. mandrillaris* encephalitis, which are crucial for the rationale development of therapeutic interventions.

Oral infection with *Balamuthia mandrillaris*, amebae in immunocompetent and immunodeficient mice

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Balamuthia mandrillaris is a free-living ameba and an opportunistic agent of lethal granulomatous amebic encephalitis in humans and other mammals. We have shown that intranasally instilled *B. mandrillaris* infect the brain of immunodeficient mice via the olfactory nerve pathway, similar to *Naegleria fowleri* (1). This study addresses the question whether *B. mandrillaris* amebae may also infect via an oral/gastrointestinal pathway.
Groups of 10 female C57BL/6 wild-type and C57BL/6 rag-1$^{-/-}$ (RAG) mice aged 8–12 weeks received 1×10^4 *B. mandrillaris* organisms in 20 μl PBS into the esophagus by gavage. A third group (RAG, n=4) was exposed by co-housing with mice (RAG, n=5) that had been infected by intranasal instillation 3 days before.
All mice were weighed daily and assessed for overt signs of morbidity. Individual mice were sacrificed when moribund and the experiment terminated day 42 p.i. Stool samples were analyzed for viable amebae and antigen days 1 to 11 p.i. and before sacrifice. Stomachs, intestines, brains and affected organs were subjected to immunohistological (IF) analysis.
All four experimental groups showed signs of morbidity beginning days 14–18 p.i., foremost the intranasally infected RAG. Mice of both orally infected groups became ill and some died (RAG, 40%; WT 20%) with abundant *Balamuthia* amebae in the brain. Stools of both orally infected groups became positive for *Balamuthia* antigen day 1 p.i. which declined somewhat beginning day 8 p.i. IF-microscopy revealed *B. mandrillaris* ameba in stool samples from both groups. However, attempts to culture amebae from the stool were largely unsuccessful. 2 of 10 WT mice showed anti-*Balamuthia* serum antibody. Interestingly, all mice exposed by co-housing became ill and one died with a massive *Balamuthia* infection of the CNS. Detailed histological analysis of all major organs will be provided.
(1) Kiderlen A.F., Laube U. (2004). Parasitol. Res. 94:49–52.

First case of *Balamuthia mandrillaris* encephalitis in Portugal

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Introduction: *Balamuthia mandrillaris* is an amoebic agent recently recognized as an unusual cause of cerebral abscess. Most of the infections have been identified in immuno-

compromised hosts although a few invasive disease cases were described in young paediatric patients. *Balamuthia* infections are very rare, with only two reported cases in Europe. We present a case of *Balamuthia mandrillaris* encephalitis that occurred in a previously healthy boy who was admitted in our department and died five weeks after the onset of symptoms.

Case Report: He was brought to medical care because of headaches that evolved for two weeks and diplopia two days before. On admission a cerebral computed tomography (CT) scan showed a left frontal hyperdense lesion with a huge perilesional oedema. The image was thought to be of a tumour, so the patient was started on intravenous (IV) corticosteroids and was referred to a neurosurgery consultation. A cerebral magnetic resonance imaging (MRI) was performed, where a large left heterogeneous frontal lesion and a smaller right temporal nodule with a ring-enhancing signal could be seen. Immunological deficiency studies were negative as well as HIV-1 and -2 antibodies. Tuberculosis was ruled out. Surgery was performed, with an uneventful recovery. On day 9 after surgery he restarted sporadic headaches. Histopathologic examination revealed amoebic infection, and he was treated with antimicrobial combinations (fluconazole, trimethoprim-sulfamethoxazole and rifampin). Despite aggressive antibiotic therapy his clinical status worsened without recovery. Post-mortem examination of specimen by immunofluorescence and PCR showed infection by *Balamuthia mandrillaris*. No source of infection was identified.

Conclusion: This is the first reported case of *Balamuthia mandrillaris* infection in Portugal. Although extremely rare, amoebic granulomatous abscess should be considered in differential diagnosis of intra-cranial masses, especially if multiple lesions are present on cerebral imaging. The absence of risk factors and immunodepression should not keep physician's awareness away from this entity.

Morphologic, isoenzyme electrophoretic and Western blot studies on three strains of *Acanthamoeba* isolated from three Spanish patients with *Acanthamoeba* keratitis

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Three Spanish patients, one male and two females, ranging in age from 40 to 70 years were diagnosed with *Acanthamoeba* keratitis. The male patient was examined for ischemic optical neuropathy of the left eye with reduced visual acuity. He underwent a cataract surgery and anterior vitrectomy with the introduction of an intraocular lens. Since he developed corneal abscess a corneal scraping was performed and examined for bacteria, fungi and *Acanthamoeba*. Cultures were positive for *Acanthamoeba* and although appropriate medication and surgery were administered the patient suffered a complete loss of vision. Of the two female patients, one wore semi-rigid contact lens and the other soft disposable contact lens. Both patients experienced foreign body sensation and painful eye. Corneal scraping yielded *Acanthamoeba* but not fungal or bacterial organisms. *Acanthamoeba* from the three cases were subsequently established in bacteria-free PYG medium containing 5% fetal bovine serum. Based on the microscopic examination of the amebas, especially the cyst stage, the amebas were identified as *Acanthamoeba*, Group II. Three isoenzymes (propionyl esterase (PE), acid phosphatase (AP) and hexokinase (HK) were resolved on high resolution polyacrylamide gradient gel electrophoresis (PGGE). The profiles obtained for three isoenzymes were compared with those obtained for *A. castellanii*, *A. culbertsoni*, *A. polyphaga* and *A. rhysodes*. Western blot profiles of PGGE separated proteins of the three Spanish isolates were compared with those obtained for the four reference isolates (*A. castellanii*, *A. culbertsoni*, and *A. polyphaga*) after reactivity with the rabbit polyclonal anti-(*A. castellanii*, *A. polyphaga* and *A. culbertsoni*) antibodies. Based on these studies we believe that one of the Spanish isolates (USP-GM-A3) is similar to *A. castellanii* while the two other isolates (USP-CC-A4 and USP-CC-A5) appeared to be different from the four reference strains. We are in the process of extracting DNA from these Spanish strains to perform PCR using specific primer sets for the amplification of *Acanthamoeba* 16S ribosomal DNA (rDNA) gene sequences to identify the genotypic classification of these isolates.

Characterization of *Acanthamoeba* sp. isolated from a human keratitic patient in the Philippines

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Acanthamoeba is an ubiquituous, free-living soil and waterborne amoeba that is potentially pathogenic to humans causing amebic keratitis or the more lethal granulomatous amebic encephalitis (GAE). Reports worldwide of amoebic keratitis and GAE caused by different species of *Acanthamoeba* are known. The organism is easily cultured in axenic conditions where both the dividing trophozoite stage as well as the cyst stage are found. Thus far, there is no satisfactory method to distinguish the different species much less identify the pathogenic species from the non-pathogenic ones. Although the first human case of amoebic keratitis in the Philippines was diagnosed in 1992, this is the first documented report of amoebic keratitis in the Philippines caused by *Acanthamoeba*.

The patient is a 68-year old male who complained of eye redness and pain in the left eye after using an old soft contact lens solution. Scrapings from the patient's cornea inoculated into non-nutrient agar supplemented with *E. coli* yielded amoebae with characteristic acanthopodia. The cysts of the amoebae were further characterized by electron microscopy. The presence of characteristic ectocyst and endocysts terminating into a cyst pore which is covered by an operculum can be clearly observed. Intranasal inoculation in Balb/c mice resulted into signs and symptoms of brain infection. Trophozoites were detected and recovered from the infected mouse brain. In addition, polymerase chain reaction was used to amplify part of its cysteine proteinase gene. PCR amplicons were purified, cloned and sequenced. A BLAST search showed that the *Acanthamoeba* cysteine proteinase is homologous to the *Entamoeba histolytica* cysteine proteinase gene fragment.

First cases of *Acanthamoeba* keratitis in Mexico

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Free-living ubiquitous amoebae of the genus *Acanthamoeba* in recent decades are under medical attention not only as etiological agent of chronic infection of the central nervous system (granulomatous amebic encephalitis), but also as causative agents of *Acanthamoeba* keratitis (AK), a serious and vision threatening disease, commonly associated with contact lens wear. Early diagnosis and treatment are essential to improve the visual outcome. Devastating ocular damage can be attributed to various factors such as misdiagnosis, incorrect treatment, excessive topical steroid before diagnosis, and resistance of the strains involved in the cases. AK in non-contact lens wearers is rare and poses a diagnostic challenge. In Mexico we do not have statistics of this pathology. We present five cases reports of AK in Mexico isolated in the Hospital Association to prevent the blindness in Mexico; four of them were contact lens wearers (soft and hard lens), the last one corresponded with a non-contact-lens wearer with an accidental exposure of foreign body. All patients were diagnosed at different clinical stages and had undergone microbiological investigations by processing of their corneal scrapings for bacteria, fungi, and *Acanthamoeba*. Corneal scrapes and contact lens solution were cultivated on 1.5% NNE (non-nutrient) with *Enterobacter aerogenes*. Plates were examined microscopically for the presence of amoeba. In all cases the strains of *Acanthamoeba* were isolated, four identified as *A. polyphaga* and one as *A. castellanii*. All patients were multi-treated but all received Tobramicine and systemic and topical Itraconazole at the end, with good prognosis. In our country Brolene it is not accessible at all; for that reason antifungical therapy (Itraconazol) is our pharmacological election. This report shows a few cases of AK diagnosed in only one hospital, but we consider that a lot of cases still remain misdiagnosed. It is important to determine the real incidence of this pathology in Mexico.

Real time PCR in diagnosis of *Acanthamoeba* keratitis

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The bad prognosis of *Acanthamoeba* keratitis (AK) justified a rapid and sensitive method of diagnosis. Clinical signs often lack of specificity particularly at the beginning of the infection or when the patients had been treated with various antibiotics. Traditional PCR assay has been proved to be a sensitive tool for diagnosis of AK, superior to direct examination and culture. Real time PCR method had been showed to improve the rapidity of PCR and its specificity by prevent post-amplification contamination.
Moreover, it allowed the quantification of microorganisms in sample. We developed a real time PCR assay to detect and quantify *Acanthamoeba* genome in corneal samples and evaluated its contribution in the diagnosis of AK.
First, 38 corneal samples from patients with AK previously diagnosed by classical PCR and mainly due to T4, T3, T11 and T2-T6 genotypes were positive with our assay.
Then, from May to December 2004, 293 corneal scrapings from patients with clinical signs suggesting *Acanthamoeba* keratitis or with a keratitis and a history of recurrent infection without improvement though antibiotic or antiviral treatments were investigated for *Acanthamoeba* species by direct real time PCR, direct examination and culture. Forty patients had a real time PCR positive corneal sample. The clinical diagnosis of AK was confirmed in six of these patients by a cure after specific treatments. Five patients presented a bacterial keratitis, one patient presented a herpetic keratitis, and the two remaining patients were lost sight of.
When comparing classical PCR using three primer sets and our real time PCR in diagnosis of AK, the last presented a higher detection rate.

Molecular identification of *Acanthamoeba* from tissue section of skin biopsy of a suspicious GAE patient

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Genus *Acanthamoeba* is one of amphizoic amoeba with ubiquitous distribution, and can cause granulomatous amoebic encephalitis (GAE), amoebic keratitis, and dermatitis. Up to date, two suspicious cases of GAE by *Acanthamoeba* were reported in Korea. One of them was 7-year-old boy received steroid therapy because of undefined granulomatous dermatitis on his face where had been lacerated by trauma 2 years ago. *Acanthamoeba* trophozites were observed in the tissue section of skin biopsy of the patient. In the present study, we tried molecular species identification of *Acanthamoeba* from paraffine block of skin biopsy of the patient by partial sequencing of 18S rDNA. Genomic DNA was extracted from amoeba trophozoites dissected from PAS stained tissue section by Laser Capture Microdissector. Two most variable regions of 18S rDNA of *Acanthamoeba* were amplified by PCR. BLAST search of the sequence revealed that the isolate was genetically closely related to 3 strains of *Acanthamoeba*. Two of them were isolated from *Acanthamoeba* keratitis patients and the other one from water system of a hospital. Phylogenetic tree with the partial 18S rDNA sequence revealed that the present isolate belong to *A. castellanii* complex, the ribotype T4.

Detection of *Acanthamoeba* sequence type T2 in the CSF and in dermal lesions of a non-AIDS patient: first GAE case in Austria?

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A 25-year old man from India presented with CNS symptoms and multiple dermal lesions. The patient had CD4 counts of only 180, but was negative for HIV.

In laboratory diagnosis non-resistant mycobacteria were detected in the CSF, and the CSF was also positive in an *Acanthamoeba*-specific PCR. Subsequently, another CSF sample and also BAL, a lung biopsy, a liver biopsy and two dermal punch specimens were investigated and, except for the liver biopsy, all samples were positive for *Acanthamoeba* in PCR. Sequencing of the PCR products revealed that they corresponded to *Acanthamoeba* genotype T2, all five sequences being identical. Cultures, however, remained negative and also histological investigation of the biopsy specimens did not reveal any amoebae, neither microscopically nor in immunofluorescence. The patient was treated with a combination of rifampicin, isoniazid, ethambutol, pyrazinamid, cephalosporin, streptomycin, flucanozole, trimethoprim/sulfamethoxazole, sulfadiazine, flucytosine and amphotericin B and, when the status of the patient did not improve, with amikacin (intrathecally and systemically) and miltefosine (systemically). Skin lesions were treated with miltefosine (miltex) and disappeared within 4 weeks. Two months after onset of symptoms the CSF was still *Acanthamoeba* positive, however, three months later the CSF was negative and it remained negative in all successive samples. This correlated to serological tests where the anti-*Acanthamoeba* IgG/IgM titres fell from 1:2000/1:1000 to 1:1000/1:500 within three months and to 1:500/1:125 in a final follow up in April 2005. After 6 months of physiotherapy the patient was discharged in March 2005 with a hearing impairment as a sequelae.

Incidences of oral cavity infection with *Acanthamoeba* sp. in the patients with gingivitis

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Different functions played by Protozoa colonizing human oral cavity in pathogenesis of periodontal changes remains still a subject of discussion however recent evidences of the pathogenic impact of these microorganisms are undoubted. In this report we present results of our studies on pathological changes in the periodontal tissues of patients infected with Protozoa. Pre-treatment status of the oral cavity of 49 patients (41–52 years old) treated in the 2nd Maxillofacial Surgery Clinic, with and without systemic diseases, was assessed clinically. Periodontal changes were designed as light gingivitis, including up to 3 teeth, and wide gingivitis, involving 4 and more teeth. Simultaneously, swabs taken from each patient of 10 places of periodontium for protozoan detection were examined. Among 24 control patients, 25% were infected with *Entamoeba gingivalis* and /or *Trichomonas tenax*. In 37.5% of the infected control patients a light gingivitis was observed. No *Acanthamoeba* sp. were found in these patients. Among 25 patients with stomatognatic abnormalities connected with congenital systemic diseases, 80% were infected with oral protozoans; near 90% of them showed the wide gingivitis. Additionally, trophozoites and cysts of *Acanthamoeba* sp. were found in four patients, infected with oral protozoans, which showed clear symptoms of wide gingivitis. Our results indicate that the protozoans colonizing human oral cavity play an important role in pathogenesis of gingivitis and they represent major risk for subsequent *Acanthamoeba* infection in patients with systemic diseases.

Detection of *Acanthamoeba* spp. in tissues of experimentally infected mice by PCR

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Acanthamoeba spp. are opportunistic pathogens that can cause Granulomatous Amebic Encephalitis and cutaneous acanthamoebiasis in immune suppressed patients. We have used a mouse model to study the pathogenesis of *Acanthamoeba* infections in immune suppressed and immune competent hosts. Mice were immune suppressed using delta-9-tetrahydrocannabinol (THC), the major psychoactive and immune suppressive component of marijuana. Mice were exposed to 50 mg/kg of THC by the intraperitoneal route prior to exposure to amoebae by the intranasal route. A first group of mice were sacrificed after day 14 and day 30 to determine whether amebae could be detected by PCR. A second group of mice were sacrificed after day 3 and day 6 of exposure to *Acanthamoeba* to determine whether amebae were present in organs other than the brain and to determine the primary site of infection. Organs including brain, lungs and liver were removed and a PCR assay was performed using primers designed specifically for *Acanthamoeba* to determine its presence in infected hosts. PCR is a sensitive diagnostic method to identify disease caused by *Acanthamoeba*. In the first group of mice amoebae were detected in brains as well as in lungs of immunesuppressed animals. In the second group, all lungs were PCR positive in vehicle-treated and drug-treated mice. In THC-treated mice, brain tissue as well as liver were PCR positive, indicating that an immune suppressed condition allows for dissemination of amebae to other organs. Additionally, brain and lung tissue from mice exposed to *Acanthamoeba* that survived greater than 50 days also were PCR positive which may suggest that amebae remain in a dormant stage in host tissue.

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Identification and properties of a novel metalloprotease, isolated from an *Acanthamoeba* granulomatous encephalitis isolate belonging to T1 genotype

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Granulomatous amoebic encephalitis due to *Acanthamoeba* is a serious human disease that almost always proves fatal. However, the pathogenesis and pathophysiology of *Acanthamoeba* encephalitis remain unclear. Since proteases may play a role in

the central nervous system pathology, we used spectrophotometric, cytopathic, and zymographic assays to assess proteolytic activities of *Acanthamoeba*. Using an encephalitis isolate (belonging to T1 genotype), we observed two major proteases with approximate molecular weights of 150 and 133KD on SDS-PAGE gels using gelatin as substrate. The 133KD protease was inhibited with phenylmethylsulfonyl fluoride (PMSF) suggesting it is a serine protease, while the 155KD protease was inhibited with 1,10-phenanthroline suggesting it is a metalloprotease. The proteolytic activities were observed in gels over a pH range of 5 to 9.5 with maximal activity at pH 7.5. Optimum activity was observed between 37–42°C indicating their physiological relevance. Both proteases exhibit properties to degrade extracellular matrix (ECM), which provide structural and functional support to the brain tissue. This is shown by their ability to degrade collagen I and III (major components of collagenous ECM), elastin (elastic fibrils of ECM), plasminogen (involved in proteolytic degradation of ECM), as well as casein and haemoglobin. The proteases were successfully purified using ion-exchange chromatography and their effects tested in vitro using human brain microvascular endothelial cell (HBMEC) monolayers. Our findings reveal that neither serine nor metalloproteases exhibit HBMEC cytotoxicity. However, these data do suggest that *Acanthamoeba* proteases digest ECM, which is critical for invasion of the brain tissue. Further characterisations of *Acanthamoeba* proteases will allow us to dissect the precise molecular mechanisms associated with *Acanthamoeba* encephalitis.

Determining the number of *Legionella* multiplied in a single amoeba cell

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While free-living amoebae are well documented to play a key role in the presence and persistence of *Legionella* in the environment, the significance of *Legionella*-harboring amoebae in transmission of Legionnaire's disease to human has yet to be fully discussed. *Legionella* is frequently found in natural and man-made aquatic environments in the complete absence of disease. There have been several hypotheses to account for the differences in incidence of disease observed. One possible interpretation may be that there is a threshold number of the bacteria required for infection to occur. In host amoebae, legionellae undergo multiplication within a vesicle and rupture it at the end. Thus, they can be the prime vehicle, although not ruptured, for the transmission of legionellae through inhaling a vesicle but not inhaling free legionellae in water. The individual could then receive many hundreds or more legionellae at one time. In the present study, we attempted to determine the number of bacteria multiplied in a single amoeba cell.

The JAC/E1 strain of *Acanthamoeba castellanii*, a clinical isolate from human eye, was co-cultured with *L. pneumophila* (SG1), originally isolated from cooling tower water. During 2–3 days of co-culturing, vesicles filled with legionellae were observed in most of *Acanthamoeba* cells. To quantify number of legionellae in a vesicle, single vesicles with diameters measured 15 to 25 μm were carefully transferred by micromanipulation to known volume of sterilized distilled water. They were stood for a few minutes till the bacterial cells were liberated from the vesicle. They were then mixed thoroughly and an aliquot volume of the legionella suspension were inoculated onto the BCYEαmedium. The results of bacterial culture showed that approximately 1,000 or more cfu of culturable legionellae were recovered from individual vesicles.

Parasite-parasite interactions: who is the beneficiary in *Escherichia coli* interactions with *Acanthamoeba*?

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The ability of *Acanthamoeba* to feed on Gram negative bacteria as well as to harbour potential pathogens such as *Salmonella*, *Legionella*, *Chlamydia*, and *Mycobacterium* suggests that amoebae and bacteria are involved in complex interactions, which play important roles in the environment and the human health. In this study, we used *A. castellanii* (keratitis isolate belonging to T4 genotype) and studied their interactions with *Escherichia coli* (a CSF isolate, O18:K1:H7 from meningitis patient and a K12 laboratory strain, HB101). The invasive K1 isolate exhibited significantly higher association with *A. castellanii* as compared with non-invasive K12 isolate. These differences suggest that the virulence of *E. coli* plays an important role in their interactions with *A. castellanii*. The bacterial invasion and/or uptake by *A. castellanii* were determined using gentamicin protection assays. A significantly higher number of intracellular K1 were recovered as compared with K12 suggesting that K1 exhibit increased invasion and/or increased intracellular viability. Longer incubations of up to 72 h showed that, following entry, *E. coli* K1 remains viable within *A. castellanii*, while K12 are killed. Using several K1 mutants including rough lipopolysaccharide (LPS) mutant as well as single-gene deletion mutants, we observed that LPS and outer membrane protein A (OmpA) play important roles in *E. coli* K1 viability within *A. castellanii*. Next, we determined the long-term effects of *A. castellanii* and *E. coli* interactions in the absence of nutrients. Our findings revealed that *E. coli* K1 while growing exponentially exhibit amoebastatic effects. In contrast, co-incubations of *A. castellanii* and *E. coli* K12 resulted in growth and viability of both organisms. Future studies will identify the precise mechanisms associated with *E. coli-Acanthamoeba* interactions leading to bacterial invasion/uptake and survival within *Acanthamoeba*.

RNA interference (RNAi) for molecular analysis of genes related to the pathogenesis of *Acanthamoeba*

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In order to confirm that extracellular serine proteases are directly involved in the pathogenesis and virulence of *Acanthamoeba*, the silencing of extracellular serine protease genes was undertaken by interference RNA (RNAi). Chemically synthesized, small interfering RNA (siRNA) were highly

specific and efficient in silencing the catalytic domain of extracellular serine proteases of *Acanthamoeba*. In order to confirm the silencing phenomenon, the extracellular serine protease activities in RNAi-treated parasites were compared to non-treated parasites, using zymography profiles, *Acanthamoeba*-conditioned medium (ACM) protease activity, cytotoxicity assays and extracellular serine protease mRNA levels analysis. Zymography profiles showed a decrease in the extracellular protease levels in the moderate pathogenic and pathogenic strains, after treatment with siRNA. These results were supported after the ACM protease activity and CPE assays were performed in all studied isolates, showing a lower protease activity or cytotoxicity both in the pathogenic and moderate pathogenic strains treated with RNAi. These results support that extracellular serine proteases are directly involved in the pathogenesis and virulence of *Acanthamoeba*.

Cannabinoid inhibition of macrophage and macrophage-like cell migration to *Acanthamoeba*

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Acanthamoeba are causative agents of granulomatous amebic encephalitis (GAE), a fatal human disease of the central nervous system (CNS) in immune suppressed individuals. Recent reports indicate increased incidence of infection among drug abusers and individuals harboring the human immunodeficiency virus. Many of these individuals purportedly use marijuana, a substance with immunosuppressive properties, and could be at increased risk of infection. Using a murine infectivity model, we have demonstrated that delta-9-tetrahydrocannabinol (THC), the major psychoactive and immunosuppressive component in marijuana, inhibits macrophage responsiveness to *Acanthamoeba*. $(B_6C_3)F_1$ mice treated intraperitoneally with vehicle and inoculated intranasally with *A. culbertsoni* (1 LD_{50}) exhibited foci of amebae in the rostral area of the brain that were circumscribed with microglial-like cells, resident macrophages in the CNS. In contrast, well-formed granulomas around amebae were not observed in mice receiving THC (50 mg/kg). In vitro chemotaxis assays using peritoneal macrophages from mice administered THC (50 mg/kg) indicated a decrease in macrophage migration to amebic extract. Similar results were obtained when peritoneal macrophages from non-drug treated mice were exposed in vitro to THC (10^{-5}M – 10^{-9}M). The inhibitory effect on macrophage migration was shown to be chemotactic rather than chemokinetic. Collectively, these results suggest that THC affects chemotaxis of macrophage-like cells to focal sites containing amebae, a condition that could contribute to amebic dissemination in the host.

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Cytotoxic activities of N-Chlorotaurine (NCT) on *Acanthamoeba* spp.

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Acanthamoebae are the causative agents of *Acanthamoeba* keratitis (AK) and of granulomatous amoebic encephalitis (GAE) and disseminated acanthamoebiasis in immunosuppressed individuals. AK is usually treated with a combination of propamidine isethionate and polyhexamethylene biguanide. However, they can only be administered in low concentrations due to their high toxicity. Moreover, there is no effective treatment for GAE or disseminated acanthamoebiasis.

The aim of this study was to investigate the effects of N-Chlorotaurine (NCT), the main representative of long-lived oxidants found in the supernatant of granulocytes, on *Acanthamoeba* spp. Trophozoites and cysts of different strains were exposed to NCT in different concentrations (0.5 mM to 10 mM). To exclude possible interferences caused by the cultivation medium all tests were also carried out in PBS and NaCl, respectively. Effects were recorded after 1, 6 and 24 hours by microscopical screening and viability staining.

After an incubation time of 24 hours and at an NCT concentration of 10 mM a two log reduction of trophozoites was recorded. The incubation of cysts with NCT was shown to cause an inhibition of excystation. These results are promising since concentrations up to 55 mM NCT are tolerated very well e.g. in the human eye.

Inactivation of *Acanthamoeba palestinensis* by photodynamic treatment of trophozoites and cysts

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Despite major advances in medicine, microbiologically-caused diseases continue to present enormous global health problems. The effectiveness of photodynamic technology (PDT), using a combination of a photosensitiser, light and molecular oxygen to achieve selective destruction of a biological target, as a novel modality for treating diseases due to bacterial and eukaryotic microorganisms by means of porphyrins and related compounds, has been demonstrated. However, the effects of visible light and photosensitising dyes on protozoa has not been studied in depth so far. Owing to the increasing importance of *Acanthamoeba* spp. infections and to the frequent unsatisfactory results of antimicrobial therapies, we studied *Acanthamoeba palestinensis* in order to assess the potential of PDT in inactivating pathogenic protozoa in both vegetative and cystic stages.

Cultures were treated with tetracationic Zn(II)-phthalocyanine (RLP068), which was readily accumulated by both trophozoites and cysts, even at doses as low as 0.5 μM, and showed no dark toxicity up to 5 μM concentration. Complete inhibition of trophozoite growth was induced by 1 h incubation with 2 μM RLP068, followed by 10 min irradiation with 600–700 nm light (50 mW/cm^2). After incubation with 3 and 5 μM RLP068 and 20 min irradiation, cysts lost their excystment ability even at day 5. The extensive cellular damage observed may reflect the high reactivity of activated oxygen species such as singlet oxygen, which induces oxidative modifications in several cell constituents. These data suggest the promising use of phthalocyanines to phototreat diseases caused by pathogenic amoebae and in initial disinfection of wastewaters by means of inexpensive light sources, such as full-spectrum visible lamps or sunlight.

Comparative study of adhesion of four strains of *Acanthamoeba* spp. to epithelial cells and contact lens

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It has been established that the first step of corneal infection by *Acanthamoeba* involves the adhesion of trophozoites to the corneal epithelium, as well as to the contact lens. However there are no reports that show interspecific differences in the adhesion to diverse substrates and evasion to IgA antibodies. This study was undertaken to determine quantitative and qualitative differences of trophozoite adherence of 4 strains of *Acanthamoeba* (*A. lenticulata*, *A. polyphaga* and 2 strains of *A. castellanii*) of different origin, 2 types of contact lens (Etafilcon A, Hefilcon) MDCK cells and the effect of IgA (human colostrum) in this process. The strains were axenically cultured and interacted with MDCK cells (1:1 ratio) and segments of soft contact lens at different times. The inhibitory role of IgA in the adherence of *Acanthamoeba* was evaluated coincubating trophozoites with IgA (1:5 dilution) with contact lens and MDCK cells at different times. Interstrain significative differences in trophozoites adherence were observed. In addition, main differences of each strain with MDCK cells, contact lens and IgA inhibition were observed. The adherence of trophozoites of all strains was more efficient to MDCK cells since 90% of them were adhered at earlier interaction times (5 min). All strains migrated and penetrated the MDCK monolayer but at different times. The adherence to contact lens was considerable low in all strains, but in general there was higher affinity to Etafilcon A, than Hefilcon lenses. We found that the greatest adherence was to the edge of both type of lenses. Only one strain was inhibited at the concentration used of IgA at the beginning of the interaction (5–30 min) after the trophic forms were reestablished. The capacity of adherence to the contact lens, the ability to produce cytopathic effect and the capacity to evade the immune response are important factors in the pathogenesis of *Acanthamoeba* keratitis.

Role of human tear fluid in *Acanthamoeba* interactions with the human corneal epithelial cells

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Acanthamoeba keratitis is a painful and progressive sight-threatening infection. *Acanthamoeba* binding to the corneal epithelial cells is a crucial first step in the onset of the disease. Aims of this study were 1) to investigate the effects of tear fluid on *Acanthamoeba* binding to and subsequent cell death of human corneal epithelial cells (HCEC), and 2) to identify factor(s) responsible for host cell protection against *Acanthamoeba*. Using in vitro assays, we observed that *Acanthamoeba* exhibited significantly less binding to HCEC in the presence of whole human tears ($P<0.05$). The adhesion-inhibitory effects of tears were heat-labile suggesting the protective factors are proteinaceous in nature. More importantly, tears collected from an *Acanthamoeba* keratitis (AK) patient had minimal effects on *Acanthamoeba* binding to the host cells. Next, we determined the role of sIgA, lysozyme and lactoferrin in *Acanthamoeba*-HCEC interactions. Interestingly, our results demonstrated that sIgA plays a role in protecting HCEC against *Acanthamoeba* adhesion. Pre-treatment of normal tears with anti-IgA antibodies abrogated adhesion-inhibitory effects of tears. In contrast, lysozyme or lactoferrin had no significant effects on *Acanthamoeba* binding to HCEC. Next, we tested the effects of tears on *Acanthamoeba*-mediated HCEC cytotoxicity. We observed that both normal and AK tears increased *Acanthamoeba*-mediated HCEC cytotoxicity. However, pre-treatment of tears with anti-IgA or anti-lysozyme antibodies exhibited cytoprotective effects against *Acanthamoeba*. Overall, these findings suggest that sIgA inhibit *Acanthamoeba* binding to HCEC, while sIgA and lysozyme promote *Acanthamoeba*-mediated HCEC cytotoxicity. This was further confirmed using purified lysozyme, which increased *Acanthamoeba*-mediated HCEC cytotoxicity in a concentration-dependent manner. Further studies will continue to identify factors that are involved in the pathogenesis of *Acanthamoeba* keratitis, which may help develop therapeutic interventions and/or pre-emptive measures.

Effects of human serum on virulence properties of *Acanthamoeba* (encephalitis isolate belonging to T1 genotype)

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Acanthamoeba are the causative agents of fatal granulomatous encephalitis (AGE). AGE is limited to immunocompromised patients suggesting that host natural immune response is sufficient to control and/or eradicate these pathogens, but the molecular mechanisms remain unclear. In this study we determined the effects of serum from healthy individuals on the virulence properties of *Acanthamoeba castellanii* (isolated from an AGE patient). We determined that normal human serum exhibited amoebacidal effects, i.e., up to 50% trophozoites were killed within 60 min. Further incubations for up to 48 h had minimal effects on the viability of *Acanthamoeba* but showed amoebastatic effects. As expected, serum inhibited *Acanthamoeba* binding to and cytotoxicity of human brain microvascular endothelial cells (HBMEC) in vitro. However, these effects are most likely due to the amoebacidal activities of serum. Zymography assays revealed that, in the presence of serum, *Acanthamoeba* exhibited decreased extracellular protease activities. Of interest, protein A-treated serum (antibody-free serum) abrogated these effects. Interestingly, serum enhanced the phagocytic ability of *Acanthamoeba*, as measured by bacterial uptake. Overall, our results demonstrate that human serum has inhibitory effects on *Acanthamoeba* growth and viability, protease secretions, binding to and subsequent cytotoxicity of HBMEC, while *Acanthamoeba* phagocytosis is stimulated by serum.

Amoebic gill disease in cultured Tasmanian Atlantic salmon (*Salmo salar* L.) caused by the marine amoeba *Neoparamoeba* sp.

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Amoebic gill disease (AGD) affects the marine culture phase of Atlantic salmon (*Salmo salar* L.) in Tasmania. Since the industries inception during the mid 1980's, the pathological development of this disease was not adequately understood. Consequently, using a combination of experimental and field trials, infection of Atlantic salmon by *Neoparamoeba* sp. was investigated to elucidate the progressive nature of AGD development. Experimentally, it was observed that AGD only occurred when fish were exposed directly to viable trophozoites. A progressive host response and significant increases in the numbers of attached amoebae were apparent over a duration of 48 hours. Initial host responses included eodema, epithelial desquamation, hyperplasia and immunocyte infiltration juxtaposed to trophozoite attachment. Sequential histopathological observations of AGD under field conditions over many weeks were likewise investigated. Similar initial host responses were observed with a subsequently pronounced immunocytic infiltration and epithelial hyperplasia noted as the infection progressed. Results suggested that development of AGD was linked to a retraction of the estuarine halocline and increases in water temperature. In conclusion, it is suggested that disease is initiated by attachment of individual trophozoites of *Neoparamoeba* sp. Subsequent disease progression is then dependant upon proliferation and migration of the parasite throughout the gill environment. The rate of proliferation and migration is subject to a suite of intrinsic (host) and extrinsic (environmental) factors.

Relationship between the presence of *Neoparamoeba* sp. in the environment and Amoebic Gill Disease in cultured Atlantic salmon in Tasmania

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Amoebic Gill Disease (AGD) is the most serious health problem affecting Atlantic salmon cultured in Tasmania. A survey was done in 2004 to determine the relationship between the presence of *Neoparamoeba* sp. in the environ-ment and AGD in cultured Atlantic salmon in Tasmania. Two sites were sampled in January and March and before the commencement of the survey in November 2003 (baseline sediment samples). Two other sites were sampled only once, in March 2004. Fish were sampled terminally and the standard scientific case definition for AGD was used: a fish was considered positive if the histological section from this fish had AGD lesions in association with amoebae. Two net samples and three sediment samples were collected for each cage, and amoebae were recovered by culture and identified using an indirect fluorescent antibody test (IFAT) and PCR.

Most sediment samples contained *Neoparamoeba* sp. before fish were introduced to the site. Mean AGD prevalence in fish sampled from cages with nets containing *Neoparamoeba* sp. was 13.75% and for cages without *Neoparamoeba* sp. 16.67% (t-test, df=13, P=0.8057). There was no significant correlation between AGD prevalence and presence of *Neoparamoeba* sp. in nets (r=-0.1505, n=14) or sediment samples (r=-0.2533, n=14). We could not find any relationship between AGD prevalence and presence of *Neoparamoeba* sp. in net or sediment samples. This may be due to imprecise quantification (present/absent only) and a low number of samples (n=2–4). Currently, there is no method available for quantitative detection of *Neoparamoeba* sp. in water. Methods investigated for quantitative detection of *Neoparamoeba* sp. in environmental samples and their limitations will be discussed.

Characterization of surface antigens of *Neoparamoeba* sp.

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Amoebic gill disease, the main disease of concern to the salmon farming industry in Tasmania, is caused by the free-living amoeba, *Neoparamoeba* sp. The infection can be induced by exposure to parasites freshly isolated from the gills of infected fish, however long-term cultured amoebae are non-infective. To identify potential candidate antigens (Ag) for vaccine development we produced monoclonal antibodies (Mab) against surface Ag expressed on infective parasites. To maximise the generation of Mab specific for Ag expressed only by infective parasites a subtractive immunisation method was employed. Mice inoculated with non-infective parasites were treated with cyclophosphamide to deplete reactive lymphocytes. This cycle was repeated a further 3 times, after which mice were immunised with either whole infective parasites, membrane preparations or deglycosylated membrane preparations from infective parasites. Analysis of the antibody response in these mice demonstrated that the immunodepletion protocol is effective, although the proportion of infective parasite-specific Mab generated from mice immunised under this regime was variable. When whole parasites were used for boosting the immune response, the percentage of WT unique Mabs was very high (86%) as was the percentage of Mabs specific for carbohydrate epitopes (89%). When deglycosylated membranes were used the numbers of Mabs specific for peptide epitopes increased slightly (11% to 29%) while the total number of WT unique Mabs was reduced (86% to 37%). Using an untreated membrane preparation the number of Mabs unique to surface antigens on the infective parasites increased, but all these antibodies recognised carbohydrate epitopes. The total number of Mabs recognising carbohydrate epitopes on the surface of the wild type parasites is very high (97%) suggesting that the dominant epitopes on the surface molecules unique to WT parasites are carbohydrate in nature. Characterisation of the reactivity of selected Mab and their target antigens will be presented.

Molecular quest for a diagnostic target for *Neoparamoeba pemaquidensis*

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The amphizoic marine amoeba *Neoparamoeba pemaquidensis* is the etiologic agent of numerous diseases in cultured finfish (Amoebic Gill Disease in salmonids) and has also been associated with mortalities in invertebrates (Blue Crabs, Green Sea Urchins and American Lobsters). Characterizing host specificity, geographical origin or potential pathogenicity of *N. pemaquidensis* isolates requires the use of a discriminative tool. The lack of consistent morphological characters has lead us to direct our efforts towards molecular diagnostics. Current protocols permit identification to the species level but do not differentiate variation at the sub-species level. Investigations of the ITS region (Internal Transcribed Spacer) of *N. pemaquidensis* indicated significant nucleotide variation among isolates. A multicopy locus comparison revealed unexpected and unreported intra-genomic variability (microheterogeneity) which introduces the issue of a "moving target" for the diagnostic. A second diagnostic target was explored to evaluate the usefulness of incorporating the *Neoparamoeba* endosymbiont (*Perkinsiella amoebae*-Like Organism) ITS. The PLO ITS region has sufficient discriminative variability and lacks the microheterogeneity observed in the host genome. Additionally, phylogenetic comparisons of *N. pemaquidensis* and its endosymbiont confirmed their coevolution. Therefore absence of microheterogeneity and the permanency of the PLO within the amoeba, identify the PLO ITS region as a target to develop a molecular diagnostic.

Refining the challenge model for amoebic gill disease in Atlantic salmon

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Amoebic gill disease (AGD) has affected the marine culture phase of Atlantic salmon (*Salmo salar* L.) in Tasmania since the early 1980's. To understand and more effectively control AGD a multifaceted research program has been established to investigate several aspects of the disease including: host-pathogen interactions, prophylaxis and novel treatment strategies. The research is underpinned by access to a reliable supply of the pathogen. Although as yet not proven to be the sole aetiological agent of AGD in Tasmania one species, *Neoparamoeba pemaquidensis*, has been commonly isolated from Atlantic salmon with AGD and has been shown to induce the disease in tank-housed salmon. The virulent organism cannot be cryopreserved nor can it be sourced through culture methods as virulence is lost after relatively short periods in culture. Consequently supplies of virulent *N. pemaquidensis* come from the gills of moribund salmon kept in an infection tank where AGD is perpetuated by regular addition of naïve salmon. At present amoebae are collected using an adherence technique (Morrison et al. 2004) which yields a relatively pure and clean suspension of cells. Once collected amoebae are used for various experiments including: initiation of controlled infections or challenges, in vitro assays and antigen characterisation. Systematic development of an AGD challenge model is essential to the success of the research in particular the efficacy testing of prospective vaccines. This paper reports on the development of a challenge model to date including amoebae viability determination using vital dyes and adherence and variation in virulence of individual batches of amoeba.

Morrison R.N., Crosbie P.B.B., Nowak B.F. 2004: The induction of laboratory based amoebic gill disease (AGD) infection revisited. J. Fish Dis. 27: 445–449.

Role of salmonid gill bacteria in amoebic gill disease

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The relationship between *Neoparamoeba* sp., the aetiological agent of amoebic gill disease (AGD) and salmon gill bacteria is not clearly defined. Amoeba might typically only colonize the gills of partially immunosuppressed fish (Lom & Dyková 1992). Likewise, gill bacteria might play a direct role by predisposing the fish to amoebic gill disease (Bowman & Nowak 2004). The aim of this study is to provide information on salmonid gill bacteria and its role in AGD.

Naegleria fowleri: partial characterization and comparison of total extracts and conditioned medium proteases

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Naegleria fowleri is the etiologic agent of the Primary Amoebic Meningoencephalitis (PAM). Up to the present, the pathogenic mechanisms involved in the invasion and tissue destruction are still poorly understood. Proteases have been suggested to play an important role in the pathogenesis of PAM. In this work, we analyzed the complete proteolytic activity profiles of the total crude extracts of *N. fowleri* trophozoites and the conditioned culture media. Using 5% and 7.5% SDS-PAGE-gelatin gels, we found different proteolytic patterns in either total extracts and conditioned medium. The patterns varied accordingly to the pH tested (pH 3.0, 5.0, 7.0 and 9.0). In total extracts at pH 3.0, two bands of proteolytic activities of 73 and 62 kDa were observed; at pH 5.0 and 7.0 a single 130 kDa band was observed, and an additional 110 kDa band was observed at pH 9.0. In conditioned medium no proteolytic activities were detected at pH 3.0. At pH 5.0 three different protease activities of 164, 178, and 310 kDa were observed and, at pH 7.0 and 9.0 the same proteolytic patterns plus an additional band of 147 kDa were observed. All proteases detected in the total extracts and the 147 kDa band observed in the conditioned medium were inhibited with cysteine protease inhibitors, whereas the remaining bands of conditioned medium were inhibited only with serine protease inhibitors. Regarding to the effect of temperatures, the optimal proteolytic activity of total crude extracts was observed at 40°C, while in the conditioned medium was at 35°C. Our results showed that *N. fowleri* has a variety of proteases that have not been previously reported and are active at different pHs and temperatures and as a free-living organisms, these factors allow the amoebae to capture nutrients from many different sources. Moreover, these protease activities may contribute on the degradation of host substrates giving access to the nervous system producing PAM.

Primary amebic meningoencephalitis: a case report in Mexico and characterization of isolated *Naegleria fowleri* strain

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A 9 year old male presenting headache, nausea, vomiting, photophobia and somnolence, was admitted to the Hospital. Seven days previous to the symptoms the child had a history of swimming in a ditch of Villa Zapata, Mexicali, Baja California (Mexico). A tomography study was reported normal. Three days later a sample of cerebrospinal fluid (CSF) was taken for diagnostic purposes. Motile amoebic forms were observed in fresh samples at light microscopy, and a treatment with fuconazole (200mg/day) and amphotericin B (1mg/kg/day) was started immediately. However the patient entered in coma soon after with episodes of apnea and died at the next day. The sample of CSF was submitted to Mexico city and cultivated in 2% bactocasitone medium supplemented with 10% FBS for further analysis. The strain characterization was achieved by virulence test in Balb/c mice. Ultrastructural analysis and protein and protease electrophoresis patterns and PCR with *N. fowleri* specific primers were performed as confirmatory tests. As a reference test we used *N. fowleri* ATCC (30808). For virulence, mice were by nasally instilled with 10^2, 10^3, 10^4 and 10^5 trophozoites/20μl. The dose of 2.5×10^4 killed 100% animals at six days, in contrast with ATCC strain, which required larger doses. A brain sample was taken at 5 days post-inoculation for histology. Light microscopy showed trophozoites surrounded by a high number of inflammatory cells with extensive lytic and hemorrhagic areas. The ultrastructure of trophozoites showed typical features for this genus, which were very similar to the reference strain. The protein and proteolytic patterns were equal to the ATCC strain with only a slight difference in the expression degree of some protein bands. A predictable 1500 bp product was found in the isolated strain as well as ATCC reference strain. Based in all studies performed we concluded that the etiologic agent in the present case belonged to *N. fowleri*.

Isoenzyme and Western blot profiles of twenty-four isolates of *Naegleria* belonging to 5 different species

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During the past 30 years we have isolated and established in axenic culture more than 30 isolates of *Naegleria fowleri* from the cerebrospinal fluid (CSF) of primary amebic meningoencephalitis (PAM) patients and water samples from hot springs, rivers, lakes and municipal water supply to which the PAM patients were exposed to or had swum. Most of these isolates have been previously identified as belonging to different genotypes based on the information of the ITS1, 5.8S, and ITS2 sequences. In this report we have compared the isoenzyme and immunoblot profiles of 18 *N. fowleri* isolates belonging to three genotypes and one isolate each of *N. gruberi*, *N. jadini*, *N. italica* and *Naegleria* sp. Of the 18 isolates of *N. fowleri* 14 originated from the CSF or brain tissue of PAM patients and four from water samples of hot springs, rivers, lakes and municipal water supply. Five isoenzymes (leucine aminopeptidase (LAP), glucosephosphate isomerase (GPI), propionyl esterase (PE), acid phosphatase (AP) and malate dehydrogenase (MDH) were resolved on high resolution polyacrylamide gradient gel electrophoresis (PGGE). Except for some minor differences all of the *N. fowleri* isolates, whether originated from CSF/Brain or water and irrespective of the genotypes to which they belonged, appeared to be similar in all of the isoenzymes tested. The four other species of *Naegleria* had bands that were different and could be easily separated from *N. fowleri*. Western blot profiles of PGGE separated proteins of the various isolates after reactivity with the monoclonal antibody 35 and the rabbit polyclonal anti-*N. fowleri* antibody as well as the human serum obtained from one of the infected patients revealed that all *N. fowleri* isolates reacted intensely with all of the tested antibodies and produced multiple bands that were similar, if not identical, whereas the four other species of *Naegleria* reacted minimally with all of the three antibodies and could be easily differentiated from *N. fowleri*. These studies indicate that all *N. fowleri* isolates whether isolated from humans originating from different geographic locales or from water appear to be homogeneous.

Intracellular localization and trafficking of AhSub, a serine proteinase and AhCP, a cysteine proteinase in *Acanthamoeba*

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Proteinases have been known to play important roles in pathogenesis and various biologic actions of *Acanthamoeba*. A serine proteinase, AhSub purified from culture supernatant of *A. healyi* showed strong activity against extracellular matrix protein and high degree of cytotoxicity against human corneal epithelial cells. In addition, the gene sequence of AhCP, a cysteine proteinase of *A. healyi* has been previously characterized. In the present study, we analyzed the intracellular localization and trafficking of AhSub and AhCP of *A. healyi* by transient transfection. Full length AhSub-EGFP fusion protein was found in intracellular vesicle like structures of transfected amoeba. Time-lapse photographs confirmed the secretion of the fluorescent material of the vesicle toward extracellular space. The mutated AhSub of which pre or pre-pro region was deleted was found out to localize diffusely throughout the cytoplasm of the amoeba rather than concentrated in the secretory vesicle. Transfection of the construct containing the pre region only showed the same localization and trafficking to the full length AhSub. A cysteine proteinase AhCP-EGFP fusion protein was also localized in the vesicle like structure in the amoeba. However, using Lysotracker analysis, these vesicular structures of AhCP were confirmed as lysosome and not secretory vesicles. On the other hand, the AhCP construct with the deleted pre-pro region showed a dispersed distribution of fluorescence in the cytoplasm of the cells. These results indicated that AhSub and AhCP would play different roles in *Acanthamoeba* biology and the pre and pro region are important for proper intracellular localization and trafficking of the proteinases.

Comparison of specific activity and cytopathic effects of purified 33-kDa serine proteinase from *Acanthamoeba* strains with different degree of virulence

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The pathogenic mechanism of granulomatous amoebic encephalitis (GAE) and amoebic keratitis (AK) by *Acanthamoeba* has yet to be clarified. Protease has been recognized to play an important role in the pathogenesis of GAE and AK. In the present study, we have compared specific activity and cytopathic effects of purified 33-kDa serine proteinases from *Acanthamoeba* strains with different degree of virulence (*A. healyi* OC-3A, *A. lugdunensis* KA/E2, *A. castellanii* Neff). Trophozoites of three strains revealed different degree of cytopathic effect on human corneal epithelial (HCE) cells. The effect was reduced remarkably by adding PMSF, a serine proteinase inhibitor. This result indicated that PMSF susceptible proteinase activity is the main component to cause cytopathy to HCE cells by *Acanthamoeba*. The purified 33 kDa serine proteinase showed strong activity toward HCE cell and extracellular matrix proteins. The specific enzyme activity was higher in the purified proteinase from OC-3A, the most virulent strain, than KA/E2, an ocular isolate, or Neff, a soil isolate. Antibody against the purified 33 kDa serine proteinase inhibit almost completely the proteolytic activity of culture supernatant of *Acanthamoeba*. In the light of these results, the 33 kDa serine proteinase may play an important role in pathogenesis and be the main component of virulence factor of *Acanthamoeba*.

Mixed infection with *Acanthamoeba* sp., parasitic oral Protozoa and opportunistic bacteria in mouth of patients with genetic diseases

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Recent studies showed direct interrelationship between occurrence of *Entamoeba gingivalis* and *Trichomonas tenax* (Protozoa) and pathogenic changes in human oral cavity. In this study we examined and compared the prevalence of the oral protozoans and bacteria in mouth of 100 mentally retarded patients of different age group (14–52 years old). Swabs taken from dental plaques, pockets and other places of periodontium were used for microscopical studies and bacterial culture examinations. Live protozoans were found in the wet preparations; most were identified on the stained slides as *T. tenax* and *E. gingivalis*. It was striking that among the twenty five oldest patients (41–52 years old), mixed infections with the parasitic oral Protozoa and free-living amoebae, identified as *Acanthamoeba* sp., occurred in four cases. Both, the trophozoites and numerous cysts of the amoeba were found in oral cavities of the somatically and mentally disordered patients, suffering from phenylketonuria or Down syndrome. In mouth of three of those patients simultaneous infection with *Staphylococcus aureus* was detected. Various local and general factors influencing the occurrence and maintenance of the mixed *E. gingivalis*, *T. tenax* and free-living amoebae infections are assessed and discussed.

Effect of selected chemical compounds on the cyst stages of *Acanthamoeba castellanii*

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The infections with *Acanthamoeba* species are reported with increasing frequency in various part of the world. Despite advances in chemotherapy, treatment is difficult, and results are often disappointing. Due to development of drug resistance, toxicity of high drug concentration, contradictory results of treatment, further studies on antiacantamoebic activity of different chemical agents are needed. Recent studies indicate that particularly cysts of the amphizoic amoebae are extremely resistant to different chemical agents. In our study we tested in vitro effects of different concentrations of metronidazoele (MT) (4μg/ml and 8μg/ml) and antiseptic agent, chlorhexidine (CHX) (4μg/ml, 10μg/ml and 98μg/ml) on 4-day grown population of the Neff strain of *A. castellanii* after 24 h and 5 day expositions. Status of the surviving amoebae, their number calculated for 1ml of the culture medium and percentage of cysts were assessed microscopically and compared with those observed in the control culture. It was striking that in spite of the general amoebicidal effect, observed after 24 h exposure to CHX, significant increase of the cyst level occurred, particularly at higher concentrations of the compound: at 10μg/ml the % cysts was 5-times higher than in the control assays. After 5 days, the trophozoite and cyst levels were similar as in the control culture. Tendency toward induction of encystment was apparent also when MT was applied that was less effective in reducing general number of amoebae than CHX. Our studies showed some amoebicidal effect of CHX and MT, depending on kind and concentration of the agents tested. However, a risk of repeated development of *Acanthamoeba* population may occur because of activation cysts, particularly when common antiseptic agent, CHX is applied. It confirms that, apart from amoebicidal effect, cysticidal efficacy of different tested agents should be assessed.

In vitro effect of acyclic phosphonate analogs of purines against pathogenic free-living amoebae of the genera *Acanthamoeba* and *Balamuthia*

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Pathogenic free-living amoebae of the genera *Acanthamoeba* and *Balamuthia* are causative agents of serious human infections. Granulomatous encephalitis is usually a fatal disease, a sight-threatening keratitis is treatable, but the treatment often fails. Absence of effective casual drugs is one of the reasons. Therefore, there is a continuing search for agents that could affect key systems within the amoeba cells. In *Acanthamoeba*, synthesis of nucleic acids mostly depends on salvage utilizing external precursors of purines. Nucleic acid metabolism in closely related *Balamuthia* might be similar. Acyclic phosphonate analogs of purines could, therefore, serve as metabolic inhibitors. These compounds represent a group of chemical inhibitors of DNA synthesis with various, not entirely clarified mechanisms of action. They were originally developed as selective inhibitors of viral DNA synthesis for antiviral chemotherapy. However, it has been shown recently that they also exert activity against important protozoan pathogens of humans such as African trypanosomes and *Plasmodium falciparum*. We used a panel of acyclic phosphonate analogs to test their potential in vitro effects on both *Acanthamoeba* and *Balamuthia*. Of 13 nucleoside phosphonates tested, two analogs of guanine, (S)-HPMPG and PMEG, displayed amoebicidal activity. For six axenically growing isolates of *Acanthamoeba* from human cases of keratitis, MLC defined as a minimal concentration of the compound in which no acanthamoebae survive after 72 hours of exposure ranged between 10–2.5 μg/ml and 15–7.5 μg/ml for (S)-HPMPG and PMEG, respectively. Even more pronounced efficacy was found for (S)-HPMPG against *Balamuthia mandrillaris* (isolate CDC-V194) with MLC being 0.1 μg/ml. The MLC was defined as a minimal concentration of the analog that completely blocks proliferation of *Balamuthia*s and their cytopathogenic effect on Vero cells. Results of our in vitro studies indicate that the acyclic monophosphate analogs of guanine represent very promissing compounds against pathogenic acanthamoebae and, in particular, against *Balamuthia*.

Encystation and excystation process in asynchronous cultures of *Acanthamoeba castellanii*: an ultrastructural study

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Acanthamoeba castellanii is an opportunistic human pathogen capable of causing infection in Central Nervous System and eye. The cysts play an important role ensuring the persistence of the parasite in the environment, being resistant to diverse biocides. Many aspects related to the encyst- and excystment remain to be studied. Structures related to the cyst wall formation have not been described. Similarly, elements involved in the excystation process have not yet been identified. Therefore, in this work we present our finding by fluorescence and transmission electron microscopy on the study of this process in asynchronous axenic culture of *A. castellanii*, isolated from a human keratitis case in Mexico City. Cytoplasmic vesicles containing a dense fibrous material very similar in appearance to the cyst wall were observed in trophozoites induced to encyst. When these trophozoites were incubated with calcofluor white m2r. fluorescence was observed in cytoplasmic vesicles, suggesting that the material contained in these vesicles corresponded to cyst wall precursors. Semithin cryosections of mature cyst with the same treatment showed fluorescence in the ectocyst and a less intense fluorescence in the endocyst. Suggesting the presence of cellulose in both structures of the cyst. In mature cysts induced to excystation, small structures very similar to electron-dense granule (EDG) previously described in other amoebae were frequently observed. The EDGs were either sparsely distributed in the cytoplasm or associated with the cytoplasmic face of the plasma membrane. Many of them were located near the ostiole. In advanced phases of excystation, endocytic activity was suggested by the formation of endocytic structures and the presence of vacuoles with fibrous content similar to that of the cyst wall. EDGs in the process of dissolution were also observed in these vacuoles. Furthermore, the formation of a pseudopodia suggest a displacement of the amoeba toward the ostiole.

Encysting *Acanthamoeba castellanii* exhibit time-dependent changes in their protein profiles

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Potentially pathogenic amoebae of the genus *Acanthamoeba* are among the most prevalent protozoa found in the environment. Their ubiquity it largely due to their ability to form dormant, double layered cysts, which are not only extremely resistant against desiccation or prolonged starvation, but also against various therapeutic agents and disinfectants. This poses a serious problem for the treatment of *Acanthamoeba* keratitis (AK), where massive encystment in the corneal stoma is a precondition for severe forms and recurrent infections. The encystation process encompasses three phases: induction (1), wall-synthesis (2), which is divided into two marker steps as each wall is synthesised separately and (3) the dormant cyst. While the outer wall, the exocyst, consists mainly of protein, the major component of the inner wall (endocyst) is cellulose.

The aim of the study is to investigate changes in the protein profile during the encystation process of *Acanthamoeba castellanii* Neff strain. Synchronous encystment was induced with defined encystment medium, cells were harvested and homogenised at different stages of encystation and proteins were precipitated and resolublised. Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) was performed and was qualitatively and quantitatively analysed by means of Melanie proteomics software. Encysting *A. castellanii* cells exhibit dramatic changes in their protein profile during the course of encystation. Several protein spots disappear during encystment and conversely new spots could be detected. Moreover, various spots increased or decreased in intensity throughout the encystation process. The identification and characterisation of these proteins could provide a basis for a greater insight in the molecular mechanisms of the encystation process of *Acanthamoeba*.

Comparison of the population of free living amoebae in three wastewater treatment systems

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Objective of the study was to compare the population of free-living amoebae in wastewater treated by three systems. Monthly samples were taken from the outflow of three biological treatment systems: activated sludge (AS), biological contactor rotators (BCR), and tinkling filters (TF). Biological contactor rotators displayed the greatest diversity of amoeba species over the study. In the cases of tinkling filters and activated sludge systems, TF displayed higher diversity from January through April, while AS displayed higher diversity from May through October. The diversity of species show a similar behavior pattern in AS and TF systems during almost the entire study period. The maximum diversity was registered in August with 13 species and the minimum in January with one species. Thirty-six species of free living amoebae belonging to 21 genera were obtained from the treated wastewater by the three systems. Of the species present, 16 were in AS, 27 in BCR, and 21 in TF. Most frequent genera were *Vannella*, *Vahlkampfia* and *Hartmannella*. With respect to abundance, BCR registered the greatest abundance, followed by AS and TF. No seasonal variation pattern was evident in any of the systems. A similarity was observed between BCR and TF systems, only from January through April. The maximum abundance of FLA was present in the months of February and May with 460,000 NMP/100 ml each; the minimum abundances were registered in June with 900 NMP/100 ml and in October with 700 NMP/100 ml. It is interesting, that even though BCR and TF are both treatment systems of biological film, no similar behavior, whether in diversity or abundance, was noted between them. In contrast, TF system displayed a diversity behavior pattern similar to AS system. BCR stood out from the three systems, presenting both the greatest diversity and abundance.

Amoebae of the genera *Vannella* Bovee, 1965 and *Platyamoeba* Page, 1969 isolated from fish and their phylogeny inferred from SSU rRNA gene and ITS sequences

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Twenty strains of flattened amoebae including 17 isolated from fish were characterised morphologically both at light microscopical and ultrastructural levels and assigned to either the genus *Vannella* Bovee, 1965 or the genus *Platyamoeba* Page, 1969. Sequence-based phylogenetic analyses of SSU rRNA genes from a data set representing a total of 29 strains of flattened amoebae strongly indicated that morphological features discriminating between these genera do not reflect phylogenetic relationships of representative strains. Contrary to a previous study, strains of this expanded assemblage formed clusters that did not reflect their environmental origin. Monophyletic groups were of mixed origins and contained freshwater as well as marine strains of both genera isolated in geographically distant localities of various continents. These findings were supported by results of phylogenetic analyses of selected strains based on ITS sequences. However, topologies of acquired ITS trees were not congruent with results inferred from SSU rRNA analyses.

Protacanthamoeba bohemica sp. n., isolated from the liver of tench, *Tinca tinca* (Linnaeus, 1758)*

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A new species of amphizoic amoeba, *Protacanthamoeba bohemica* sp. n., isolated from the liver of *Tinca tinca* (Cypriniformes: Cyprinidae) is described. Trophozoites typical of the genus *Protacanthamoeba* Page, 1981 differ distinctly from those of the type species of the genus in having constantly more numerous and much longer acanthopodia. No relevant ultrastructural distinctions were observed. SSU rRNA gene sequence acquired for *P. bohemica* (the first within the genus) have been deposited in the GenBank database under accession number AY960120. A phylogenetic analysis based on SSU rRNA gene sequences assigned *P. bohemica* to the *Acanthamoeba* and *Balamuthia* clades.

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Fish-isolated strains of *Hartmannella vermiformis* Page, 1967: morphology, phylogeny and molecular diagnosis of the species in tissue lesions*

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Based on morphological and molecular characterisation, four amoeba strains isolated from organs of freshwater fish were identified as *Hartmannella vermiformis* Page, 1967. Small subunit rRNA gene sequences of these strains expand the set of corresponding complete and almost complete sequences of this species to twelve. A new species specific oligonucleotide probe inferred from recently available SSU rRNA gene sequences was designed and successfully tested in tissue lesions produced by one strain of *H. vermiformis* in experimentally infected fish.

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Balamuthia and *Balamuthia*-like amebas from soils

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Balamuthia mandrillaris amoebas are recognized as a causative agent of the usually fatal granulatomatous amoebic encephalitis first recognized in a mandrill baboon and since have been isolated from a number of human cases. Identification of

the amoebas in the environment had been illusive. They have now been isolated from the soils around two potted plants in different and unrelated locations. A comparison of them with the amoebas from the clinical specimens of a child who died in Northern California has shown them to be identical in their growth pattern and by DNA sequence analyses.

We have expanded the search for the *Balamuthia* amoebas in the environment. After examining some 50 soil samples from a variety of locations, we have identified amoebas that are *Balamuthia*-like in their slow rate of growth, their location within agar cultures, their growth on cultured cells or on other amoebas, their size and their morphology. The amoebas pictured here are from the edge of a duck pond, soil from a barren area, and soil near a drainage pipe. They have not yet been isolated, cloned and fully characterized, however, the morphologic similarities to those amoebas that have been fully characterized indicate that the *Balamuthia* amoebas likely have a wide environmental distribution.

Comparing the cytopathogenicity of different species and isolates of *Acanthamoeba* and *Balamuthia mandrillaris* with a simple, non-radiometric assay

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Introduction. As some *Acanthamoeba* species may cause keratitis or even lethal encephalitis, it is important to differentiate cytopathic from non-cytopathic isolates. Amebic proteases are involved and extracellular protease activity has been found enhanced in pathogenic compared to non-pathogenic *Acanthamoeba*. However, protease detection is complicated and for clinical diagnosis a simpler assay to assess amebic pathogenicity is needed.

Materials and methods. *Acanthamoeba*: Eight isolates were kindly provided by Dr. J. Walochnik, Vienna; one belonging to group-I (*A. comandoni* Pb30/40), 6 to group-II (*A. castellanii* 9GU, 4CL, 1BU; *A. hatchetti* 11DS, 3ST, 2HH), and one to group-III (*A. lenticulata* 72/2). *Balamuthia mandrillaris*: The isolate CDC-C039 originated from a deceased mandrill baboon and was kindly provided by Prof. F.L. Schuster, San Francisco. All amebae had been adapted to axenic culture. Target cells: The murine mastocytoma line P815 (ATCC TIB-64) had been transfected for constitutive expression and cytoplasmic accumulation of bacterial ß-galactosidase as reporter enzyme. ß-gal-release assay: The assay is based on the enhanced release of ß-gal by damaged target cells. Amebae and $P815_{ß\text{-}gal}$ were co-incubated for 4 h at 28°C or 37°C before extracellular ß-gal activity was detected by a chemiluminescent substrate-conversion which was then related to total releasable ß-gal and ß-gal spontaneously released by target cells alone.

Results. At 37°C, *B. mandrillaris* showed the highest cytolytic activity, followed by the *A. hatchetti* isolates 2HH and 11DS. However, all *Acanthamoeba* isolates were much less active than *B. mandrillaris* at this temperature. At 28°C, the cytolytic activities of the *Acanthamoeba* isolates were generally higher than at 37°C. Again *B. mandrillaris* and the *A. hatchetti* isolates 11DS and 3ST revealed the highest activities of all tested amebae. *A. comandoni* Pb30/40 showed no cytolytic activity under these conditions.