Burkholderia cepacia association with Acanthamoeba isolated from an AIDS patient with cutaneous acanthamoebiasis.

Francine Marciano-Cabral¹, Gregory Booton³, Daryl J. Kelly³, Tammy Ferguson¹, Eric Powell², and Paul A. Fuerst³

¹Department of Microbiology & Immunology
Medical College of Virginia Campus
Virginia Commonwealth University
Richmond, VA

²Department of Pathology
Johns Hopkins Hospital
Baltimore, MD

³Department of Evolution, Ecology, and Organismal Biology
The Ohio State University
318 W. 12th Avenue
Columbus, Ohio 43210
Abstract

_Acanthamoeba_ are free-living amebae found throughout the environment. Several species have been identified as causative agents of amebic keratitis, cutaneous acanthamoebiasis, and granulomatous amebic encephalitis. A clinical isolate of _Acanthamoeba_, designated JH2, cultured from a cutaneous lesion of an HIV positive IV drug abuser, was investigated. Western immunoblot analysis using rabbit polyclonal anti-sera generated against known species of _Acanthamoeba_ showed that JH2 had an antigen profile similar to _A. castellanii_. Analysis of the sequence for the nuclear small ribosomal subunit RNA gene identified the _Acanthamoeba_ isolate as a Type 4 that includes _A. castellanii_, _A. polyphaga_, and _A. rhysodes_. Electron microscopy of the isolate revealed the presence of bacteria in cytoplasmic vacuoles. Analysis of the bacterial 16S ribosomal RNA identified the bacterium as a member of _Burkholderia cepacia_ complex. Ribonuclease protection assays were employed to investigate the ability of the JH2 isolate of _Acanthamoeba_ to induce expression of mRNAs for specific cytokines in EOC murine microglia-like cells. Results indicate that co-culture of the isolate with EOC cells induced increases in the mRNA levels of the pro-inflammatory cytokine, interleukin-1β, along with the anti-inflammatory cytokine, interleukin-1 receptor antagonist. Messenger RNA for the chemokines MCP-1, MIP-1α, MIP-1β, and MIP-2, also were increased in EOC cells co-cultured with JH2 compared to EOC cells cultured alone. These data indicate that the _Acanthamoeba_ isolate was capable of inducing an immune response; however, the contribution of intracellular bacteria within amoebae to the host response is unknown.
**Introduction**

*Acanthamoeba* *spp.* are free-living amebae found ubiquitously in soil, air, and water worldwide that are the causative agents of granulomatous amebic encephalitis, amebic keratitis and cutaneous lesions (26,29,31). Cutaneous acanthamoebiasis, caused by various species of the opportunistic pathogen, *Acanthamoeba*, is a disease characterized by intradermal or subcutaneous erythematous or tender nodules that can become ulcerative and necrotic (24,32, 48). The disease is more frequent in immune suppressed individuals such as organ transplant patients and in human immunodeficiency virus-infected (HIV) infants and adults (12, 24, 31, 34). Trophozoites in skin lesions can disseminate to the central nervous system (CNS) and cause fatal infections (13, 16, 46). The mortality rate of cutaneous acanthamoebiasis has been reported as 73% in patients without CNS involvement and 100% in patients with CNS disease (48).

Identification of *Acanthamoeba* is problematic because the morphological characteristics are similar between species. Diagnosis of *Acanthamoeba* infections is difficult because the organisms are often mistaken for yeast or fungi in tissue sections (9, 15, 41, 46). Cutaneous acanthamoebiasis, also, has been misdiagnosed as Kaposi’s sarcoma or cytomegalovirus inclusions in tissue (9, 47). Treatment of cutaneous acanthamoebiasis is not clearly established but combination therapies have resulted in improvements. Successful treatment using combinations of itraconazole, pentamidine, 5-flucytosine, topical chlorhexidine gluconate, and ketoconazole cream, has been reported (24, 40). Very little is known concerning the host immune response in cutaneous or CNS infections. Histologic examination of cutaneous lesions generally shows inflammatory cells but well-formed granulomas present in immune competent individuals are lacking in immune suppressed patients (31).
In the present study, we examined a clinical isolate of *Acanthamoeba* from an HIV-infected IV drug abuser for identification of ameba genotype, the presence of associated bacteria, and for cytokine message induction in EOC cells, a continuous microglial cell line (49). The patient was admitted to the hospital for painful cutaneous nodules of 2 weeks duration. Sections of a skin biopsy of a cutaneous lesion were reported to show an inflammatory infiltrate (37). *Acanthamoeba* trophozoites and cysts were observed in the infiltrate. The patient was treated with pentamidine, 5-flucytosine, sulfadiazine and HAART therapy and improvement in the patient’s condition was reported (37). The purpose of this study was to identify the ameba and to determine whether the clinical isolate contained bacteria since a number of clinical isolates appear to contain symbiotic bacteria (14). The ameba isolate was assigned to the T4 genotypic group of *Acanthamoeba* by molecular methods. Further it was found to harbor a beta-proteobacteria of the *Burkholderia cepacia* complex. Additionally, the amebae induced high levels of chemokine message for MIP-1 and MIP-2 (macrophage inflammatory protein) in EOC microglial cells in vitro.

**Materials and Methods**

**Ameba.** Amebae were obtained from an HIV positive IV drug abuser (37). Ameba cultures were initially plated on 1.5% non-nutrient agar plates to isolate trophozoites from bacteria, fungi, or other organisms that may have been present. Amebae isolated from the lesions were sent to our laboratory and cultured axenically at 37 °C in Oxoid medium supplemented with donor calf serum and hemin and lacking antibiotics. Morphologically the amebae were identified as a species of *Acanthamoeba* based on cyst structure. The amebae were designated *Acanthamoeba sp. JH2* since they had been isolated from a patient transferred to Johns Hopkins Hospital in Baltimore, Maryland (37). The initial cultures of amebae were used for Western blot analysis and electron microscopy. These amebae, also were cocultured with EOC microglial cells for studies on the induction of
cytokine and chemokine messenger RNA. Three week old B₆C₃F₁ mice (Taconic Farms, Inc., Hudson, N.Y) were inoculated by the intranasal route to determine whether the clinical isolate was pathogenic for experimental animals in accordance with institutional policies for animal research.

Another set of amebae was fed heat-killed Enterobacter aerogenes as a bacterial food source on nonnutrient agar plates. Following migration to the periphery of the petri plate, an individual ameba of Acanthamoeba isolate JH2 was cut from the plate and placed in sterile Bacto-Casitone/Serum (BCS) media, modified with the substitution of adult bovine serum for rabbit serum, in 25 cm² culture flasks at 27 °C (8). When Acanthamoeba trophozoites became confluent and/or when encystment of the amebae was observed, a 10µl aliquot of the culture was transferred to a new 25 cm² culture flask containing 5mls of fresh BCS medium and maintained axenically. Total DNA extractions described below were performed on multiple cultures of Acanthamoeba sp. JH2 over a number of months of continuous axenic culture.

**Electron Microscopy.** Transmission (TEM) and scanning electron microscopy (SEM) were performed on ameba cultures one week after axenic culture at 37 °C. For TEM, trophozoites of JH2 cultured in Oxoid medium were plated in 25 cm² tissue culture flasks. Amebae cultured alone or cocultured with EOC microglia cells were harvested by centrifugation, fixed in 2.5% glutaraldehyde, and post fixed with OsO₄ as previously described (38). Samples were dehydrated through a graded series of ethanol washes, transferred to propylene oxide, and embedded in Epon-Araldite mixture. Ultrathin sections were cut and stained with saturated aqueous uranyl acetate followed by lead citrate. Grids were examined in a Zeiss EM10 electron microscope operating at 80 kV. For scanning electron microscopy (SEM), trophozoites of JH2 were placed onto sterile 12 mm round cover glasses in Oxoid medium and allowed to adhere at 37 °C for 1 h. A parallel set of sterile glass coverslips was seeded with EOC microglia cells and were incubated at 37°C in 5% CO₂
for 6-12 h. Amebae that had been suspended in DMEM were added to the coverslips containing EOC cells at a 1:10 ameba to EOC cell ratio and incubated at 37 °C. Cover slips containing ameba alone or cocultures of EOC cells with JH2 amebae were fixed with warm 2.5% glutaraldehyde, post-fixed with 2% OsO₄ and dehydrated in a graded series of ethanol. The cover glasses were subjected to critical point drying, coated with gold, and viewed with a JEOL scanning electron microscope operating at 15kV.

**Identification of ameba by Western immunoblot analysis.** Amebic extracts were made by glass bead disruption of trophozoites of the JH2 strain of *Acanthamoeba* as well as laboratory-maintained cultures obtained from the American Type Culture Collection (ATCC). Extracts of *A. castellanii* (ATCC 30010), *A. polyphaga* (ATCC 30146), *A. astronyxis* (ATCC 30137), *A. culbertsoni* (ATCC 30171), and another clinical isolate of *Acanthamoeba* (JH1) (27, 44) were used as the antigen source for Western immunoblot analysis using SDS-PAGE. The amebic lysates containing 10 μg of protein were separated on a 12% acrylamide gel, then transferred to nitrocellulose membranes. Rabbit polyclonal anti-serum to each of four *Acanthamoeba* species was used as the source of primary antibody for each of four blots. Antibodies to the *Acanthamoeba* proteins were detected using goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma-Aldrich, St. Louis, MO) followed by enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ).

**Identification of amebae by DNA extraction and PCR.** Total DNA was extracted from confluent cultures of *Acanthamoeba* sp. JH2 using the DNeasy kit (Qiagen, Inc., Valencia, CA). Following DNA extraction, PCR was used to amplify the *Acanthamoeba* nuclear *Rns* sequences using genus-specific primers JDP1 (5’-GGCCCAGATCGTTTACCGTGAA-3’) and JDP2 (5’TCTCACAAGCTGCTAGGGGAGTCA-3’), which amplifies a region of the *Rns* that permits genotypic identification of an *Acanthamoeba* isolate following sequence analysis. PCR conditions
for this reaction were as follows: initial denaturing step of 95 °C for 7 min, followed by 35-40 cycles of 1 min @ 95 °C, 1 min @ 60 °C, and 2 min @ 72 °C (4, 39). The PCR reaction (10µl of a 50µl product) was visualized on a 1% agarose gel. The remainder of the PCR product was prepared for sequencing using the Qiaquik PCR clean-up kit (Qiagen, Inc., Valencia, CA). Three microliters of Qiaquik prepared PCR product was used directly in subsequent sequencing reactions.

**Identification of intracellular bacteria.** PCR was performed, also, using universal bacterial primers that amplified any bacterial 16S rDNA genes present in the total DNA sample. This was done using bacterial universal primers 16S-uni5’ (5’-AGAGTTTGATYMTGGCTCAG-3’) and 16S-uni3’ (5’-ACGGCTACCTTGTTACGACTT-3’). This primer set amplifies any bacterial 16S ribosomal genes present in the total DNA sample screened. This PCR reaction was done using the total DNA extracted from *Acanthamoeba* sp. JH2. PCR reaction conditions for this primer set were the following: an initial 10 min denaturing step @ 94 °C, followed by 40 cycles of 1 min at 94 °C, 2 min @ 50 °C, and 3 min @ 72 °C. This was followed by a final extension step of 15 min @ 72 °C. PCR amplimers produced in this reaction were pooled, prepared using the Qiaquik kit, and re-suspended in 40µl of elution buffer. However, unlike the direct sequencing procedures used to sequence the *Acanthamoeba Rns* products, bacterial 16S amplimers were cloned into a T/A cloning vector so that the possibility of multiple bacterial sequences that may have been present in the *Acanthamoeba* isolate could be examined. Specifically, 1µl of a 1:10 dilution of the Qiaquik elution product was used in a T/A cloning system (Invitrogen, Inc, Carlsbad, CA) following the manufacturer’s instructions. Kanamycin antibiotic selection produced putatively positive clones. Multiple clones were selected, grown in Kanamycin/LB media overnight, and prepared using the Qiaprep plasmid preparation kit (Qiagen, Inc., Valencia, CA). Three microliters of re-suspended
plasmid DNA was then directly sequenced as described above using M13 forward primer located in the T/A cloning vector.

**DNA sequence analysis.** DNA sequencing of the *Rns* was done with an ABI 310 automated fluorescent sequencing system (Applied Biosystems, Foster City, CA.) using a set of conserved primers and methods that have been used previously in our phylogenetic studies (5, 23, 39, 45). *Acanthamoeba* sp. JH2 sequence that was obtained was aligned to other *Acanthamoeba* spp. sequences in our *Rns* database using the alignment program XESEE (7). *Acanthamoeba* sp. JH2 *Rns* sequence and *Burkholderia cepacia* 16S small subunit ribosomal RNA gene sequence obtained in this study have been deposited in GenBank under the following accession numbers, DQ239700 and DQ239701, respectively.

**Identification of bacteria.** The primary sequence of cloned bacterial 16S small subunit ribosomal gene sequences was compared to other sequences in GenBank by using the blastn program available at the NCBI website (http://www.ncbi.nlm.nih.gov/). This was done to determine the phylogenetic similarity between the unknown bacterial sequences obtained from *Acanthamoeba* sp. JH2 and known bacterial 16S rDNA sequences.

**Mammalian Cell Cultures.** LADMAC (ATCC# CRL-2420) and EOC-20 (ATCC# CRL-2469) cells were obtained from ATCC (Manassas, VA). LADMAC cells were cultured in Eagle minimum essential medium (EMEM) containing 2mM L-glutamine, 0.1 mM non-essential amino acids, 1.5g/L sodium bicarbonate, and 10% heat-inactivated fetal bovine serum (FBS). Cultures were maintained at 37°C in 5% CO₂. Conditioned medium (CM) obtained from LADMAC cultures was harvested by centrifugation to remove cells and the supernatants pooled and stored at -20° until used. The conditioned medium from LADMAC cells (LCM) was used as a source of murine colony stimulating factor-1 (CSF-1) to supplement Dulbecco’s modified Eagle’s medium (DMEM)
for culture of the EOC microglial cells (42). EOC-20 cultures were maintained in DMEM with 1% each: penicillin-streptomycin, 0.1 mM non-essential amino acids, vitamins, 2mM L-glutamine, hepes buffer, 10% FBS, and 20% LCM. Cultures were maintained at 37°C in 5% CO₂ (49).

**JH2 and EOC-20 Cocultures.** EOC-20 microglial cells were harvested by centrifugation and suspended in DMEM medium supplemented with LCM. Cells were adjusted to 1 x 10⁶/ml and were plated into 12-well sterile tissue culture plates and incubated at 37°C in 5% CO₂.

*Acanthamoeba* (JH2) were harvested by centrifugation, suspended in DMEM and adjusted to 1 x 10⁵/ml. Amebae were added to each of the appropriate wells containing EOC cells at a 1:10 ameba to EOC ratio. Cocultures of *Acanthamoeba* and EOC cells were maintained for 6 h at 37°C with 5% CO₂. At the end of the incubation period, total RNA was harvested by removal of the culture medium followed by addition of 0.75 mls of TRI-zol® (Life Technology, Grand Island, NY) to each well for RNase Protection assays to determine cytokine or chemokine mRNA induction (27, 28). Each of the experimental conditions was assayed in duplicate and each experiment was conducted at least two separate times for each of the RPA probe sets utilized.

**Multiprobe Ribonuclease Protection Assay (RPA).** RNA probes to examine mRNA levels of the cytokines were prepared using the mCK-2b and mCK-5c template sets from Pharmingen (San Diego, CA). Cytokine mRNA for IL-1β, IL-1Ra, IL-18, and IL-6, or chemokine mRNA for MIP-1α, MIP-1β, MIP-2, MCP-1, TCA-3, and Eotaxin, were examined.

**Probe Synthesis:** RNA probes were prepared using In Vitro Transcription Kit according to manufacturer’s instructions. Briefly, the template set was incubated RNAsin, GACU pool, DTT, 5X transcription buffer, [α³²P] UTP (MP Biomedicals, Aurora, OH), and T7 RNA polymerase. Transcription was terminated after 1 h at 37°C by addition of RNase-free DNase. Following a 30 min incubation at 37°C, the radio-labeled probes were isolated using the phenol:chloroform
extraction method. The probes were then precipitated using ammonium acetate and ethanol. Glyco-blue was added to act as a carrier (Ambion, Austin, TX). The RNA pellet was washed once in ethanol and air dried. The probes were then solubilized in hybridization buffer and the Cherenkov counts per μl were determined for duplicate samples using a scintillation counter.

**RNA Preparation & Hybridization:** To isolate total RNA, samples in TRI-zol® were thawed and chloroform extraction was performed followed by isopropyl alcohol precipitation. The supernatants were extracted and the RNA pellets were air dried. Assay controls included yeast tRNA as a negative control and mouse splenic mRNA as a positive control. Hybridization buffer was added to the controls and the samples to be assayed and the pellets were solubilized. Radiolabeled probe was added to each of the tubes that were incubated briefly at 90°C. The samples and radiolabeled probes were allowed to hybridize overnight at 56°C.

**RNase Treatments:** Following an overnight incubation, RNase A and RNase T1 was added to each of the samples which were incubated at 37°C to degrade unhybridized, single stranded RNA. Following RNase digestion, Proteinase K was added to each sample to degrade the remaining RNase. Intact RNA was then isolated by phenol and chloroform: isoamyl alcohol (50:1) extraction. The upper aqueous phase of each sample was carefully extracted and transferred to new tubes containing 1 μl of Glyco-blue. The RNA was precipitated using ammonium acetate and ethanol. The tubes were mixed by inversion then incubated for 45 minutes at -70°C. When the -70°C incubation was complete, RNA was isolated from the samples by centrifugation. The supernatants were extracted and the pellets were washed once with ethanol and the pellets were dried completely. Loading buffer was added to the samples, which were then boiled and loaded into a 5% acrylamide gel with 8M urea. The protected fragments were imaged using a Molecular Dynamics 445SI PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The pixel intensity of each band was
quantification using Imagequant, 5.2 software. The amount of cytokine mRNA was normalized by dividing the pixel value for the cytokine fragment by the sum of the pixel values for the mRNA of two housekeeping genes, L32 and GAPDH (11).

**Statistics:** Differences of the mRNA densities for the cytokines or chemokines of interest for the different groups were averaged and compared to each other using SigmaStat 3.0 statistical software (SPSS, Chicago, IL).

**Results**

Cultures of amebae were examined by SEM and TEM to determine whether the amebae were morphologically similar to *Acanthamoeba*. Trophozoites with acanthapodia and wrinkled cysts consistent with morphological characteristics of *Acanthamoeba* were observed (Fig. 1A –1D). TEM revealed that bacteria were present in trophozoites. Bacteria were not observed in *Acanthamoeba* cysts. Western immunoblot analysis suggested that the amebae were closely related to *A. castellanii* since the greatest reactivity was with anti-*A. castellanii* antiserum (Fig. 2).

Amebae were recovered from the brains of B₆C₃F₁ mice inoculated with *Acanthamoeba* JH2 that died. The LD₅₀ was 1 X 10⁵ amebae.

*Acanthamoeba* sp. JH2 genotype identification. PCR amplification using *Acanthamoeba* genus-specific *Rns* primers JDP1 and JDP2 produced a single band of the expected size from *A.* sp. JH2 (Fig. 3A if we want to include a pic of the gel, to be decided). Comparison of the primary sequence from *A.* sp. JH2 with the *Acanthamoeba Rns* database shows that *A.* sp. JH2 is identical in primary sequence to *Acanthamoeba* sp. Rodriguez, which is a genotype T4 *Acanthamoeba* isolate (45). Genotype T4 *Acanthamoeba* is a large assemblage of *Acanthamoeba* isolates from both clinical and natural environments. This is a phylogenetically closely related genotype (<5% sequence dissimilarity within genotype T4) that contains nearly ten, or more, species of *Acanthamoeba*. A.
sp. Rodriguez is found within a sub-clade of seven isolates of *Acanthamoeba* genotype T4 that is called T4-6, which is strongly supported by bootstrap analysis (6). The seven isolates of T4-6 are phylogenetically very closely related; the average dissimilarity within T4-6 is 0.5%.

**Acanthamoeba-associated bacterial identification.** PCR amplification of DNA extracted from *Acanthamoeba* sp. JH2 produced amplimers of the expected size, supporting the premise that bacterial DNA was present and could be used as a template by these primers ([Fig 3B](#), again if we want to include gel pics here). Because of the possibility of PCR products from multiple bacterial sources, these PCR products were cloned prior to sequence analysis.

Sequencing of multiple clones that were produced using the universal bacterial 16S small subunit ribosomal gene primers identified two bacterial species, following a blast search of the GenBank database. One, *E. aerogenes* was to be expected since A. sp. JH2 was fed killed *E. aerogenes* as a bacterial food source during isolation of amebae. The second bacterial species that was identified was *Burkholderia cepacia*, specifically it was identical with *B. cepacia* complex genomovar IV and IX.

Electron microscopy studies of EOC cells cocultured with *Acanthamoeba* (JH2) revealed amebae in contact with EOC cells by means of a finger-like projection ([Fig. 4A](#)). EOC microglia-like cells were capable of ingesting the ameba ([Fig. 4B](#)).

RPA analysis utilizing total amebic RNA showed no hybridization of the radioactive probes to *Acanthamoeba sp.* (JH2) RNA or to RNA from other *Acanthamoeba* species which have been examined previously (27, 28). JH2 did not induce changes in mRNA levels for the TGF-β family of cytokines or TNF-α compared to constitutive levels produced by the EOC cultures alone (data not shown). IL-6 was increased during co-incubation with JH2 although the levels were not significantly different than EOCs cultured in the absence of amebae. Although IL-18 was not
significantly different in the cocultures, IL-1β and the IL-1 receptor antagonist (IL-1 RA) were significantly different in the cocultures. Examination of cocultures of EOC cells and *Acanthamoeba* strain JH2 showed a greater induction of chemokine mRNA for MCP-1, MIP-2, MIP-1α and MIP-1β rather than proinflammatory cytokines (Fig. 5A -C).

**Discussion**

Amebae isolated from an HIV positive IV drug abuser with cutaneous acanthamoebiasis were identified as *Acanthamoeba castellanii* by Western immunoblot analysis and as a T4 genotype. The identification of *Acanthamoeba* sp. JH2 as a genotype T4 from human infection is consistent with previous results demonstrating that the majority of isolates examined from various human tissues including lungs, nasal sinuses, and brain belong to the genotype T4 (6, 45). Occasionally, other genotypes have been observed.

SEM and TEM confirmed the isolate as *Acanthamoeba* based on the cyst structure and the presence of acanthapodia on the trophozoites. TEM of the amebae also revealed the presence of bacteria in the trophozoites. The bacteria were identified as belonging to the *Burkholderia cepacia complex* by 16S rDNA analysis. Members of the *B. cepacia* complex are beta-proteobacteria described originally as plant pathogens, but now recognized as opportunistic pathogens in patients with cystic fibrosis (10, 25, 35) and chronic granulomatous disease (43).

The association of free-living amebae with bacteria in the environment has been reported (14,17). Although *Burkholderia* has not been found in free-living amebae isolated from environmental sources or from patients, *Ralstonia pickettii*, a closely related species has been found in *Acanthamoeba* (33). Michel and Haurder (33) initially showed the association of intracellular *Ralstonia pickettii* (formerly *Burkholderia pickettii*) with *Acanthamoeba* isolated from a hospital environment. This observation led to a number of studies to determine whether *Burkholderia* could
survive in amebae. Marolda et al (30) demonstrated in laboratory infections that *B. cepacia* is capable of intracellular survival in a number of clinical and environmental isolates of *Acanthamoeba*. These investigators reported that *B. cepacia* display a low level of intracellular replication following infection and concluded that the bacteria can survive at low levels intracellularly but that replication of the bacteria is primarily extracellular. Landers et al (22) reported that *B. cepacia* not only survive within *A. polyphaga* but suggested that *A. polyphaga* supported the growth of *B. cepacia* in laboratory infections. More recent studies indicate that intracellular *B. cepacia* can reside within acidified membrane-bound compartments distinct from lysosomes without bacterial replication (21).

Recently, *B. pseudomallei* the causative agent of melioidosis a fatal infection with septicemia has been shown to survive as a transient bacterial symbiont of *Acanthamoeba astronyxis*, avoiding destruction following phagocytosis (19). Invasion of *A. astronyxis* by *B. pseudomallei* appears to be regulated by the *Burkholderia* flagella (20). Intraamebic survival of *B. pseudomallei* in *A. astronyxis* in amebic vacuoles with eventual escape into the environment was reported. However, small numbers of intracellular bacteria were noted. These authors suggested that the relationship between *Acanthamoeba* and *B. psuendomallei* was commensal in shared environmental habitats but not endosymbiotic (19,20).

The results of the current study show that a genotype T4 isolate of *Acanthamoeba* from a human infection is able to harbor a *Burkholderia* species (*B. cepacia*). Although bacteria were present initially, it appears that the amebae were harboring *B. cepacia* as a transient passenger since not all trophozoites contained bacteria. Furthermore, after prolonged culture of the amebae in enriched Oxoid medium, bacteria were neither observed by electron microscopy nor by Rns analysis. Alternatively, *Acanthamoeba* may be in the process of forming a more long-term
relationship with *Burkholderia* in the environment. To address this question, following *Acanthamoeba* with *Burkholderia* over a longer period of time in a laboratory infection, which would encompass multiple generations of both the ameba and the bacteria, may offer some insight into this potentially important ameba/bacterium relationship.

The paucity of bacteria seen in the JH2 isolate may be due to the enriched ameba growth medium or the temperature at which the amebae were cultured. Upon receipt of the cultures, the amebae were maintained in Oxoid medium at 37 °C. Marolda et al (30) noted that at 37 °C trophozoites infected with *Burkholderia* were lysed while at 20°C to 30 °C infections proceeded normally. Also, different species of *Acanthamoeba* may allow greater numbers of bacteria to survive in intracellular vacuoles (*A. astronyxis* vs *A. castellanii*).

High levels of proinflammatory cytokine mRNA were not induced by JH2 isolate in EOC microglial cells in contrast to other *Acanthamoeba* (2, 27, 28). Studies in our laboratory with another clinical isolate containing an alpha-proteobacterium (44) indicate that high levels of the proinflammatory cytokine TNF alpha were induced when the amebae were cultured with macrophages (27). The low level of bacteria could explain the lack of induction of proinflammatory cytokines. However, chemokine message for MIP-1, MIP-2, and MCP were induced. Barnes et al (1) noted that patterns of chemokine induction and expression including MIP-2 and MCP1 in experimental *B. pseudomallei* infection in mice reflected bacterial loads.

The course of most HIV positive patients with cutaneous acanthamoebiasis is rapid with dissemination to the brain and a fatal outcome. Although the clinical isolate was pathogenic for mice, the patient from which JH2 was isolated survived the infection. Administration of antivirals along with multi-drug therapy consisting of 5 flucytosine and sulfadiazine was recommended for a favorable prognosis (37). A successful treatment protocol, a less virulent strain of ameba, or
induction of MIP-2 by this strain of *Acanthamoeba* may have contributed to survival of the patient. MIP-2, a chemokine chemotactic for neutrophil migration, plays an important role in clearing *Acanthamoeba* infections of the cornea in experimental animals (36). Hurt et al (18) reported that resolution of *Acanthamoeba* keratitis coincides with corneal production of MIP-2 and anti-MIP-2 antibody treated animals failed to resolve their ocular lesions. In the present study, the patient was lost to follow up. Thus, the eventual outcome of infection is unknown. Further studies are needed to determine the effect of bacterial symbionts or passenger bacteria on the outcome of infection with *Acanthamoeba*.

**Literature Cited.**


Acknowledgements

This work was supported in part by NIDA grant
Figure 1. Scanning and Transmission electron micrographs of *Acanthamoeba* isolate. JH2 trophozoites (A and C) and cysts (B and D). SEMs of JH2 (A and B) are x 3,300 and x 4,300, respectively. TEMs of JH2 (C and D) are x 10,200 and x 10,800, respectively. Arrow head (→) indicates intracellular bacterium (C).
Figure 2: Western Immunoblot Analysis of JH2 *Acanthamoeba* isolate. Whole cell lysates from known *Acanthamoeba* species, *A. culbertsoni*, *A. astronyxis*, *A. castellanii*, *A. polyphaga*, an additional clinical *Acanthamoeba* isolate, and the JH2 *Acanthamoeba* isolate, were separated by SDS-PAGE on a 12% acrylamide gel. The proteins were transferred to nitrocellulose then incubated in rabbit polyclonal antiserum raised against *A. castellanii* (1:30,000 dilution). HRP labeled goat anti-rabbit IgG was used at 1:15,000 dilution to detect the primary antibodies by enhanced chemiluminescence.
Figure 4. Electron micrographs of the murine microglia cell line, EOC-20, co-cultured with the JH2 clinical isolate of *Acanthamoeba*. (A) SEM of JH2 co-cultured with the EOC-20 cell line (x 3,300). Arrow (▼) indicates digipodia extending from a trophozoite (T) into a neighboring EOC cell (E). (B) TEM of JH2 *Acanthamoeba* trophozoite (T) surrounded by EOC cells (E) (x 3,220). Note dying EOC (D) in close apposition to trophozoite.
Figure 5?. RPA analysis of mRNA levels for specific cytokines and chemokines induced in EOC cells co-cultured with JH2 Acanthamoeba. EOC-20 cells were incubated with JH2 Acanthamoeba for 6 hours prior to harvest of total RNA in TRI-zol®. Panel A shows a typical RPA gel. Undigested probe RNA (U) is run on either side of samples to act as size markers. Murine splenic RNA (+) and yeast tRNA (-) are included as a positive and negative control, respectively. Band densities were quantified using Imagequant® software and graphs from the mCK-2b (Panel B) and mCK-5c (Panel C) probe sets were prepared after normalization of density levels of target RNA compared to density levels of housekeeping genes L32 and GAPDH (*P $\leq$ 0.05, **P $\leq$ 0.001).