Detection of Bacterial Endosymbionts in Clinical Acanthamoeba Isolates

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Purpose: To determine the presence of 4 clinically relevant bacterial endosymbionts in *Acanthamoeba* isolates obtained from patients with *Acanthamoeba* keratitis (AK) and the possible contribution of endosymbionts to the pathogenesis of AK.

Design: Experimental study.

Participants: *Acanthamoeba* isolates (N = 37) recovered from the cornea and contact lens paraphernalia of 23 patients with culture-proven AK and 1 environmental isolate.

Methods: *Acanthamoeba* isolates were evaluated for the presence of microbial endosymbionts belonging to the bacterial genera *Legionella*, *Pseudomonas*, *Mycobacterium*, and *Chlamydia* using molecular techniques (polymerase chain reaction and sequence analysis, fluorescence in situ hybridization) and transmission electron microscopy. Corneal toxicity and virulence of *Acanthamoeba* isolates with and without endosymbionts were compared using a cytopathic effect (CPE) assay on human corneal epithelial cells in vitro. Initial visual acuity, location and characteristics of the infiltrate, time to detection of the infection, and symptom duration at presentation were evaluated in all patients.

Main Outcome Measures: Prevalence and potential pathobiology of bacterial endosymbionts detected in *Acanthamoeba* isolates recovered from AK.

Results: Twenty-two (59.4%) of the 38 cultures examined contained at least 1 bacterial endosymbiont. One isolate contained 2 endosymbionts, *Legionella* and *Chlamydia*, confirmed by fluorescence in situ hybridization. Corneal toxicity (CPE) was significantly higher for *Acanthamoeba*-hosting endosymbionts compared with isolates without endosymbionts ($P<0.05$). Corneal pathogenic endosymbionts such as *Pseudomonas* and *Mycobacterium* enhanced *Acanthamoeba* CPE significantly more than *Legionella* ($P<0.05$). In the presence of bacterial endosymbionts, there was a trend toward worse initial visual acuity ($P>0.05$), central location ($P<0.05$), absence of radial perineuritis ($P<0.05$), delayed time to detection ($P>0.05$), and longer symptom duration at presentation ($P>0.05$).

Conclusions: Most *Acanthamoeba* isolates responsible for AK harbor 1 or more bacterial endosymbionts. The presence of endosymbionts enhances the corneal pathogenicity of *Acanthamoeba* isolates and may impact detection time and clinical features of AK.

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Acanthamoeba keratitis (AK) is a painful, sight-threatening, and difficult-to-treat corneal infection caused by pathogenic *Acanthamoeba.*[1,2] Although it is considered a rare corneal affection, the incidence of AK has increased exponentially over the past 2 decades as a consequence of the increasing use of contact lenses.[3] *Acanthamoeba* keratitis primarily affects otherwise healthy contact lens wearers and patients with a history of trauma.[1,2] Clinical features often are misleading, and the clinical course usually is protracted, despite aggressive treatment with antiamebic drugs.[2]

The genus *Acanthamoeba* encompasses at least 15 species of free living amoebae that have been isolated from a wide range of environments ranging from natural habitats like soil, salt water, and fresh water, to domestic sources like tap water, air conditioning units, and sewage systems.[1,4–7] *Acanthamoeba* undergoes 2 stages during its life cycle: a vegetative trophozoite stage and a dormant resistant cyst stage.[1,8] During the trophozoite stage, *Acanthamoeba* actively feed on bacteria, fungi, yeasts, algae, or small organic particles.[8] However, a wide range of bacteria have developed strategies to resist phagocytosis, survive intracellularly, and exploit *Acanthamoeba* for multiplication and therefore are defined as endosymbionts.[9–11] These bacterial endosymbionts usually are able to survive encystment of the amoeba, and the intracellular lifestyle protects the bacteria from adverse environmental conditions.[11] This adaptation makes the amoeba a potential vehicle of virulence for pathogenic bacteria.[9,12]

The association between bacterial endosymbionts and their amoeba hosts can be either transient (in the case of facultative intracellular bacteria) or stable (in the case of obligate intracellular bacteria).[9] Stable associations of bacteria with amoebae leading to long-term symbiotic interactions have been described for members of 4 evolutionary

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lineages within the domain bacteria: the *Alpha Proteobacteria*, the *Beta Proteobacteria*, the *Bacteroidetes*, and the *Chlamydiae*.[13–17] None of these bacterial endosymbionts have the ability to survive and cannot be cultured outside their amebic host cells. Such interactions may be of clinical relevance, because *Acanthamoeba* may be able to protect bacterial endosymbionts and release them under certain conditions. In fact, coinfections with other microorganisms have been reported in patients with culture-proven AK.[18] These include herpes simplex virus, adenovirus, and *Pseudomonas* species.[19–21] Because of the relationship of bacterial communities and free-living amoebae in the environment, the potential for dual human infections is increased. The purpose of this study was to determine the prevalence of bacterial endosymbionts in *Acanthamoeba* isolates recovered from keratitis and to assess their potential in the pathogenesis of the disease.

Materials and Methods

Isolates

Thirty-eight *Acanthamoeba* isolates were recovered and examined for the presence of endosymbionts. Thirty-seven (97%) of the 38 were cultured from corneal scrapings, corneal biopsies, corneal buttons, contact lenses, or lens cases from 23 patients with AK seeking treatment at the authors' institution between January 2006 and February 2008. One environmental sample was cultured from tap water obtained at the laboratory.

All cultures were grown on agar–agar plates seeded with heat-killed *Escherichia coli* or Peptone Yeats glucose broth. Subsequently, amoebae were grown axenically for 2 weeks in 1X Page's saline solution (NaCl, 120 mg; $MgSO_4$, 4 mg; Na_2HPO_4, 142 mg; KH_2PO_4, 136 mg; $CaCl_2$, 4 mg; 100 ml H_2O).

DNA Isolation and Genotyping

Acanthamoeba samples were rinsed in phosphate-buffered saline (pH, 7.4), and amoeba and bacterial DNA were extracted using the UNSET method.[22] Amplification and sequencing of the 16S-23S internally transcribed spacer with primers Sp1 (5′-ACCTCCTTTCTAAGGAGCACC-3′) and Mb23S.44n (5′-TCTCGATGCCAAGGCATCCACC) were used to detect *Mycobacterium* endosymbionts.[23,24] *Legionella* and *Pseudomonas* endosymbionts were detected by amplification and sequencing with rRNA primers targeting the variable 23S-5S intergenic spacer: 23S (5′-TGAAGCCCGTTGAAGACTAC-3′) and 5S (GGAAGCCTCACACTATCAT-3′).[25] The 23S primer was not an exact match to the *Pseudomonas* genus with 2 mismatches and an insertion, all at the 5′ half of the primer. Detection of endosymbionts belonging to the *Chlamydiaceace* family used primer set Momp1 (5′-ATGAAAAAACTCTTGAAATCGG-3′) and Momp2 (5′-GCTCCTAAAGTTGCACA-3′) that target the major outer membrane protein gene.

Sequencing, Nucleotide Alignment, and Phylogenetic Reconstruction

Sequences derived from the strains used in this study were analyzed along with sequences from strains available in GenBank. Several of the isolates are linked epidemiologically by being isolated from the same individual at different times or from contact lens paraphernalia of the same individual. The nucleotide sequences reported in this study were deposited in the GenBank database under accession numbers FJ444796 to FJ444819.

Alignments and phylogenetic reconstructions were performed using the phylogenetic computer program MEGA4 (Molecular Evolutionary Genetic Analysis software, version 4; http://www.megasoftware.net; accessed February 2, 2008).[26] Gene trees were generated using the maximum-parsimony, neighbor-joining, unweighted pair group method with arithmetic mean or minimum evolution methods in MEGA4. The evolutionary distances were computed using the Kimura 2-parameter distance algorithm and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons.[27] Confidence levels for the branching pattern were estimated by a bootstrap resampling of the data. Bootstrap values for the trees were obtained from a consensus tree based on 1000 replicates.[28]

Fluorescent In Situ Hybridization

For fluorescent in situ hybridization, a protocol previously described was used.[10] Briefly, *Acanthamoeba* samples (>95% trophozoites) harvested from axenic cultures were washed and resuspended in 100 μl 1X Page's saline. Twenty-microliter aliquots of amebic suspension were incubated on poly-L-lysine slides for 20 minutes at 45° C to allow attachment of amoebae and were fixed with 20 μl 4% paraformaldehyde for 20 minutes at room temperature. The slides were washed subsequently 3 times in 1X phosphate-buffered saline and were dehydrated in ethanol (96%, 70%, 50%) for 3 minutes. Slides then were incubated for 1 hour at 45° C with an aliquot (20 μl) of hybridizing buffer (20% formamide, 0.9 M NaCl, 0.01% SDS, 20 mM Tris/HCl, pH 7.6) containing 100 nanograms of the specific probe (*Pseudomonas*, 5′-GGTTAGCTCAACGCCTCACAACGCTTACACACCCA-3′; *Legionella*, 5′-CGCTATGGTCGCCAGGAAAACTGGTTT-3′; *Mycobacterium*, 5′-TCACGACCAAGCTTTCCAG-3′; *Chlamydia*, 5′-CGATTTCAAGAGTTTTTTCAT-3′). Slides then were washed gently with washing buffer (20 mM Tris/HCl, pH 7.6, 180 mM NaCl e 0.01% SDS), were reincubated for 15 minutes at 45° C and were covered with 500 μl washing buffer. Slides then were washed with distilled water, were dried at room temperature, and were mounted (Vectashield mounting medium; Vector Laboratories, Burlingame, CA). Images were acquired using a confocal microscope (Leica TC S SP5; Leica Microsystems, Inc, Wetzlar, Germany).

Transmission Electron Microscopy

Acanthamoeba cysts and trophozoites harvested from axenic cultures were fixed in 2% glutaraldehyde in 0.1M PO_4/100 mM sucrose overnight at 4° C. Then, they were rinsed 3 times with 0.15M PO_4. The samples were postfixed in 2% phosphate buffered osmium tetroxide for 1 hour, followed by 3 rinses with 0.15M PO_4. Samples then were dehydrated in an ascending series of ethanol up to 100%, infiltrated overnight with a 1:1 mixture of propylene oxide:epon-araldite resin, and then were embedded in the resin. Images were acquired with a Philips CM-10 transmission electron microscope (Philips Co., Eindhoven, Holland).

In Vitro Corneal Pathogenicity Assay

The cytopathic effect (CPE) of *Acanthamoeba* isolates was tested on human corneal epithelial cells (HCECs) in vitro as described elsewhere.[29] Briefly, 500 μl 2×10^4 colony-forming units/ml *Acanthamoeba* (>95% trophozoites) suspended in Keratynocyte serum free medium (KSFM) were added to confluent HCECs in a 24-well plate and were incubated at 37° C for 8 hours. After

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