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Ophthalmology. Author manuscript; available in PMC 2011 March 1.

Published in final edited form as:
Ophthalmology. 2010 March ; 117(3): 445–452.e3. doi:10.1016/j.ophtha.2009.08.033.

Detection Of Bacterial Endosymbionts In Clinical Acanthamoeba Isolates

Alfonso Iovieno, MD[1], **Dolena R. Ledee, PhD**[1], **Darlene Miller, DHSc, MPH**[1], and **Eduardo C. Alfonso, MD**[1]

[1]Department of Ophthalmology, Bascom Palmer Eye Institute, University of Miami - Miller School of Medicine, Miami, FL

Abstract

Purpose—To determine the presence of four 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 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*, *Mycobacteria* and *Chlamydia* using molecular techniques (Polymerase chain reaction and sequence analysis, fluorescent 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 of human corneal epithelial cells *in vitro*. Initial visual acuity (VA), location and characteristics of the infiltrate, time to detection of the infection and symptoms 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 of the 38 (59.4%) cultures examined contained at least one bacterial endosymbiont. One isolate contained two endosymbionts, *Legionella* and *Chlamydia*, confirmed by fluorescence in situ hybridization. Corneal toxicity (CPE) was significantly higher for *Acanthamoebae* hosting endosymbionts compared to 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 VA ($p>0.05$), central location ($p<0.05$),

Corresponding author: Darlene Miller, DHSc, MPH, CIC, Bascom Palmer Eye Institute, Miller School of Medicine-University of Miami, 900 NW 17th Street, Miami, Fl 33136.

Part of this work has been presented at:
Association for Research in Vision and Ophthalmology (ARVO) meeting, May 2008
Ocular Microbiology and Immunology Group (OMIG) meeting, November 2008;
Harry Hirsch Leiter Award winning presentation
American Academy of Ophthalmology (AAO) meeting, November 2008

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absence of radial perineuritis ($p<0.05$), delayed time to detection ($p>0.05$) and longer symptoms duration at presentation ($p>0.05$).

Conclusion—The majority of *Acanthamoeba* isolates responsible for AK harbors one or more bacterial endosymbionts. The presence of endosymbionts enhances the corneal pathogenicity of *Acanthamoeba* isolates and might impact detection time and clinical features of AK.

Introduction

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 exponentially increased over the past two decades as a consequence of the increasing use of contact lenses.[3] AK primarily affects otherwise healthy contact lens wearers and patients with a history of trauma.[1, 2] Clinical features are often misleading and clinical course is usually protracted despite aggressive treatment with anti-amoebic 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 two stages during its life cycle: a vegetative trophozoite 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 are therefore defined as endosymbionts.[9-11] These bacterial endosymbionts are usually 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 four evolutionary lineages within the domain *Bacteria*: the *Alphaproteobacteria*, the *Betaproteobacteria*, the *Bacteroidetes* and the *Chlamydiae*.[13-17] None of these bacterial endosymbionts have the ability to survive and cannot be cultured outside their amoebic host cells. Such interactions may be of clinical relevance, since *Acanthamoeba* might be able to protect bacterial endosymbionts and release them under certain conditions. In fact, co-infections with other microrganisms have been reported in patients with culture proved AK.[18] These include HSV, 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 of the 38 (97%) were cultured from corneal scrapings, corneal biopsies, corneal buttons, contact lenses, or lens cases from 23 patients presenting with AK at

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our institution between January 2006 and February 2008. One environmental sample was cultured from tap water taken at the laboratory. All cultures were grown on agar-agar plates seeded with heat-killed *Escherichia coli* or Peptone Yeats Glucose (PYG) broth. Subsequently, amoebae were grown axenically for two weeks in 1× 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

Acanthamoebae were rinsed in phosphate-buffered saline (pH 7.4), and amoeba and bacterial DNA extracted using the UNSET method.[22] Amplification and sequencing of the 16S-23S internally transcribed spacer (ITS) with primers Sp1 (5′-ACCTCCTTTCTAAGGAGCACC-3′) and Mb23S.44n (5′-TCTCGATGCCAAGGCATCCACC) was 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 (IGS): 23S (5′-TGAAGCCCGTTGAAGACTAC-3′) and 5S (GGAAGCCTCACACTATCAT-3′).[25] The 23S primer was not an exact match to the *Pseudomonas* genus with two mismatches and an insertion all at the 5′ half of the primer. Detection of endosymbionts belonging to the *Chlamydiales* family utilized primer set Momp1 (5′-ATGAAAAAACTCTTGAAATCGG-3′) and Momp2 (5′-GCTCCTAAAGTTGCACA-3′) that target the major outer membrane protein (MOMP) 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 epidemiologically linked 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, ver. 4; http://www.megasoftware.net. Accessed February 2, 2008).[26] Gene trees were generated using maximum-parsimony, neighbor-joining, Unweighted Pair Group Method with Arithmetic mean (UPGMA) 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 1,000 replicates.28

Fluorescent in situ hybridization (FISH)

For FISH, a protocol previously described was used.[10] Briefly, *Acanthamoebae* (>95% trophozoites) harvested from axenic cultures were washed and resuspended in 100 μl of 1× Page's saline. 20 μl aliquots of amoebic suspension were incubated on poly-L-lysine slides for 20 minutes at 45°C to allow attachment of amoebae and fixed with 20 μl of 4% paraformaldeyde for 20 minutes at room temperature. The slides were subsequently washed for three times in 1× PBS and dehydrated in ethanol (96%, 70%, 50%) for 3 minutes. Slides were then incubated for and hour at 45°C with an aliquot (20 μl) of hybridizing buffer (20% formamide, 0.9 M NaCl, 0.01% SDS, e 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 were then gently washed with washing buffer (20 mM Tris/HCl, pH 7.6, 180 mM NaCl

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e 0.01% SDS), re-incubated for 15 minutes at 45°C covered with 500 μl of washing buffer. Slides were then washed with distilled water, dried at room temperature and mounted (Vectashield mounting medium, Vector Laboratories, Burlingame, CA). Images were acquired using a confocal microscope (Leica TC S SP5, Leica Microsystems Inc.).

Transmission electron microscopy (TEM)

Acanthamoeba cysts and trophozoites harvested from axenic cultures were fixed in 2% glutaraldehyde in 0.1M PO_4/100mM sucrose overnight at 4°C. Then, they were rinsed with 0.15M PO_4 for three times. The samples were post fixed in 2% phosphate buffered osmium tetroxide for 1 hour, followed by 3 rinses with 0.15M PO_4. Samples were then 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 embedded in the resin. Images were acquired with a Phillips CM-10 transmission electron microscope.

In vitro corneal pathogenicity assay

The cytopathic effect (CPE) of *Acanthamoeba* isolates was tested on human corneal epithelial cells (HCEC) *in vitro* as described elsewhere.[29] Briefly, 500 μl of 2×10^4 CFU/mL *Acanthamoebae* (>95% trophozoites) suspended in KSFM were added to confluent HCECs in a 24-well plate and incubated at 37° for 8 h. After incubation, all plates were washed three times with PBS, stained with Giemsa (Fisher Scientific Company, Kalamazoo, MI) and solubilized with 400 μl of 5% SDS. Optical density (OD) was read at 590 nm in a microplate reader. Percent of CPE was calculated according to the following formula: %CPE= 100-[(OD experimental well − OD amoebae alone/OD control HCEC alone) × 100].

Clinical data

Clinical data were available for 22/23 patients. Initial visual acuity (VA), location and characteristics of the infiltrate, time to detection (defined as the time between first presentation at our institution and culture positivity) and symptoms duration at presentation (defined as the time between the symptoms onset and the first presentation at our institution) were examined and compared between patients infected by *Acanthamoebae* with endosymbionts and without endosymbionts.

Statistical analysis

Statistical analyses were performed using SPSS software version 15.0 (SPSS Inc, Chicago, Illinois, USA). Tests of significance were two-tailed with $p \leq 0.05$ for all tests.

Results

Endosymbionts

Twenty-two of the 38 (59.4%) *Acanthamoeba* cultures from 12/23 patients (52.2%) examined yielded endosymbionts (Table 1, available at http://aaojournal.org).

Legionella—*Legionella* endosymbionts were found in 3 patients and in one environmental sample. *Legionella* endosymbionts had highest sequence similarity to *Legionella cherrii* ORW (Z30537) between 94-95%, and greater than 99% similarity to each other (Table 1 and 2, available at http://aaojournal.org). Phylogenetic analysis supported this grouping with a bootstrap value of 98% (Fig. 1A, available at http://aaojournal.org).

Pseudomonas—*Pseudomonas* species were documented in 6 patients (13 *Acanthamoeba* isolates) (Table 1, available at http://aaojournal.org). Ten of the endosymbionts showed highest sequence similarity to *P. aeruginosa* UCBPP-PA 14 (CP000438) at 99% or more. All but one

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endosymbiont sequences from this group were identical (Table 2, available at http://aaojournal.org). The phylogenetic grouping of these endosymbionts with members of the *P. aeruginosa* clade was supported by a bootstrap value of 100% (Fig.1B, available at http://aaojournal.org). The remaining three endosymbionts were identical to each other and had the closest sequence similarity to *P. putida* W619 (CP000949) at 96.4% sequence identity (Table 2, available at http://aaojournal.org). The phylogenetic grouping of these endosymbionts with members of the *Pseudomonas putida* clade was supported by a bootstrap value of 92% (Fig. 1B, available at http://aaojournal.org).

Mycobacterium—Four patients (5 *Acanthamoeba* isolates) and one environmental isolate with endosymbionts had sequences similar to bacteria in the *Mycobacterium* genus (Table 1, available at http://aaojournal.org). Two of the endosymbionts (BP:P2:CS and BP:P20:LCS) were identical to each other and showed highest sequence similarity to *M*. sp. 1371 (AY646435) at 98% sequence identity (Table 2, available at http://aaojournal.org). Phylogenetic analysis grouped these endosymbionts in the *M. mucogenicum* clade with 96% supported value (Fig. 2A, available at http://aaojournal.org). Endosymbionts of isolates BP:P19:RCS and BP:C:TW were 99.2% similar to each other and identical to *M. gordonae* Tropicalis-2 (EU497913) and *M. gordonae* (L42258), respectively (Table 2, available at http://aaojournal.org). The remaining *Acanthamoeba* isolates BP:P7:LCL and BP:P7:RCL contained endosymbionts with sequences at 98% identity to each other and closest sequence similarity to *M. fortuitum* K7594-03 (AM709726) at 97-98% sequence identity (Table 2; Fig. 2A, available at http://aaojournal.org).

Chlamydia—A single isolate from one patient (BP:P2:CB) was identified as a member of the *Chlamydophila* family The endosymbiont was most similar to *C. trachomatis* DK-K40 (AM901173) serovar D at 99% sequence identity (Table 2, available at http://aaojournal.org). Phylogenetically it grouped among other members of the *C. trachomatis* serovar D clade (Fig. 2B, available at http://aaojournal.org). This isolate was unique in that it also contained *Legionella*-like bacterial endosymbionts.

Isolates of BP:C:TW

Two *Acanthamoeba* sequences were detected in tap water cultured from the laboratory; and two endosymbiont sequences were amplified from the genomic DNA of this culture, *Legionella* and *Mycobacterium* (Table 1, 2, available at http://aaojournal.org). The origin of the two endosymbionts could not be determined by PCR and images from FISH and TEM were not available.

Fluorescence in situ hybridization (FISH)

Positive hybridization reactions were obtained with the specific fluorescent DNA probes for all four species of bacterial endosymbionts (Fig 3). Bacterial endosymbionts were dispersed throughout the cytoplasm of the *Acanthamoeba* host, and were present in all amoeba cells in the population. Co-localization of phylogenetically different endosymbionts strains was also detected by FISH in the amoeba isolate that had *Legionella* and *Chlamydia* colonization (Fig 3D).

Transmission electron microscopy (TEM)

The ultrastructure and intracellular niche of endosymbionts were further investigated by transmission electron microscopy (Fig 4). Bacteria were observed inside both trophozoites and cysts. None of the endosymbionts was enclosed in phagosomal or phagolysosomal vacuoles, as observed by other laboratories.[30-33] *Pseudomonas* endosymbionts (0.7-0.9 × 0.4 μm in size; Fig. 4 A,B) had a typical rod-shaped appearance, and were surrounded by an electron-

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translucent area probably corresponding to their capsule. They formed small clusters randomly distributed within the host cytoplasm. *Legionella* (0.8-1 × 0.3 μm; **Fig. C,D**) and *Mycobacterium* (1.3-1.6 × 0.2-0.3 μm; Fig. 4 E,F) endosymbionts were also rod-shaped, and were randomly distributed within cysts and throphozoites cytoplasm. No EM image was available for the one isolate that showed presence of a *Chlamydia* endosymbiont.

Cytopathic effect

CPE on HCEC was significantly higher for *Acanthamoeba* hosting endosymbionts compared to isolates without endosymbionts ($p<0.05$, T-test, Fig. 5). Also, *Pseudomonas* and *Mycobacterium* enhanced *Acanthamoeba* CPE significantly more than *Legionella* ($p<0.05$, T-test).

Clinical data

A trend towards a worse initial VA ($p>0.05$) and a preferred central location of the infiltrate ($p<0.05$) was observed in patients affected by *Acanthamoeba* with bacterial endosymbionts (Table 3). Interestingly, radial perineuritis was only reported in patients with Acanthamoeba isolates without endosymbionts (60% vs 0%, $p<0.01$). In the presence of endosymbionts, a delayed time to culture positivity and presentation to our clinic were also noted ($p>0.05$, Table 3).

Discussion

The presence of a variety of endosymbiont bacteria in *Acanthamoeba* hosts has been known for a long time.[34] In this study, we examined 37 *Acanthamoeba* isolates obtained from patients with AK and one tap water isolate for the presence of four medically important bacterial agents: *Legionella*, *Mycobacterium*, *Pseudomonas* and *Chlamydiae*.

On a total of 23 AK patients, 12 were found infected by *Acanthamoebae* hosting bacterial endosymbionts (52.2%). Of the 38 isolates examined, 22 (59.4%) were found to possess at least one bacteria as an endosymbiont, a twofold greater number than the 26% endosymbiont presence observed by Fritsche et al.[30] The higher endosymbiont presence may be due to the sensitive detection method of PCR. Additionally, the 59% observance may be low due to the restricted search for endosymbionts of interest to our laboratory.

The predominant endosymbiont of the four genera examined belonged to the genus *Pseudomonas* with 13 of the 22 endosymbionts (6/12 patients) reported here. *Pseudomonas* species are commonly responsible for acute-onset and highly destructive keratitis.[35] Previously, it was assumed that *Pseudomonas aeruginosa* and *Acanthamoeba* were mutually exclusive ocular pathogens, since *Pseudomonas* expressed amoebicidal activity in co-cultivation.[36] More recently, in vitro studies have showed that *Pseudomonas* can actually cooperate in the pathogenesis of contact lens related AK: in fact, it increases the resistance of *Acanthamoeba* to contact lens disinfecting solutions and creates a biofilm on contact lenses surface that enhances amoebae retention.[37] *Pseudomonas-Acanthamoeba* co-infections have also been described in keratitis patients, along with the presence of *Pseudomonas aeruginosa* intracellularly in environmental *Acanthamoebae*.[20, 38]

The second most common endosymbionts belonged to the *Mycobacteria* family. Corneal infection by atypical *Mycobacteria* is a relevant clinical challenge, mostly occurring after laser in situ keratomileusis (LASIK) procedures.[39] *Mycobacteria* have been previously reported as endosymbionts in an environmental *Acanthamoebae*, but this study is the first to report on *Mycobacteria* in clinical samples.[33] *Mycobacteria* can penetrate the amoeba cell, multiply inside the cytoplasm and resist amoeba encystation.[40] It has also been shown that

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Acanthamoeba can protect *Mycobacteria* from chlorine and antibiotics and enhance *Mycobacteria* virulence.[41, 42] Five clinically related Acanthamoeba isolates and the tap water sample harbored *Mycobacteria* species. The *Mycobacterium* endosymbionts observed segregated into to 3 different *Mycobacteria* species cluster: *M. gordonae, M. fortuitum and M. mucogenicum*. Patient BP:P7 was unusual in that the *Acanthamoeba* isolated from the right and left contact lens cases were closely related, but not identical (unpublished data). Interestingly, both housed *Mycobacteria* that were very closely related.

Legionella type endosymbionts were also identified in 3 out of 12 patients. The symbiosis between *Acanthamoeba* and *Legionella* has been extensively characterized.[43] In fact, intracellular replication within amoeba is considered a prerequisite to infection in humans in *Legionella pneumophila*, a facultative intracellular pathogen causing a severe pneumonia called Legionnaire disease.[44]

A *Chlamydia* type endosymbiont was observed in only one *Acanthamoeba* host (one patient), BP:P2:CB, from this dataset. This was surprising since endosymbionts belonging to the *Chlamydiales* family are quite common in free-living amoeba, and may reflect a lower affinity of *Chlamydia* endosymbionts for clinically relevant amoebae.[12] Moreover, the identification of an intra-amoebal form of *Chlamydia trachomatis* opens the door to a possible role of *Acanthamoeba* in the developmental cycle and spreading of this important ocular pathogen, responsible in humans for the occurrence of inclusion conjunctivitis and sight-threatening trachoma.[45]

Interestingly, the BP:P2:CB isolate was also the only isolate observed to contain two of the bacteria types investigated, *Chlamydia* and *Legionella*. Occurrence of multiple endosymbionts in a clinical isolate has never been reported. Recovery of more than one endosymbiont has only recently been documented in an environmental *Acanthamoeba* strain.[46] Co-colonization of different bacteria may result from the ability of each bacterial type to exploit a different niche within the amoeba. In fact, *Proteobacteria*, like *Legionella*, survive within the cytoplasm of amoeba, while *Chlamydiales* symbionts are sequestered within vacuoles.[46]

Patient BP:P2 was interesting in that two *Acanthamoeba* isolates isolated from the cornea at different times hosted different endosymbionts. This could be explained by the fact that the two isolates grew from a corneal scraping done before starting specific amoebicidal topical therapy (chlorexidine and polyhexamethylbiguanide) and from the corneal button subsequently obtained during therapeutic keratoplasty. The anti-amoebic therapy and/or the change in microenvironment could have determined loss or selection of specific endosymbionts. A reversal of this observation is also seen, in that *Acanthamoeba* from different patients possess endosymbionts with identical sequences. For example, *Acanthamoeba* sp. BP:P3:RCL and *Acanthamoeba* sp. BP:P16:RCS possess *Pseudomonas* sp. type endosymbionts with identical sequences. Although bacteria-*Acanthamoeba* symbiosis has been reported and analyzed in multiple studies, the real contribution of endosymbionts to the pathogenesis of amoebal infections has not been clarified yet.

Endosymbionts may influence *Acanthamoeba* pathogenicity, virulence or susceptibility to antiamoebic drugs. An enhancement of *Acanthamoeba* cytopathogenicity following the acquisition of bacterial endosymbionts has been described in vitro.[47] The presence of the endosymbiont may modify *Acanthamoeba* phenotype making the protozoa more pathogenic or resistant to therapy. Changes in gene expression and protein profiles have been observed resulting from the amoeba/bacteria interaction in *Hartmannella* and *Naegleria*.[48, 49] In our study, we described an increased pathogenicity of *Acanthamoebae* harboring bacterial endosymbionts on corneal epithelial cells. Moreover, amoeba isolates hosting corneal

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pathogenic bacteria (*Pseudomonas* and *Mycobacteria*) as endosymbionts were significantly more pathogenic than *Legionella*.

Our preliminary clinical observations also suggested that endosymbionts may influence *Acanthamoeba* virulence and AK clinical features. In AK patients, the worse VA at presentation and the central location of the infiltrate observed in the presence of endosymbionts could be considered as a consequence of the increased corneal pathogenicity of *Acanthamoeba*. The absence of radial perineuritis and the delay in culture positivity of amoebae with endosymbionts might be due to modification of *Acanthamoeba* life cycle or AK pathogenesis induced by the endosymbionts. Moreover, the fact that endosymbionts were most frequently found in cases where the interval between symptoms onset and first medical examination was longer could indicate that a longer persistence of *Acanthamoeba* in the eye increases the chances of bacterial uptake and endosymbiosis. Unfortunately, only a small number of patients were included in this study due to the low prevalence of this infection, and most of them were only referred to our tertiary center for diagnosis and subsequently lost to follow-up. Further studies with a larger cohort of patients and longer follow-up are needed to better understand the contribution of endosymbionts to the natural history of AK.

Conversely, *Acanthamoeba* can affect endosymbionts pathogenicity.[11] In fact, intracellular growth of *Mycobacteria* and *Legionella* in *Acanthamoeba* enhances invasiveness and virulence of the bacteria.[41, 50, 51] *Acanthamoeba* can also protect endosybiont bacteria from antibiotics, disinfectant and hostile environmental conditions.[40, 42, 52] Detection of bacterial and fungal contaminants in contact lens cases of patients with AK is common. In a contact lens-related keratitis scenario, *Acanthamoeba* may be able to uptake bacteria from the contact lens surface or an improperly cleaned case, protect them from the disinfecting solutions and antibiotics through encystation, and deliver them to the cornea. The release of bacterial endosymbionts upon the demise of the amoeba host is an equally important point of interest. Bacterial endosymbionts may be not be capable of inducing an infectious keratitis themselves, but the presence of pro-inflammatory bacterial components in a compromised cornea may boost corneal inflammation and further exacerbated the outcome of keratitis.

Obviously, there is a definite need to better understand the interactions between amoebae and their endosymbionts. More data on the effect of amoebic endosymbionts influence on virulence factors, clinical outcome, drug susceptibility and role in human/host interactions need to be acquired. Additionally, to what degree is the amoeba/endosymbiont relationship important in dissemination of medically important bacteria worldwide? These are only a few of the questions that need to be addressed to better understand and develop measures for disease control.

Acknowledgments

Financial support: Research to Prevent Blindness (P30 grant EY014801)

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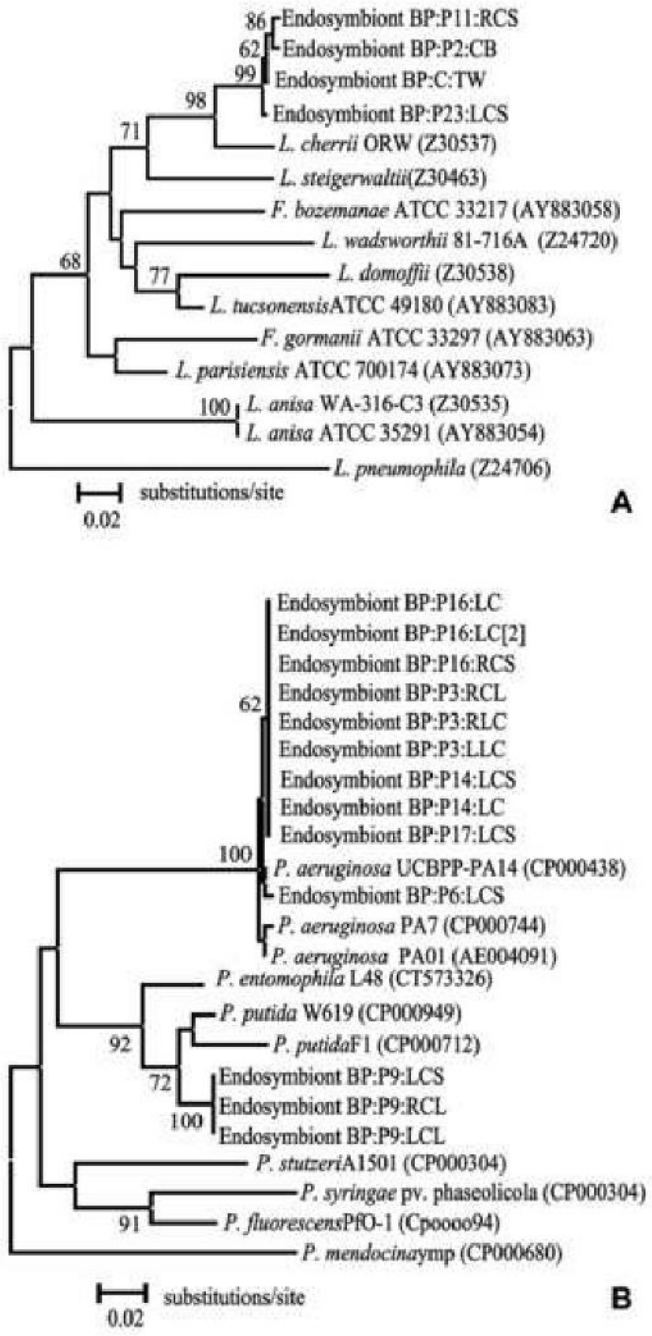

Figure 1.

A Distance Neighbor-joining tree based on 23S-5S IGS analysis showing relationship of *Acanthamoeba* endosymbionts to representative members of the *Legionella* genus. The tree was rooted by using the *L. pneumophila* (Z24706) strain. **B** Distance Neighbor-joining tree based on 23S-5S IGS analysis showing relationship of *Acanthamoeba* endosymbionts to representative members of the *Pseudomonas* genus. The tree was rooted by using the *P. mendocina* ymp strain. The scale bar represents percent difference per average site. Bootstrap values based on 1000 replicates are given at nodes to which they apply, values below 50 are not shown.

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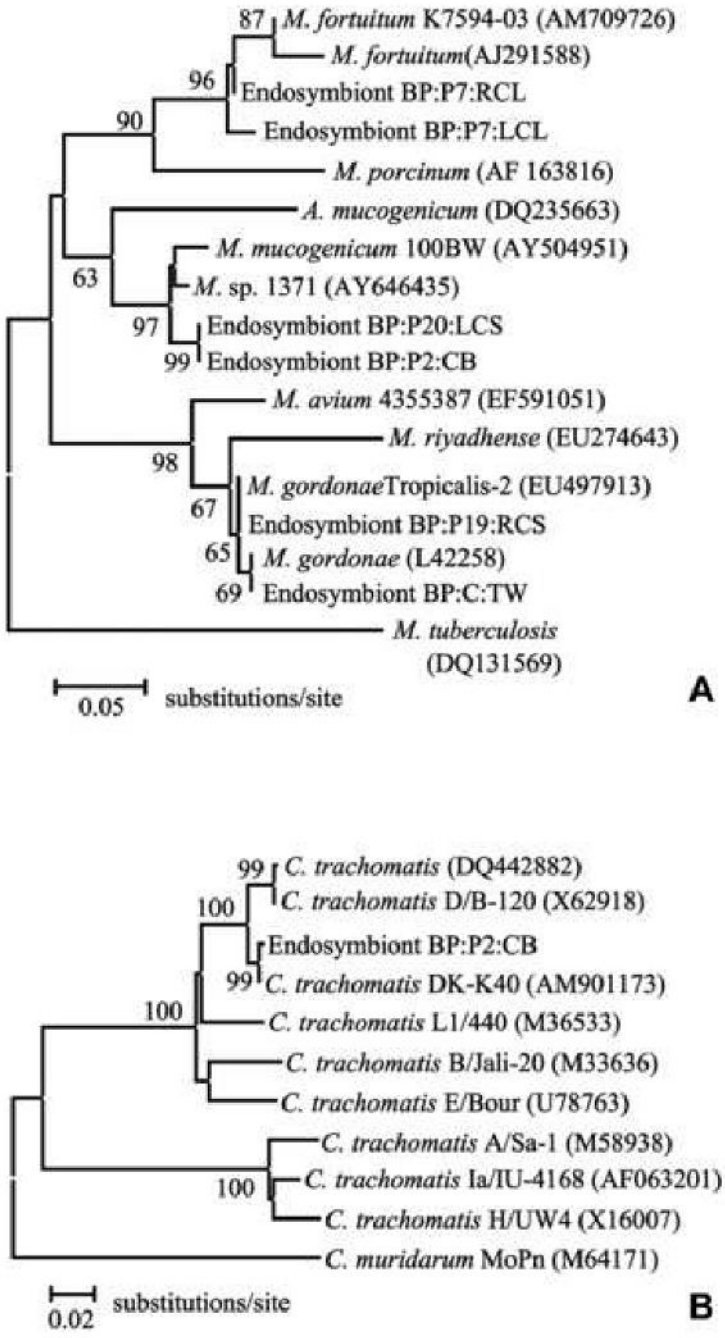

Figure 2.

A Distance Neighbor-joining tree based on 16S-23 ITS analysis showing relationship of *Acanthamoeba* endosymbionts to representative members of the *Mycobacterium* genus. The tree was rooted by using the *M. tuberculosis* (DQ131569) strain. The scale bar represents percent difference per average site. Bootstrap values based on 1000 replicates are given at nodes to which they apply, values below 50 are not shown. **B** Distance Neighbor-joining tree based on analysis of partial sequence of the MOMP gene, showing relationship of *Acanthamoeba* endosymbionts to representative members of the *Chlamydia* genus. The tree was rooted by using the *C. muridarum* MoPn strain. The scale bar represents percent difference

per average site. Bootstrap values based on 1000 replicates are given at nodes to which they apply, values below 50 are not shown.

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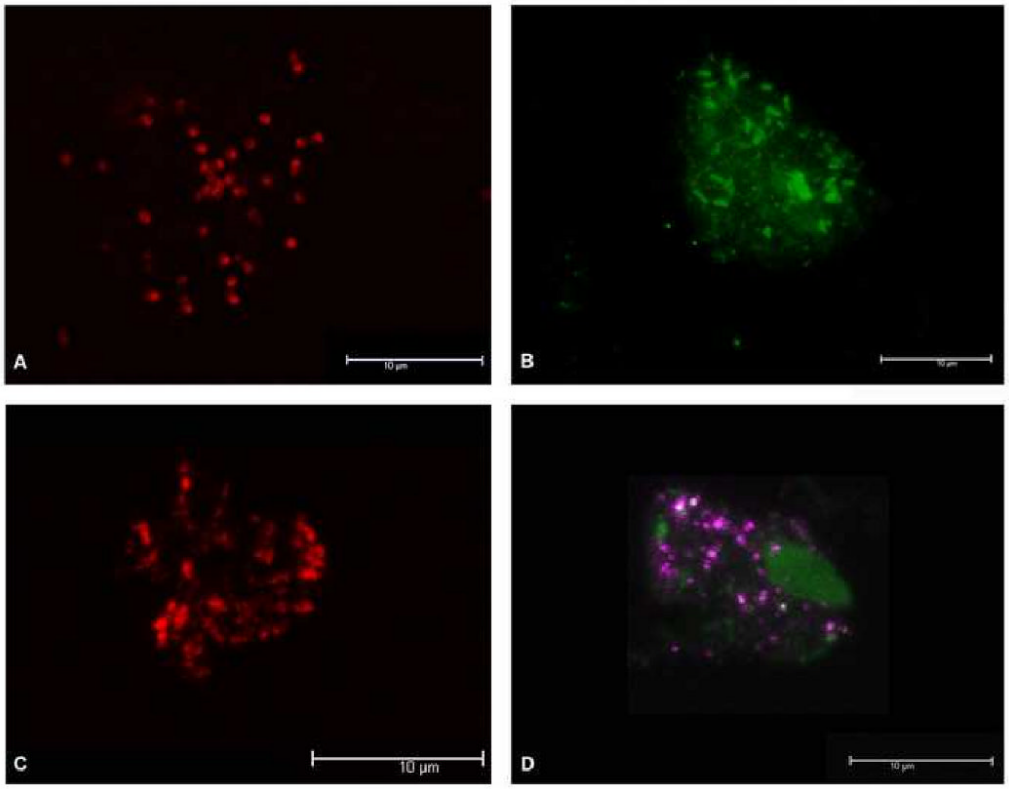

Figure 3.
Specific fluorescent DNA probes targeting *Pseudomonas* species (**A**, Texas Red, in red, 63×), *Legionella* species (**B**, FAM labeling, in green, 63×), *Mycobacterium* species (**C**, Cy3, in red, 63×). **D** Co-existence of *Legionella* and *Chlamydia* species in a single *Acanthamoeba* isolate DNA probes marked with, respectively (FAM/*Legionella* and Cy5/*Chlamydia* labeling, in green and purple respectively, 63×).

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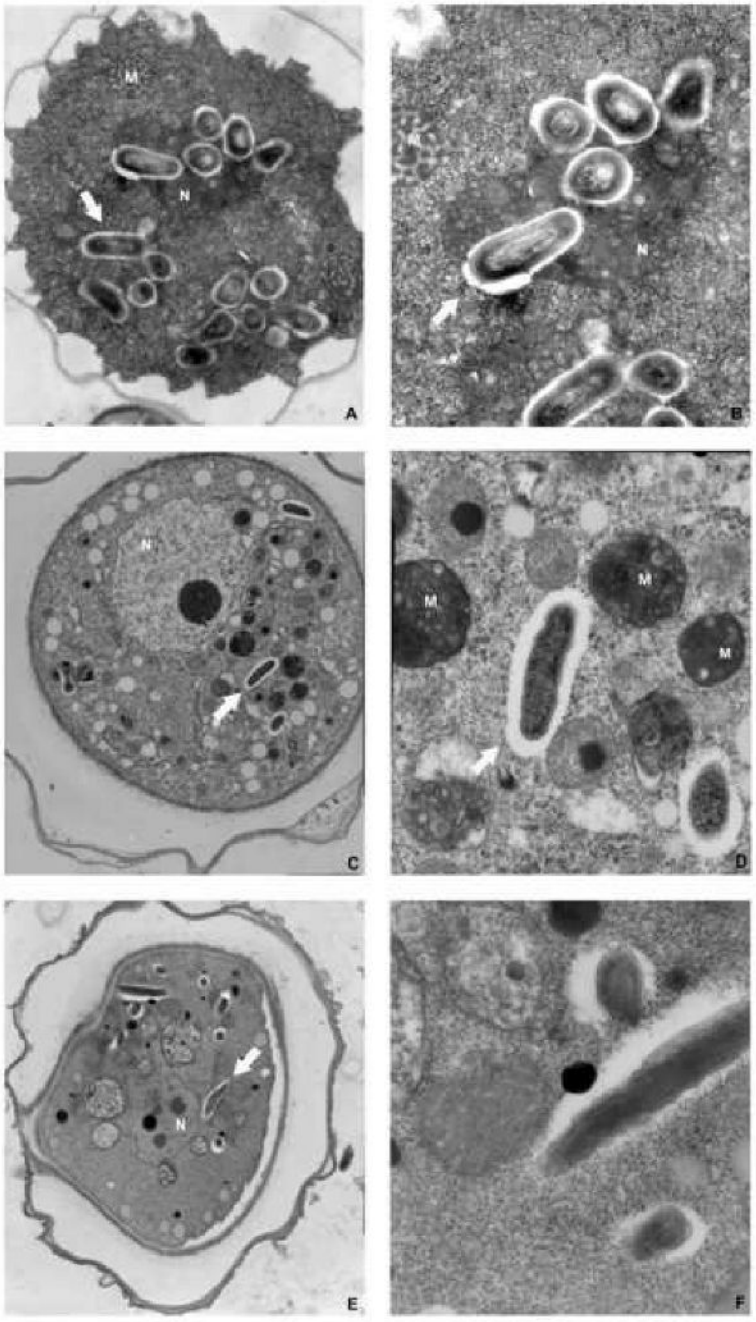

Figure 4.

Transmission electron micrographs of *Acanthamoeba* isolates with endosymbionts. *Acanthamoeba* cysts containing *Pseudomonas* (**A**), *Legionella* (**C**) and *Mycobacteria* (**E**). Ultrastructural feature of *Pseudomonas* (**B**), *Legionella* (**D**) and *Mycobacteria* (**F**). Notice absence of phagolysosomal membrane surrounding the endosymbiont bacteria. N: nucleus; M: mytocondria.

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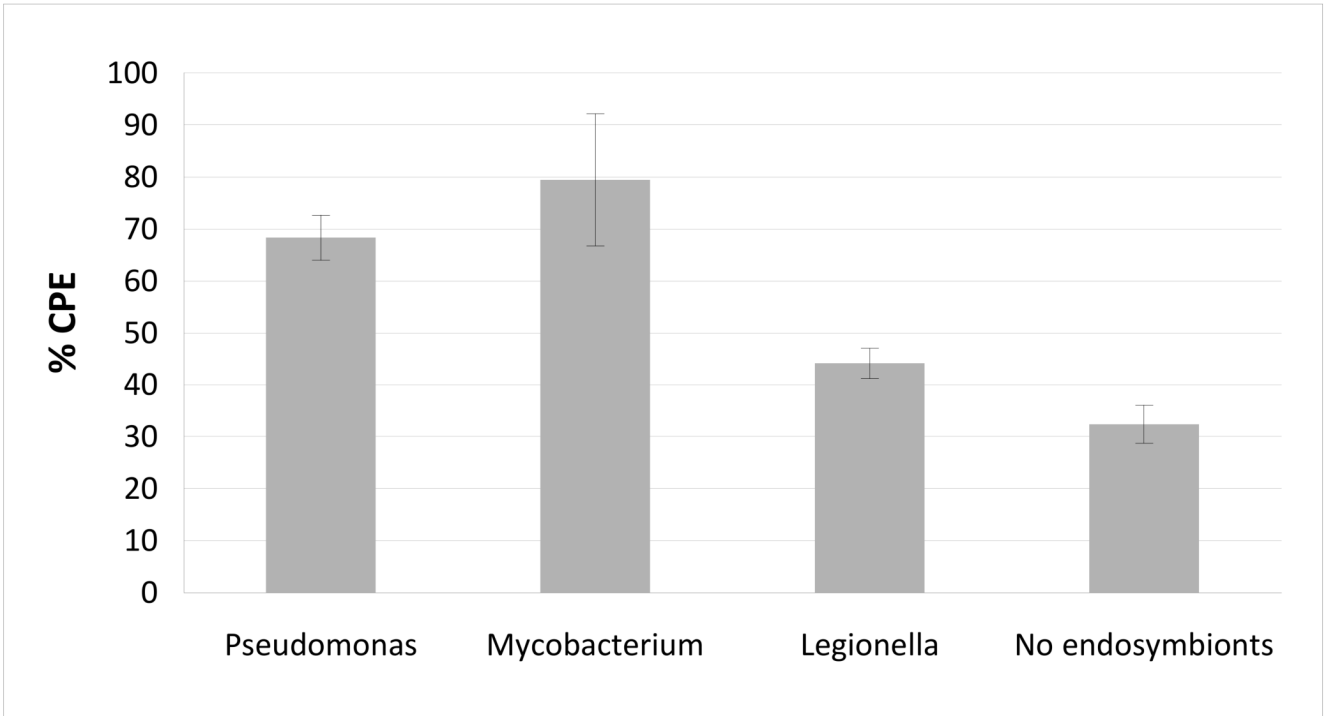

Figure 5.
Cytopathic effect (CPE) on human corneal epithelial cells for *Acanthamoebae* hosting different endosymbionts. Amoebae harboring bacterial endosymbionts were more pathogenic than isolates without endosymbionts ($p<0.05$). Bacteria capable of corneal infections, such as *Pseudomonas* and *Mycobacteria*, were able to confer to *Acanthamoebae* additional pathogenicity when compared to *Legionella* ($p<0.05$). Data are expressed as mean ±SD results of triplicate experiments.

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Table 1
Acanthamoeba Isolates with detected endosymbionts

Culture Designation[a]	Culture Source	Endosymbiont designation	Endosymbiont GenBank Accession Number
BP:P2:CB	Corneal Button	*Legionella*	FJ444813
		Chlamydia	FJ444796
BP:P2:CS	Corneal Scrape	*Mycobacterium*	FJ444817
BP:P3:RCL	Right Contact Lens	*Pseudomonas*	FJ444800
BP:P3:RLC	Right Lens Case	*Pseudomonas*	FJ444805
BP:P3:LLC	Left Lens Case	*Pseudomonas*	FJ444804
BP:P6:LCS	Left Corneal Scrape	*Pseudomonas*	FJ444806
BP:P7:LCL	Left Contact Lens	*Mycobacterium*	FJ444814
BP:P7:RCL	Right Contact Lens	*Mycobacterium*	FJ444815
BP:P9:LCS	Left Corneal Scrape	*Pseudomonas*	FJ444807
BP:P9:RCL	Right Contact Lens	*Pseudomonas*	FJ444808
BP:P9:LCL	Left Contact Lens	*Pseudomonas*	FJ444809
BP:P11:RCS	Right Corneal Scrape	*Legionella*	FJ444810
BP:P14:LCS	Left Corneal Scrape	*Pseudomonas*	FJ444802
BP:P14:LC	Lens Case	*Pseudomonas*	FJ444803
BP:P16:RCS	Right Corneal Scrape	*Pseudomonas*	FJ444797
BP:P16:LC	Lens Case	*Pseudomonas*	FJ444798
BP:P16:LC[2]	Lens Case	*Pseudomonas*	FJ444799
BP:P17:LCS	Left Corneal Scrape	*Pseudomonas*	FJ444801
BP:P19:RCS	Right Corneal Scrape	*Mycobacterium*	FJ444818
BP:P20:LCS	Left Corneal Scrape	*Mycobacterium*	FJ444816
BP:P23:LCS	Left Corneal Scrape	*Legionella*	FJ444811
BP:C:TW	Tap Water	*Mycobacterium*	FJ444819
		Legionella	FJ444812

[a]Culture designation: BP, Bascom Palmer: P, Patient: Acronyms - RCS, Right Corneal Scrape; CB, Corneal Button; LCS, Left Corneal Scrape; LLC, Left Lens Case; RLC, Right Lens Case; LC, Lens Case; LCL, Left Contact Lens; RCL, Right Contact Lens; TW, Tap Water: C, control.

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Table 2

Differences in the sequences between the endosymbiont isolates

		[1]	[2]	[3]	[4]	[5]						
[1]	BP:P11:RCS	*	2	3	2	17						
[2]	BP:P2:CB	99.3	*	3	2	17						
[3]	BP:P23:LCS	99	99	*	1	16						
[4]	BP:C:TW	99.3	99.3	99.7	*	15						
[5]	*L.cherrii* ORW	94.3	94.3	94.6	94.9	*						
		[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]
[6]	BP:P3:RCL	*	0	0	3	0	0	0	0	0	0	1
[7]	BP:P3:RLC	100	*	0	3	0	0	0	0	0	0	1
[8]	BP:P3:LLC	100	100	*	3	0	0	0	0	0	0	1
[9]	BP:P6:LCS	99	99	99	*	3	3	3	3	3	3	2
[10]	BP:P14:LCS	100	100	100	99	*	0	0	0	0	0	1
[11]	BP:P14:LC	100	100	100	99	100	*	0	0	0	0	1
[12]	BP:P16:RCS[2]	100	100	100	99	100	100	*	0	0	0	1
[13]	BP:P16:LC	100	100	100	99	100	100	100	*	0	0	1
[14]	BP:P16:LC[2]	100	100	100	99	100	100	100	100	*	0	1
[15]	BP:P17:LCS	100	100	100	99	100	100	100	100	100	*	1
[16]	*P. aeruginosa* UCBBPP-PA 14	99.7	99.7	99.7	99.3	99.7	99.7	99.7	99.7	99.7	99.7	*
		[17]	[18]	[19]	[20]							
[17]	BP:P9:RCL	*	0	0	10							
[18]	BP:P9:LCL	100	*	0	10							
[19]	BP:P9:LCS	100	100	*	10							
[20]	*P. putida* W619	96.4	96.4	96.4	*							
		[21]	[22]	[23]	[24]	[25]	[26]	[27]	[28]	[29]	[30]	
[21]	BP:P2:CS	*	0	4								
[22]	BP:P20:LCS	100	*	4								
[23]	*M. sp.* 1371	98	98	*								

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		[1]	[2]	[3]	[4]	[5]					
[24]	BP:P7:LCL				*	6	7				
[25]	BP:P7:RCL				98	*	2				
[26]	*M. fortuitum* K7594-03				97	98.8	*				
[27]	BP:P19:RCS							*	0	1	1
[28]	*M. gordonae* Tropicalis-2							100	*	1	1
[29]	BP:C:TW							99.2	99.2	*	0
[30]	*M. gordonae* (L42258)							99.2	99.2	100	*

		[31]	[32]
[31]	BP:P2:CB	*	13
[32]	*C. trachomatis* DK-K40	98.9	*

Culture designation: BP, Bascom Palmer: P, Patient: Acronyms - RCS, Right Corneal Scrape; CB, Corneal Button; LCS, Left Corneal Scrape; LLC, Left Lens Case; RLC, Right Lens Case; LC, Lens Case; LCL, Left Contact Lens; RCL, Right Contact Lens; TW, Tap Water: C, control. Numbers above the diagonal are nucleotide differences. Numbers below the diagonal are percent similarities. Numbers not shown comparing the different *Mycobacterium* species were greater than 15% and 70 base pairs different.

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Table 3
Comparison of patients outcomes with and without endosymbionts

	Endosymbionts Present (12)	Endosymbionts Not Present (10)	P value (95 CI)
Initial VA	1.11± 0.5 (20/260)	0.56 ± 0.2 (20/72)	0.9[a]
Location of the infiltrate	Central (91.7%, 11/12)	Central (50%, 5/10)	0.04[b]
Radial perineuritis	0% (0/12)	60% (6/10)	0.002[b]
Time to detection (days) Less than 7 days	7.6 ± 2.5 33% (4/12)	5.4 ± 1.6 70% (7/10)	0.8[a] 0.08[b]
Symptoms duration at presentation	50.2 ± 30.4	21 ± 5.8	0.9[a]

Data are expressed as mean ± standard error of the mean (SEM). VA: visual acuity; CI: confidence interval.

[a] T-test.

[b] Fisher exact