Laboratory Investigation of Acanthamoeba lugdunensis from Patients with Keratitis

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METHODS. Morphologic features of amebic cysts were evaluated with a microscope with differential interference contrast (DIC) optics. Restriction fragment length polymorphisms (RFLP) of mitochondrial DNA (mtDNA), riboprinting of small subunit ribosomal RNA gene (18S rDNA), and DNA sequences of 18S rDNA were analyzed. mtDNA and PCR-amplified 18S rDNA of the ocular isolates were digested with restriction enzymes, and the restriction patterns were compared with those of reference strains purchased from American Type Culture Collection (ATCC, Manassas, VA). PCR products of 18S rDNA were cloned and subjected to sequencing. The complete sequence of approximately 2300 bp obtained from the isolates and reference strains were compared with each other and those registered in GenBank.

RESULTS. Three ocular isolates (KA/E2, KA/E12, and KA/E16) of Acanthamoeba revealed the identical mtDNA RFLPs and riboprint patterns with Acanthamoeba L3a, the type strain of A. lugdunensis. The other isolate (KA/E15) had riboprint patterns very similar to A. lugdunensis L3a but quite different mtDNA RFLP patterns from those of all the other strains. A dendrogram based on the riboprint data showed that three ocular isolates were digested with restriction enzymes, and the restriction patterns were compared with those of reference strains purchased from American Type Culture Collection (ATCC, Manassas, VA). PCR products of 18S rDNA were cloned and subjected to sequencing. The complete sequence of approximately 2300 bp obtained from the isolates and reference strains were compared with each other and those registered in GenBank.

CONCLUSIONS. This is the first report of Acanthamoeba keratitis in Korea caused by A. lugdunensis, which was originally isolated from a freshwater pool in France. Riboprinting can be used as a simple and rapid tool for putative identification of unknown Acanthamoeba ocular isolates. (Invest Ophthalmol Vis Sci. 2004;45:1418–1426) DOI:10.1167/iovs.03-0433

The Acanthamoeba has been a relatively uncommon causative organism of chronic infectious keratitis in contact lens users since the first report of Acanthamoeba keratitis in 1975.1 The number of cases of Acanthamoeba keratitis has grown substantially in recent years, and several Acanthamoeba spp. have been found to cause keratitis. Among Acanthamoeba isolates from the environment, some isolates that grow at higher temperatures preferably are known to be more pathogenic.2 However, considering that Acanthamoeba strains that cause keratitis grow in contact lens storage cases at room temperature and the relative low temperature of the corneal surface, the possibility that keratitis is caused by other Acanthamoeba isolates from the environment cannot be excluded.

To reveal the ecology and prevalence of pathogenic strains, species identification of the ocular isolates is a prerequisite. Although at least 20 species of Acanthamoeba have been classified, largely into three groups, by Pussard and Pons,3 according to morphologic characteristics, the taxonomy of this small ameba species has not been well established. Alloenzyme profiles and mtDNA RFLP studies have been widely applied, but the results were too polymorphic to be the criteria for species identification.4,5 Recently riboprinting and 18S rDNA gene sequence analyses have been applied for the species identification of the ameba strains.6–8 To date, six species of Acanthamoeba—A. castellanii, A. polyphaga, A. rhysodes, A. culbertsoni, A. batcetti, and A. griffini—have been identified in ocular infections.9

This article presents the results of molecular characterization of four ocular isolates that were finally identified as A. lugdunensis and compares the 18S rDNA sequences among these isolates and reference strains.

METHODS

Case Examples

Case 1. In a 17-year-old boy, a daily-wear soft contact lens user, ocular pain, tearing and conjunctival injection developed in the left eye after he wore contact lenses that had been stored in a contact lens case for a couple of weeks. At first the condition was diagnosed as herpes simplex keratitis and treated with 1% trifluridine, acyclovir ointment, and 0.12% prednisolone acetate at a local clinic for 2 months. On the first examination of the affected eye, there were a round central epithelial defect and dense stromal infiltrates. Numerous keratic precipitates existed, and a moderate degree of anterior chamber reaction was shown (Fig. 1, case 1). Visual acuity was FC/50 cm in his left eye. Corneal scrapings contained double-walled and orange flouresced cysts visualized with acridine orange stain. Amebas were grown in a non-nutrient agar (NNA)—coated plate with an Escherichia coli overlay. Treatment was begun with topical polyhexamethylene biguanide (PHMB, 0.02%, Baquacl, Zeneca, Inc., Wilmington, DE), hexamidine (0.1% Desomedine; Chauvin-Blache, Montpellier, France), and systemic itraconazole. After medical treatment for 4 months, the epithelium was covered, and a round central stromal opacity with stromal thinning remained.

Case 2. A 26-year-old woman was referred with an uncontrolled corneal ulcer in her right eye after wearing daily-wear soft contact lenses.
lenses. She had been treated for herpes simplex keratitis for the past 3 months. She reported severe ocular pain and loss of visual acuity. On slit lamp examination, a 6-mm round, elevated corneal inflammation with neovascularization was visible (Fig. 1, case 2). For suspected Acanthamoeba keratitis, PHMB (0.02%), ofloxacin (0.3%), and cycloplegic eye drops were started with systemic itraconazole after smear, culture, and biopsy. Smear and culture were negative, but biopsy specimens revealed amebic cysts. After 6 months of medical treatment, she underwent penetrating keratoplasty, and corrected visual acuity of 20/25 was restored.

**Case 3.** A 26-year-old man was referred with uncontrolled contact-lens–induced keratitis. He had worn daily-wear soft contact lens for several years, but keratitis had developed 2 months before his visit to the clinic. Even with topical antibiotics, ocular signs and symptoms...
gradually intensified. On the first ocular examination, there was a marked ciliary injection, corneal epithelial defect, and stromal infiltrates in the paracentral area (Fig. 1, case 3). The anterior chamber reaction was 2-positive (+ +), and keratic precipitates existed on endothelial side of corneal lesion. Acanthamoeba infection was suspected, and topical PHMP (0.02%) was started with systemic itraconazole. Corneal scrapings revealed many Acanthamoeba cysts, and cultures from a corneal specimen and the contact lens case were also positive. After antiamebic treatment for 2 months, the corneal lesion changed to mild stromal opacity with fine neovascularization. Vision corrected with glasses improved to 20/25.

**Case 4.** A 24-year-old female college student visited an eye clinic with severe ocular pain, tearing, and decreased vision. She occasionally used disposable soft contact lenses but sometimes cleaned them just like daily-wear soft contact lens. On slit-lamp examination, scattered ring-shaped corneal infiltrates were visible at the central cornea. The anterior chamber reaction was mild, and ocular tension was normal (Fig. 1, case 4). With a clinical diagnosis of Acanthamoeba keratitis, antiamebic treatment was initiated after smear and culture. Six months after treatment, the corneal lesion cleared up and barely visible stromal haze was left. The patient’s corrected vision was restored to 20/20.

**Protozoology**

The corneal epithelial scrapings from a patient were obtained by using sterile scalpels, and samples were inoculated onto 1.5% NNA-coated plates covered with heat-treated Escherichia coli. The plates were incubated at 30°C and were examined daily for viable trophozoites. After 96 hours, some encysted amebas were transferred into new agar-coated plates, and other cysts were subjected to axenization. The isolates were designated Korean Acanthamoeba/eye series KA/E2, KA/E12, KA/E15, and KA/E16. No other pathogenic microbes were isolated on routine culture of corneal specimens.

A piece of agar-coated plate (0.5 × 1 cm) covered with cysts of each clone was treated with 0.1 N HCl for 24 hours for axenization and washed with glass-distilled water three times. The agar-coated plate was placed and incubated in protease peptone-yeast extract-glucose (PYG) or protease peptone-yeast extract-glucose-cysteine (PYGC) media at 25°C and 37°C. The size and number of arms of the cysts from the four Acanthamoeba isolates were determined for morphologic grouping.

**In Vitro Drug Sensitivity**

Acanthamoeba cysts were recovered from prolonged incubation of trophozoites on non-nutrient E. coli plates for 4 weeks. The cysts were recovered from the plates, washed, and adjusted to a concentration of 5 × 10^7/100 μL. A cyst suspension (100 μL) was inoculated on a 96-well microplate, and each well was treated with three kinds of contact lens disinfectants (100 μL each, 4, 8, 12, and 24 hours) and serial twofold diluted chlorhexidine (from 0.005%), PHMB (from 0.04%), and hexamidine (from 0.1%; 100 μL each, 8 and 48 hours). Microplates were centrifuged at 2000 rpm for three times for 5 minutes each after incubation and the supernatant removed. Each well was supplemented with an E. coli suspension and examined under an inverted microscope, and excysted viable organisms were observed until 7 days after treatment. The minimal cysticidal concentration (MCC) of each disinfectant was defined as the lowest concentration of test solution that resulted in no excystment and trophozoite replica-

**Extraction of mtDNA and RFLPs**

We extracted mtDNA of Acanthamoeba by the method described by Yagita and Endo. Acanthamoeba trophozoites harvested at the end of a logarithmic growth phase were washed with cold PBS. Amebas were lysed with fresh 1% sodium dodecyl sulfate solution in 0.2 N NaOH and potassium acetate buffer and incubated on ice for 30 minutes. The mtDNA was extracted with phenol and phenol-chloroform (1:1) and recovered by precipitation with cold absolute ethanol in the presence of sodium acetate. We digested mtDNA of Acanthamoeba isolates with four restriction enzymes: EcoRI, BglII, CiaI, and Sgrl (Promega, Madison, WI) at 37°C for 2 hours in 20 μL reaction volume with the buffers specified for each restriction enzyme. Digested DNA was electrophoresed in 0.7% agarose gel at 4 V/cm for 1 to 2 hours and stained with ethidium bromide for 15 minutes. The mtDNA RFLP patterns were observed and photographed under a UV transilluminator.

**Chromosomal DNA Extraction, 18S rDNA Amplification, and Riboprinting**

Chromosomal DNA of Acanthamoeba isolates was obtained by the method described by Kong and Chung. Briefly, Acanthamoeba trophozoites (5 × 10^5) washed with PBS were boiled with 0.1 mL of 0.1 N NaOH for 3 minutes. Supernatant collected after centrifugation at 800g for 2 minutes at room temperature was mixed with 0.2 mL of distilled water. The genomic DNA was extracted with phenol and phenol-chloroform (1:1) and recovered by precipitation with cold absolute ethanol in the presence of sodium acetate. The DNA was used as a template for PCR.

The primers P3, 5′-CCGAATTCCTGGACACCTGGTGATCCGT CCAGT-3′, and P4, 5′-GATCCAGCTGTCTCCAGGTTCACCTAC-3′, were designed to hybridize to highly conserved sequences at the extreme 5′ (P3) and 3′ (P4) termini of eukaryotic 18S rDNA. PCR was performed with a kit of premixed PCR reagents (Bioneer, Daejon, South Korea) and a thermal cycler (Perkin Elmer, Boston, MA). The PCR products of four clinical isolates were electrophoresed in a 2.5% agarose gel with DNA size standards (HindIII digested λ-phage DNA, Poscochem, Sungnam, Korea; and Amplisize, Biорad, Hercules, CA). Ten restriction endonucleases with four-base recognition sequences (HaeIII, Hhal, HinfI, MspI, Ddel, Alul, Sau96I, BsiBI, TaqI, and Tvo9I; Poscochem) were used to generate comparative riboprints. The digested DNA was electrophoresed in a 2.5% agarose gel. To differentiate small DNA fragments, which were unclear in the agarose gel, we used 12% polyacrylamide gels. The gels were stained with ethidium bromide and photographed under a UV transilluminator.

Sequence divergence estimates were calculated by the Nei and Li equation from a fragment comigration data set (an average of 15.8% of the SSU-rDNA sequence) obtained by comparison of the riboprints of four clinical isolates and 23 reference strains (Table 1). A phylo-

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Treatment Time (h)</th>
<th>PHMB</th>
<th>Chlorhexidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>KA/E2</td>
<td>8</td>
<td>9.36</td>
<td>18.75</td>
</tr>
<tr>
<td>KA/E12</td>
<td>48</td>
<td>5.50</td>
<td>7.03</td>
</tr>
<tr>
<td>KA/E15</td>
<td>48</td>
<td>9.25</td>
<td>17.89</td>
</tr>
<tr>
<td>KA/E16</td>
<td>48</td>
<td>5.34</td>
<td>6.76</td>
</tr>
<tr>
<td>KA/E15</td>
<td>48</td>
<td>5.89</td>
<td>7.65</td>
</tr>
<tr>
<td>KA/E16</td>
<td>48</td>
<td>8.97</td>
<td>18.46</td>
</tr>
<tr>
<td>KA/E16</td>
<td>48</td>
<td>5.20</td>
<td>7.25</td>
</tr>
</tbody>
</table>

Table 2. MCC of Disinfectants against Four Clinical Isolates of Acanthamoeba
A phylogenetic tree was reconstructed by the unweighted pair group method with arithmetic average (UPGMA) using a computer program (Phylip ver. 3.5).13,14

**Gene Sequencing**

PCR products were sequenced in a commercial system (dsDNA Cycle Sequencing System; Invitrogen-Gibco, Gaithersburg, MD) either directly or after cloning into pBSK/H11001 (Stratagene, La Jolla, CA) or PCR II (Invitrogen, San Diego, CA). The sequences were compared with those of other *Acanthamoeba* sequences available in GenBank (http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD).

**RESULTS**

**Morphology**

Cysts of four clinical isolates, KA/E2, KA/E12, KA/E15, and KA/E16 had typical morphologic characteristics of morphologic group 2 of Pussard and Pons:5 satellite or polygonal endocyst and wavy ectocyst (Fig. 2). They had almost the same diameter (15.3–16.0 μm) and number of arms (three to seven).

**Drug Sensitivity**

All the clinical isolates were quite sensitive to chlorhexidine and PHMB treatments for 8 and 48 hours but treatment with hexamidine for 8 hours was ineffective. Table 2 provides the findings from the in vitro drug sensitivity tests.

**mtDNA RFLP and Riboprint Patterns**

Three isolates, KA/E2, KA/E12, and KA/E16 showed the identical mtDNA RFLP patterns with *A. lugdunensis* L3a by four kinds of restriction enzymes (Fig. 3). The other isolate, KA/E15, had a phenotype of mtDNA RFLP that differed from those of any other reference strains compared.

The PCR product for 18S rDNA of four clinical isolates was approximately 2300 bp. Figure 4 shows the riboprints of these isolates by the 10 kinds of restriction enzymes. They had riboprints identical with that of *A. lugdunensis* L3a except KA/E15 which had almost the same riboprint as the type strain, L3a. We compared the riboprints of four clinical isolates with those of the reference strains reported by Chung et al.6

A phylogenetic tree (Fig. 5) based on the riboprints shows that KA/E2, KA/E12, and KA/E16 were classified as *A. lugdunensis*, whereas KA/E15 was very closely related to *A. lugdunensis*.

**18S rDNA Sequences**

We compared the 18S rDNA sequence of four isolates with that of several reference strains stored in GenBank and present the percentage differences among the sequences in Table 3. All the
FIGURE 4. Agarose (top two rows) and polyacrylamide (bottom two rows) gel electrophoretic pattern of PCR products of SSU rDNA and their restriction fragment patterns by nine kinds of restriction enzymes from Acanthamoeba four clinical isolates and two reference strains. Lane M: HindIII digested λ-phage DNA or 100-bp ladder as the DNA size standards; lane 1: KA/E2; lane 2: KA/E12; lane 3: KA/E15; lane 4: KA/E16; lane 5: A. lugdunensis L3a; lane 6: A. castellanii Castellani.
clinical isolates analyzed in this study showed less than 0.5% of sequence difference from *A. lugdunensis* L3a. They showed more than 1% differences from the other reference strains compared. The aligned 18S rDNA sequences (~2300 bp) of four isolates and *A. lugdunensis* L3a are presented in Figure 6.

When these sequences were compared with those of other *Acanthamoeba* strains stored in GenBank, they were most closely related to the sequences from *A. lugdunensis* L3a (ATCC 50240) and *Acanthamoeba* sp. Rawdon (ATCC 50479). Based on the sequence homologies (99.9%) with L3a, the type strain of *A. lugdunensis* and the pattern of mtDNA RFLP and riboprint, four isolates were identified as *A. lugdunensis*.

**DISCUSSION**

*Acanthamoeba* keratitis is a relatively uncommon infectious keratitis but is a potentially devastating corneal disease. The first case was recognized in 1973, but the disease remained very rare until the 1980s, when an abrupt increase of the infection occurred in contact lens wearers. *Acanthamoeba* is the genus of small, free-living protozoa that have been isolated from such environmental sources as fresh, marine, and chlorinated water; arctic ice; soil; vegetable matter; dust; and air. Until the present, more than 20 species of *Acanthamoeba* have been detected and classified morphologically. Among them, only six species—*A. castellanii*, *A. polyphaga*, *A. culbertsoni*, *A. batchetti*, *A. rhysodes*, and *A. griffini*—have been identified in ocular infections. Among them, the most common type is *A. castellanii*. The reason that certain species predominantly cause corneal infection is unknown, but it may relate to such intrinsic factors as tissue adhesiveness, growth rates, and possible enzymatic activities.

**TABLE 3. Percentage Difference between the 18S rDNA Sequence for Isolates KA/E2, KA/E12, KA/E15, and KA/E16 and Other Acanthamoeba Strains Representing the Three Morphological Groups**

<table>
<thead>
<tr>
<th>Species and Strain</th>
<th>Species Group</th>
<th>KA/E2</th>
<th>KA/E12</th>
<th>KA/E15</th>
<th>KA/E16</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. astronyxis</em> Ray &amp; Hayes (AF019064)†</td>
<td>I</td>
<td>27.0 (2682:2236)</td>
<td>27.1 (2682:2237)</td>
<td>27.0 (2682:2237)</td>
<td>26.9 (2682:2237)</td>
</tr>
<tr>
<td><em>A. tubiashi</em> OC-15C (AF019065)</td>
<td>I</td>
<td>25.8 (2517:2236)</td>
<td>25.7 (2517:2237)</td>
<td>25.6 (2517:2237)</td>
<td>25.7 (2517:2237)</td>
</tr>
<tr>
<td><em>A. castellanii</em> Castellani (U07413)</td>
<td>II</td>
<td>1.5 (2224:2236)</td>
<td>1.5 (2224:2237)</td>
<td>1.2 (2224:2237)</td>
<td>1.3 (2224:2237)</td>
</tr>
<tr>
<td><em>A. lugdunensis</em> L3a (AF005995)</td>
<td>II</td>
<td>0.2 (2236:2256)</td>
<td>0.5 (2236:2257)</td>
<td>0.4 (2236:2257)</td>
<td>0.1 (2236:2257)</td>
</tr>
<tr>
<td><em>A. castellanii</em> Neff (U07416)</td>
<td>II</td>
<td>2.6 (2256:2236)</td>
<td>2.6 (2256:2237)</td>
<td>2.5 (2256:2237)</td>
<td>2.5 (2256:2237)</td>
</tr>
<tr>
<td><em>A. polyphaga</em> Page (AF019061)</td>
<td>II</td>
<td>3.3 (2253:2236)</td>
<td>3.3 (2253:2237)</td>
<td>3.1 (2253:2237)</td>
<td>3.2 (2253:2237)</td>
</tr>
<tr>
<td><em>A. palestinensis</em> Reich (U07411)</td>
<td>III</td>
<td>8.8 (2243:2236)</td>
<td>8.8 (2243:2237)</td>
<td>8.8 (2243:2237)</td>
<td>8.8 (2243:2237)</td>
</tr>
<tr>
<td><em>A. beatyI</em> OC-3A (AF019070)</td>
<td>III</td>
<td>15.8 (2223:2236)</td>
<td>15.8 (2223:2237)</td>
<td>15.7 (2223:2237)</td>
<td>15.8 (2223:2237)</td>
</tr>
</tbody>
</table>

Numbers in parentheses represent length of 18S rDNA in bp (mean number of nucleotides matched : mean number of nucleotides of 18S rDNA of two strains compared).

† Genbank accession number of the 18s rDNA sequence of the strain.
FIGURE 6. Alignment of the 18S rDNA sequence from *A. lugdunensis* L3a with the clinical isolates KA/E2, KA/E12, KA/E15, and KA/E16. (•) indicates the same base as the bottom sequence and (−, fourth group from the top) is a gap introduced for alignment.
Clinically, the features of keratitis associated with Acanthamoeba infection resemble those observed with herpes simplex or, in some instances, with bacteria or fungi. These similarities often lead to inappropriate medical treatment. Subsequent culture isolation of Acanthamoeba can be rendered more difficult under such circumstances. Delayed diagnosis or misdiagnosis as bacterial or herpes simplex keratitis leads to extensive corneal inflammation and profound visual loss. Therefore, accurate and rapid diagnosis of Acanthamoeba keratitis is essential for successful treatment and good prognosis.

Successful medical treatment is the goal in management of this disease and avoids the requirement for corneal transplantation. It is our experience and that of others that Acanthamoeba keratitis diagnosed at an earlier stage can be more successfully treated medically, without resorting to keratoplasty in the setting of uncontrollable infection. With increased awareness of Acanthamoeba keratitis among clinicians and the availability of rapid diagnostic techniques, this infection is being diagnosed at an earlier stage. Laboratory diagnosis is primarily by culture of epithelial scraping samples inoculated onto NNA-coated plates spread with bacteria. Direct microscopic examination of samples is aided with stains for the cyst wall or immunostaining. A variety of topically applied therapeutic agents are thought to be effective, including propamidine isethionate, clotrimazole, PHMB, and chlorhexidine.

Some investigators found that different Acanthamoeba species and strains differ in susceptibility to other antimicrobials at practically obtainable drug levels. Theses findings normally suggest that species is important for effective treatment and favorable prognosis. Also, given that differences in efficacy were particularly striking against different Acanthamoeba species and substrates, our concern is about the potential importance of species identification and obtaining a successful clinical response.

Although species identification of Acanthamoeba isolates from clinical samples is unlikely to be critical in chemotherapy, genetic characterization is necessary for molecular epidemiology. Because of the pleomorphism of the endocyst and variation of cyst size within even a clone of Acanthamoeba, reproducible and reliable methods should be used for species identification and molecular epidemiology. Alloenzyme and mtDNA RFLP were effective for strain identification, differentiation, or characterization because of the high polymorphism from isolate to isolate but, for the same reason, these methods were not suitable for taxonomy and systematics. Recent studies have shown that the 18S rDNA sequence analysis is useful for the taxonomic and phylogenetic study of Acanthamoeba isolates. However, the generation of 18S rDNA sequence data is too labor intensive and expensive for routine identification and classification of Acanthamoeba, especially when a number of isolates must be examined. Riboprinting (i.e., PCR and RFLP of 18S rDNA), was regarded as a rapid and inexpensive method for identification of unknown Acanthamoeba isolates from clinical and environmental samples. In this study, the same result was obtained from 18S rDNA sequence analysis as from riboprinting. Furthermore, the percentage difference of the sequences among Acanthamoeba strains was nearly the same as that estimated by riboprinting. These results support subsequent classification of Acanthamoeba based on a riboprinting analysis. Nevertheless, it may be necessary to confirm the riboprinting-based identification with 18S rDNA sequence analysis.

Acanthamoeba isolates, of which 18S rDNA sequences were closely related with A. lugdunensis L3a, had already been reported as a causative agent of human amebic keratitis. However, they were designated as A. castellanii or Acanthamoeba sp. This is the first report of amebic keratitis caused by A. lugdunensis in Korea that was identified based on molecular characteristics. A. lugdunensis was the most frequently isolated from contact lens storage cases in Korea. More attention should be paid to hygienic maintenance of contact lens paraphernalia in Korea for prevention of keratitis by A. lugdunensis.

References


