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Restriction Enzyme Analysis of Mitochondrial DNA of *Acanthamoeba* Strains Isolated from Corneal Lesions

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Abstract: Although *Acanthamoeba* keratitis has been recognized as one of the important infectious diseases of the cornea, especially in contact lens wearers in recent years, its taxonomy has not been well established. We carried out mitochondrial DNA (mtDNA) analysis of the *Acanthamoeba* organisms isolated from corneal lesions in four eyes of three patients who had suffered from keratitis. The mtDNA was analyzed by restriction fragment length polymorphism (RFLP) using restriction enzymes *Bgl*II and *Eco*RI. The RFLP analyses revealed that the DNA phenotypes of the *Acanthamoeba* organisms were identical to those of the Ma strain in two patients and to the Castellani strain in one patient. **Jpn J Ophthalmol 1998;42:22–26** © 1998 Japanese Ophthalmological Society

Key Words: *Acanthamoeba* keratitis, contact lens wearer, corneal ulcer, mitochondrial DNA analysis.

Introduction

Acanthamoeba organisms are a genus of free-living protozoan that inhabit soil and aquatic environments ubiquitously. In humans, the organisms are known as the causative agents in two forms of infectious diseases—granulomatous encephalitis and keratitis. *Acanthamoeba* was first identified as the cause of keratitis by Nagington et al[1] in 1974 and by Jones et al[2] in 1975. Although for several years there were few clinical reports on *Acanthamoeba* keratitis,[3–7] the number of reports on this disease has increased markedly since the mid-1980s because of increased recognition.[8–11] Ishibashi et al[12] reported the first clinical case of *Acanthamoeba* keratitis in Japan in 1987. It has been reported that most cases of *Acanthamoeba* keratitis have been associated with contaminated contact lenses.[8–11,13] It is difficult to diagnose *Acanthamoeba* keratitis early in the course of the disease because there are few diagnostic characteristics at that time.[9,14] The visual prognosis of patients not treated adequately in the early stage of the disease was reported to be poor.[15,16]

Although the disease has been diagnosed by isolation of *Acanthamoeba* by culture or by microscopic examination of corneal lesion smears,[17] the identification of species involved in the disease has not been performed in most reported cases.[3,5,7,18,19] The taxonomic classification of the genus *Acanthamoeba* has been based on morphological characteristics of the trophozoites and cysts of the organisms.[20,21] Although morphological classification may clearly define the genus theoretically, variations in the morphology of cysts are commonly found even within cloned strains, making the classification based on the morphology of cysts or trophozoites relatively subjective and arbitrary.[22–24] The mitochondrial DNA (mtDNA) of *Acanthamoeba* is circular and composed of about 42 kilobasepairs on average. When the mtDNA of *Acanthamoeba* is digested by specific restriction enzymes, each strain shows specific and consistent electrophoretic patterns. Therefore, analysis of mtDNA by restriction enzyme digestion can

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show consistent differences between the genera at the molecular level.[18,25–28]

We carried out restriction fragment length polymorphism (RFLP) analyses of the mtDNA of *Acanthamoeba* isolated from the corneal lesions of four eyes of three patients to classify the organism on a molecular basis.

Materials and Methods

Case Reports

Case 1. A 25-year-old man, who had been using daily wear soft contact lenses for 6 years, was referred to the Branch Hospital of the Tokyo University School of Medicine in February 1992 with a 13-week history of pain and foreign body sensation in his right eye. He was initially treated with antibiotics and then with 1% prednisolone acetate eyedrops and acyclovir ointment because herpetic keratitis was presumed by his referring ophthalmologist. However, there was no significant improvement in clinical signs or symptoms. He had disinfected the contact lenses using Hydrocare™ (papain) every day and boiled the lenses 2–5 times a week. On his first visit, the visual acuity was limited to hand movement (n.c.) in the right eye and 0.05 (1.2 × −7.5 D = cyl −1.75 D Ax 175°) in the left. On slit-lamp examination, geographic-form ulcer and severe ciliary injection were observed. A moderate degree of floating cells and aqueous flare were found in the anterior chamber. Gram staining or Giemsa staining of corneal scrapings showed negative results. Cultures of the scrapings on blood, chocolate, or Sabouraud's agar were negative. Indirect immunofluorescence staining using monoclonal antibody to herpes simplex virus was also negative. As *Acanthamoeba* keratitis was presumed, we carried out culture of the corneal scrapings, contact lenses, and their solutions on nonnutrient agar plates covered with suspensions of heat-treated (60°C for 60 minutes) *Escherichia coli* DH1 that did not contain bacterial plasmid.[25] *Acanthamoeba* cysts were identified on the plates used to culture the corneal scrapings and contact lenses. Examination of the cysts using phase-contrast microscopy showed that the characteristics of their morphology were closely related to those of *A. castellanii.*

Case 2. A 27-year-old man was referred to the University of Tokyo Hospital in November 1992 with a 5-week history of foreign body sensation and visual loss in both eyes. He had been treated with antibiotics and 1% prednisolone acetate eyedrops by his referring ophthalmologist. He had worn daily wear soft contact lenses and used a commercial cleaning solution and tap water for rinsing the lenses for 3 years. His contact-lens–cleaning regimen included daily chemical disinfection with Hydrocare™ (papain) and weekly enzymatic treatment with distilled water, but he rinsed his lenses with tap water. On his first examination, the visual acuity was 0.04 (0.2 × −3.5 D = cyl −1.0 D Ax 90°) in the right eye and 0.04 (n.c.) in the left. Slit-lamp examination showed bilateral multiple corneal erosions, severe ciliary injections, and vascular invasions into the corneas. Inflammation in the anterior chambers was minimal. Results of Gram and Giemsa staining, culture for bacteria and fungi, and indirect immunofluorescent staining by monoclonal antibody to herpes simplex virus of the corneal scrapings of both eyes were negative. Culture of the corneal scrapings, contact lenses, and their solutions on nonnutrient agar plates covered with *E. coli* revealed *Acanthamoeba* cysts. The morphological characteristics of the cysts showed a close relationship to *A. hatchetti.*

Case 3. A 19-year-old man had been wearing daily wear soft contact lenses and had used a commercial cleaning solution and tap water for rinsing the lenses. He was referred to the Hospital of the University of Tokyo in August 1993 with a 5-week history of pain and visual loss in the right eye. He had been treated by his referring ophthalmologist with antibiotics and then with acyclovir ointment. His contact-lens–cleaning regimen included a daily chemical disinfection system with Hydrocare™ (papain) and weekly enzymatic treatment with distilled water, but he rinsed his lenses with tap water. On his first visit, the right visual acuity was 0.04 (n.c.), and slit-lamp examination showed a central corneal ulcer and a moderate degree of iritis. Microscopic examination of the corneal scrapings identified *Acanthamoeba* cysts. Culture of the scrapings grew *Acanthamoeba* cysts and trophozoites. The morphology of the cysts showed characteristics closely resembling those of *A. hatchetti.*

Mitochondrial DNA Analyses

The mtDNA of the isolated *Acanthamoeba* organisms obtained from the above three patients was analyzed by RFLP, and the phenotype was determined.[25] About 10 fragments resulting from mtDNA digestion with endonuclease, such as *Bgl*II or *Eco*RI, were separated by agarose gel electrophoresis. In the present study, we analyzed three strains in three cases, which had been digested with *Eco*RI and *Bgl*II.

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Results

The digestion patterns of the isolated *Acanthamoeba* mtDNA are shown in Figures 1 and 2. A mixture of *Sal*I-digested L phase DNA and *Hind*III-digested L phase DNA was used as a size marker (Figure 1, lane C; Figure 2, lane C). The strains of *Acanthamoeba* mtDNA in cases, 1, 2, and 3 were JAC/E8, JAC/L8, and JAC/L9, respectively. The digestion pattern of mtDNA in case 1 using *Bgl*II (Figure 1, lane 1) was very similar to that of the Castellani strain in the keratitis patient with *Bgl*II digestion (Figure 1, lane 2). Although the digestion pattern of mtDNA by *Eco*RI (Figure 1, lane 3) was the same as that of the Castellani strain (Figure 1, lane 4) in the first, third, fourth, and fifth fragments, it was different from that of the Castellani strain in the second, sixth, and seventh fragments. Therefore, the DNA phenotype of JAC/E8, with the morphological classification of *A. castellanii*, was exactly the same as that of the Castellani strain. The digestion pattern of mtDNA in case 2 (JAC/L8) by *Bgl*II, in case 3 (JAC/L9) by *Bgl*II, in case 2 by *Eco*RI, and in case 3 by *Eco*RI are shown in Figure 2 in lanes 7, 8, 9, and 10, respectively. The digestion pattern of the Ma strain in the keratitis patient by *Bgl*II and *Eco*RI is shown in Figure 2, lanes 5 and 6, respectively. The digestion pattern of JAC/L8 and JAC/l9 were identical to that of the Ma strain, and the morphological classification was confirmed as *A. hatchetti.*

Discussion

So far, approximately 400 cases of *Acanthamoeba* keratitis have been diagnosed and reported worldwide. It has been reported that there is a close relationship between *Acanthamoeba* keratitis and contact lens wear.[13] It is estimated that about 85% of patients with *Acanthamoeba* keratitis are contact lens wearers.[11]

In fact, all of the patients we report here had worn soft contact lenses. Although reports of bilateral keratitis, like case 2, have been few,[29,30] it should be noted that ophthalmologists must beware of bilateral

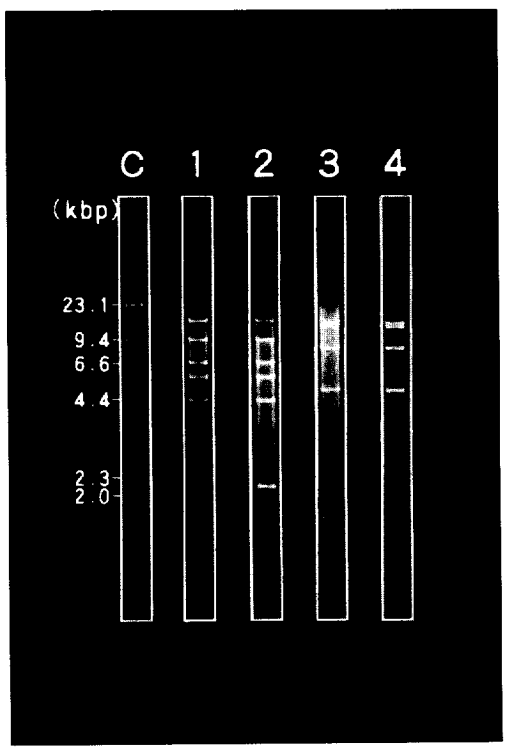

Figure 1. Representative agarose gel electrophoretic patterns for mtDNA digested with *Bgl*II or *Eco*RI. Lane C = size marker; lane 1 = case 1 (JAC/E8)/*Bgl*II; lane 2 = Castellani strain/*Bgl*II; lane 3 = case 1 (JAC/E8)/*Eco*RI; and lane 4 = Castellani strain/*Eco*RI.

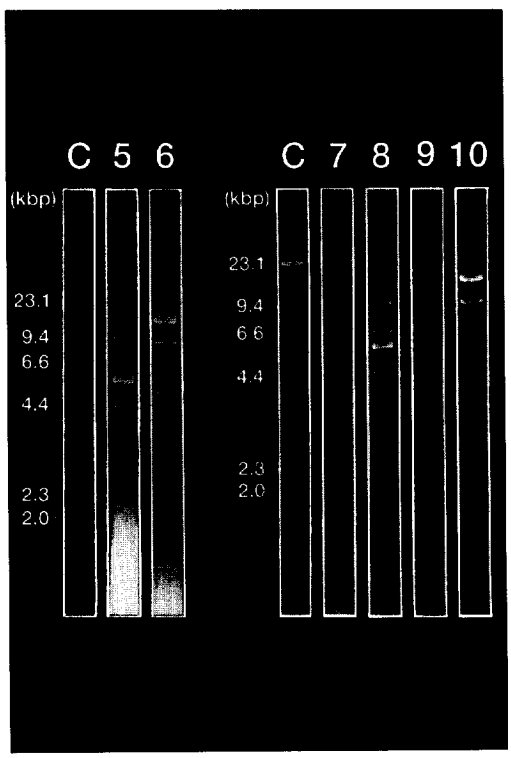

Figure 2. Representative agarose gel electrophoretic patterns for mtDNA digested with *Bgl*II or *Eco*RI. Lane C = size marker; lane 5 = Ma strain/*Bgl*II; lane 6 = Ma strain/*Eco*RI; lane 7 = case 2 (JAC/L8)/*Bgl*II; lane 8 = case 3 (JAC/L9)/*Bgl*II; lane 9 = case 2 (JAC/L8)/*Eco*RI; and lane 10 = case 3 (JAC/L9)/*Eco*RI.

infection because most patients use contact lenses in both eyes.

Diagnosis of *Acanthamoeba* keratitis in the early stage is often difficult because there are few pathognomonic signs then.[9,14] In most cases of *Acanthamoeba* keratitis, diagnoses were made only after failure of treatment for suspected bacterial, viral, or fungal infection or after penetrating keratoplasty or enucleation.[13,15] Delays in diagnosis and treatment may decrease the likelihood of successful medical treatment. Because of confusion with herpetic keratitis, acyclovir ointment and topical steroids have been frequently prescribed for patients at the time *Acanthamoeba* keratitis is diagnosed. All our patients had been treated with topical acyclovir and steroids by referring ophthalmologists. The use of topical steroid therapy in *Acanthamoeba* keratitis is controversial.[10,15] Although it has been reported that clinical improvement was noted after initiation of topical steroid therapy in many cases, deterioration of the keratitis patient's condition was noted in some cases.[15] Steroid treatment may be harmful because it may reduce the host's capacity to eradicate the infection.[31] The most reliable diagnosis of this disease is achieved by detecting *Acanthamoeba* organisms from the involved epithelium or stroma or the cornea. If the patient is a contact lens wearer, the lenses, contact lens cases, and solutions should be submitted to culture mtDNA digestion with endonucleases, such as *Bgl*II or *Eco*RI; this may the best method for taxonomic classification of the genus *Acanthamoeba*. Although the classification of *Acanthamoeba* usually has been based on subjective morphological characteristics of the cyst, there has been considerable disagreement among researchers. This method can determine the taxonomic classification of *Acanthamoeba* in detail, and its specificity for classification is high.

Fourteen phenotypes of *Acanthamoeba* mtDNA isolated from human ocular lesions have been reported so far. Yagita reported that 17.2% of mtDNA phenotypes from *Acanthamoeba* keratitis patients were identical to the pathogenic Ma strain, and 7.4% to the Castellani strain.[28,32] Kilvington[33] studied the relationship between 33 morphologically identical strains from keratitis cases using the restriction endonuclease digestion of *Acanthamoeba* whole-cell DNA, and his findings were similar to Yagita's. The Ma and Castellani strains, therefore, may indicate the types most frequently associated with keratitis because of either a greater prevalence in the environment or increased virulence for corneas. Byers et al[26] and Bogler et al[25] reported a close relationship between the mtDNA of the Ma and the Castellani strains because of their mtDNA digestion patterns. In this study, the mtDNA phenotype of the genus obtained in cases 2 and 3 was identical to that of the Ma strain; in case 1, it was identical to the Castellani strain. Because the two phenotypes obtained in the present experiment have already been demonstrated in human eye infection and isolated in various other countries, we may assume that virulence is associated with specific clusters of *Acanthamoeba*. All the amoeba in our cases were susceptible to fluconazole and miconazole. No significant difference in clinical features or drug sensitivity was noted among the patients.

The determination of the mtDNA phenotypes of *Acanthamoeba* by RFLP enables us to classify the *Acanthamoeba* genus clearly at the molecular level. These results will contribute not only to developing measures for early diagnosis and treatment but also to carrying out epidemiological studies of *Acanthamoeba* keratitis.

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