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Genetic analyses of *Acanthamoeba* isolates from contact lens storage cases of students in Seoul, Korea

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Abstract: We conducted both the small subunit ribosomal DNA (SSU rDNA) polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and mitochondrial (mt) DNA RFLP analyses for a genetic characterization of *Acanthamoeba* isolates from contact lens storage cases of students in Seoul, Korea. Twenty-three strains of *Acanthamoeba* from the American Type Culture Collection and twelve clinical isolates from Korean patients were used as reference strains. Thirty-nine isolates from contact lens storage cases were classified into seven types (KA/LS1, KA/LS2, KA/LS4, KA/LS5, KA/LS7, KA/LS18, KA/LS31). Four types (KA/LS1, KA/LS2, KA/LS5, KA/LS18) including 33 isolates were regarded as *A. castellanii* complex by riboprints. KA/LS1 type was the most predominant (51.3%) in the present survey area, followed by KA/LS2 (20.9%), and KA/LS5 (7.7%) types. Amoebae of KA/LS1 type had the same mtDNA RFLP and riboprint patterns as KA/E2 and KA/E12 strains, clinical isolates from Korean keratitis patients. Amoebae of KA/LS2 type had the identical mtDNA RFLP patterns with *A. castellanii* Ma strain, a corneal isolate from an American patient as amoebae of KA/LS5 type, with KA/E3 and KA/E8 strains from other Korean keratitis patients. Amoebae of KA/LS18 type had identical patterns with JAC/E1, an ocular isolate from a Japanese patient. Three types, which remain unidentified at species level, were not corresponded with any clinical isolate in their mtDNA RFLP and riboprint patterns. Out of 39 isolates analyzed in this study, mtDNA RFLP and riboprint patterns of 33 isolates (84.6%) were identical to already known clinical isolates, and therefore, they may be regarded as potentially keratopathogenic. These results suggest that contact lens wearers in Seoul should pay more attention to hygienic maintenance of contact lens storage cases for the prevention of *Acanthamoeba* keratitis.

Key words: *Acanthamoeba*, contact lens storage cases, genetic characterization, keratopathogens, Seoul

INTRODUCTION

Free-living amoebae of the genus *Acanthamoeba* are ubiquitous in nature and are found in diverse habitats such as soil, water, dust, air-conditioning units, contact lenses and lens cases (Marciano-Cabral et al., 2000). Several species of *Acanthamoeba* leads to serious human diseases, including vision-threatening amoebic keratitis, especially in contact lens wearers, and fatal granulomatous amoebic encephalitis (GAE) in compromised

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patients. Recent increase in the number of *Acanthamoeba* keratitis cases are resulted partly by the increased use of contact lenses (Illingworth and Cook, 1998; Schaumberg et al., 1998). A recent study showed that the contamination rate of contact lens storage cases by *Acanthamoeba* in Korea was 15.1% (Lee et al., 1997a) which the rate is much higher than that reported in the west. Lee et al. (1997a) emphasized the fact that the determination of keratopathogenicity of *Acanthamoeba* isolates from the contaminated contact lens storage cases are urgently needed. However, for the time being, there are no established animal models to evaluate the corneal virulence of *Acanthamoeba* isolates. Therefore, the evaluation of pathogenic potential of *Acanthamoeba* isolates relies on the indirect methods such as genetic characterization and comparisons of the molecular genetic data with clinical isolates from infected corneas (Lee et al., 1997b).

Acanthamoeba can be easily identified at the generic level because of their trophozoites and cysts have distinctive morphology (Chung et al., 1998). However, there have been many disputes concerning the validity of morphologic features alone as a tool for identification of *Acanthamoeba* isolates at the species level. The morphology of cyst can change according to different culturing conditions and is highly variable even in the same strain.

Several non-morphological methods such as alloenzyme analysis, restriction fragment length polymorphism (RFLP) of mitochondrial DNA (mtDNA), RFLP of small subunit ribosomal DNA (ssu rDNA) and sequence analysis of ssu rDNA have been recently applied (De Jonckheere, 1983; Byers et al., 1990; Chung et al., 1998; Stothard et al., 1998). Studies of alloenzyme patterns have suggested other groupings of *Acanthamoeba* strains which are not consistent with that devised by Pussard and Pons (1977). In addition, Kong et al. (1996) and Chung et al. (1996) reported intraspecific heterogeneity of the restriction phenotypes of mtDNA and the zymograms of several alloenzymes of *Acanthamoeba*.

The examination of restriction-enzyme-site polymorphism of ssu rDNA amplified by polymerase chain reaction (PCR) has recently been used to establish taxonomic relationships among *Entamoeba* spp. (Clark and Diamond, 1997), *Naegleria* spp. (De Jonckheere, 1994) and *Acanthamoeba* spp. (Kong and Chung, 1996; Chung et al., 1998). In the present study, the authors used rDNA PCR-RFLP and mtDNA RFLP analyses to identify and characterize the isolates from contact lens storage cases of students in Seoul, Korea, with special references to their keratopathogenic potential.

MATERIALS AND METHODS

Acanthamoeba isolation, cloning and axenization

From contact lens storage cases of students in Seoul, Korea, *Acanthamoeba* were isolated and cloned according to the method described by Lee et al. (1997a). For axenization, a piece of agar plate (0.5×1 cm) covered with the cysts of a clone was treated with 0.1 N HCl for 24 hour and washed three times with glass distilled water. it was then placed in PYG medium (Lee et al., 1997b) at 25°C.

Reference strains

Twenty-three *Acanthamoeba* strains (Table 1) were obtained from American Type Culture Collection (Rockville, MD). Twelve clinical isolates from Korean keratitis patients were also used as reference strains.

Mitochondrial DNA RFLP and ribo-printing

mtDNA of 39 *Acanthamoeba* isolates and reference strains extracted according to the method described by Yagita and Endo (1990) were digested with 1-6 units of restriction enzyme at 37°C for 2 hours, or sometimes overnight, in 20 μl reaction volume with the buffers specified for each restriction enzyme (*EcoR* I, *Bgl* II and *Xba* I, Promega, U.S.A.; *Sst* I, Gibco BRL, U.S.A., and *Sal* I, *Cla* I, *Hpa* I and *Pvu* II, Poscochem, Korea). Digested DNA was electrophoresed in 0.7% agarose gel at 4 v/cm for 1-2 hours. The gel was stained with ethidium bromide for 15 minutes and washed with glass distilled water for 30 minutes. The

Table 1. List of *Acanthamoeba* 35 reference strains

No.	Strain	ATCC No.	Virulence	Environmental source	Geographic source	Reference	Former species designation
1	Castellani	30011	+	yeast culture	England	Douglas (1930)	*A. castellanii*
2	L3a	50240	+	swimming pool	France	Pussard & Pons (1997)	*A. lugdunensis*
3	Vil3	50241	nd[a)]	swimming pool	France	Pussard & Pons (1997)	*A. quina*
4	Jones	30461	+	keratitis	U.S.A.	Jones et al. (1975)	*A. polyphaga*
5	SH621	50254	nd	human feces	France	Pussard & Pons (1997)	*A. triangularis*
6	Nagington	30873	+	keratitis	England	Nagington et al. (1974)	*A. polyphaga*
7	Singh	30973	—	soil	England	Singh (1952)	*A. rhysodes*
8	1652	50253	—	soil	Morocco	Pussard & Pons (1977)	*A. mauritaniensis*
9	AA2	50238	—	soil	France	Pussard & Pons (1977)	*A. divionensis*
10	AA1	50251	—	soil	France	Pussard & Pons (1977)	*A. paradivionensis*
11	Neff	30010	—	soil	U.S.A.	Neff (1957)	*A. castellanii*
12	Ma	50370	+	keratitis	U.S.A.	Ma et al. (1981)	*A. castellanii*
13	P23	30871	—	fresh-water	U.S.A.	Page (1967)	*A. polyphaga*
14	Chang	30898	+	fresh water	U.S.A.	Byers et al.(1990)	*A. castellanii*
15	BH-2	30730	+	ocean sediment	U.S.A.	Sawyer et al.(1977)	*A. hatchetti*
16	RB-F-1	50388	nd	ocean sediment	U.S.A.	Sawyer et al. (1993)	*A. stevensoni*
17	S-7	30731	+	beach-bottom	U.S.A.	Sawyer (1971)	*A. griffini*
18	Ray & Hayes	30137	nd	soil	U.S.A.	Ray & Hayes (1954)	*A. astronyxis*
19	OC-15C	30867	nd	river	U.S.A.	Lewis & Sawyer (1979)	*A. tubiashi*
20	A-1	30171	+	tissue culture	U.S.A.	Singh & Das (1970)	*A. culbertsoni*
21	OC-3A	30866	+	GAE[b)]	U.S.A.	Moura et al. (1992)	*A. healyi*
22	GE-3a	50252	—	swimming pool	France	Pussard & Pons (1977)	*A. pustulosa*
23	Reich	30870	—	soil	Israel	Reich (1933)	*A. palestinensis*
24	KA/E1	—	+	keratitis	Korea	—	—
25	KA/E2	—	+	keratitis	Korea	—	—
26	KA/E3	—	+	keratitis	Korea	—	—
27	KA/E4	—	+	keratitis	Korea	—	—
28	KA/E5	—	+	keratitis	Korea	—	—
29	KA/E6	—	+	keratitis	Korea	—	—
30	KA/E7	—	+	keratitis	Korea	—	—
31	KA/E8	—	+	keratitis	Korea	—	—
32	KA/E9	—	+	keratitis	Korea	—	—
33	KA/E10	—	+	keratitis	Korea	—	—
34	KA/E11	—	+	keratitis	Korea	—	—
35	KA/E12	—	+	keratitis	Korea	—	—

[a)]not determined

[b)]granulomatous amebic encephalitis

RFLP pattern of mtDNA of isolates was observed and photographed under an UV transilluminater. The *Hind* III digested λ phage DNA was used as the size marker.

The chromosomal DNA of each strain was extracted from *Acanthamoeba* trophozoites according to the method described by Kong and Chung (1996). PCR was performed as described by Chung et al. (1998). The PCR products of 39 *Acanthamoeba* isolates were checked by electrophoresis in 1.5% agarose gel at 4 V/cm for 1.5 hours. The amplified ssu rDNA was examined by digestion with eight restriction endonucleases (*Msp* I, *Hae* III, *Hha* I, *Hinf* I, *Dde* I, *Alu* I, *Taq* I, and *Sau* 96 I; Poscochem, Korea) for 2 hr. The digested DNA was electrophoresed in 2.5% agarose gel (agarose 3 : Nusieve 1) for 1.5 hr. Amplisize^{R} (Biorad, USA) was used as a size marker. The gel was stained, washed and photographed as described elsewhere. To differentiate small DNA fragments, which were unclear in agarose gel, digested samples were electrophoretically separated in 15% polyacrylamide gel using TBE buffer.

RESULTS

Mitochondiral DNA restriction phenotypes

Fig. 1 shows agarose-gel electrophoretic patterns of *Eco*R I digested mtDNA from 39 *Acanthamoeba* isolates. A total of 7 different restriction phenotypes emerged for the isolates by 8 kinds of restriction endonucleases (Table 2). The most common type was KA/LS1, 20 isolates out of 39 (51.3%), followed by KA/LS2, 8 (20.9%) and KA/LS5, 3 (7.7%). The other 8 isolates divided into 4 different types (KA/LS4, KA/LS7, KA/LS18 and KA/LS31) evenly. Fig. 2 represents representative patterns by each restriction enzyme.

rDNA PCR-RFLP

The size of PCR products was approximately 2,300 bp. A total of 7 different rDNA PCR-RFLP patterns (KA/LS1, KA/LS2, KA/LS5, KA/LS4, KA/LS18, KA/LS7 and KA/LS31) emerged for 39 isolates from contact lens storage cases of lens wearers in Seoul. Inferred from a distance matrix table (data not shown) based on the proportions of homologous fragments and estimates of genetic divergence

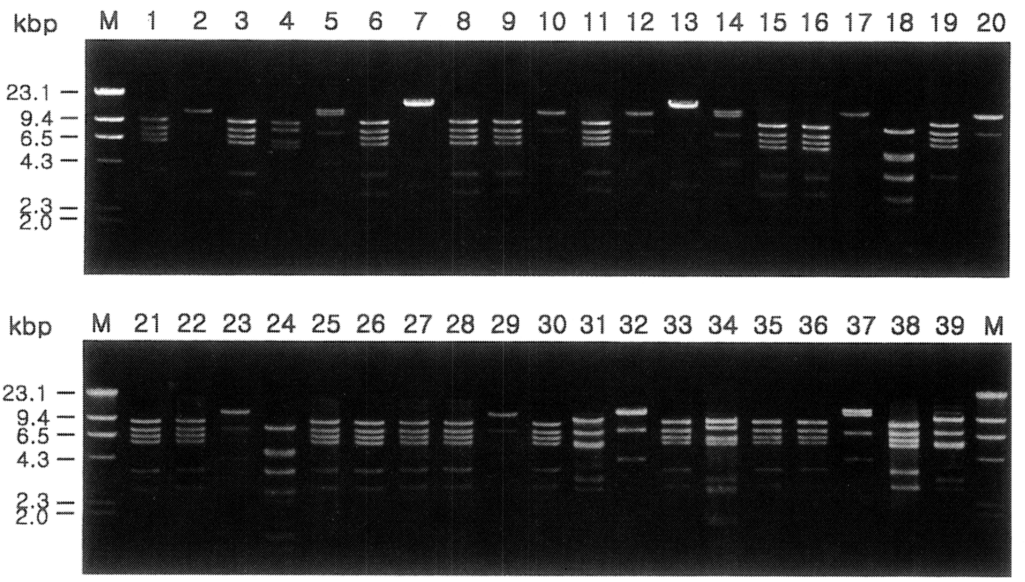

Fig. 1. Agarose gel electrophoretic restriction fragment patterns by *Eco*R I of mitochondrial DNA of *Acanthamoeba* 39 isolates. M, *Hind* III digested λ phage DNA as a DNA size standard.

Table 2. Isolates of *Acanthamoeba* spp. from contact lens paraphernalias and restriction phenotypes of SSU rDNA and mitochondrial DNA

Isolates	rDNA RFLP phenotype	Mt DNA RFLP phenotype	finally assigned species	associated pathogenic strains
KA/LS1	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/LS2	KA/LS2	KA/LS2	*A. castellanii*	Ma
KA/LS3	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/LS4	KA/LS4	KA/LS4	?	—
KA/LS5	KA/LS5	KA/LS5	*A. castellanii*	KA/E3, KA/E8
KA/LS6	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/LS7	KA/LS7	KA/LS7	?	—
KA/LS8	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/LS9	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/LS10	KA/LS2	KA/LS2	*A. castellanii*	Ma
KA/LS11	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/SL12	KA/LS2	KA/LS2	*A. castellanii*	Ma
KA/LS13	KA/LS7	KA/LS7	?	—
KA/LS14	KA/LS5	KA/LS5	*A. castellanii*	KA/E3, KA/E8
KA/LS15	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/LS16	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/LS17	KA/LS2	KA/LS2	*A. castellanii*	Ma
KA/LS18	KA/LS18	KA/LS18	*A. castellanii*	JAC/E1[a)]
KA/LS19	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/LS20	KA/LS2	KA/LS2	*A. castellanii*	Ma
KA/LS21	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/LS22	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/LS23	KA/LS2	KA/LS2	*A. castellanii*	Ma
KA/LS24	KA/LS18	KA/LS18	*A. castellanii*	JAC/E1
KA/LS25	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/LS26	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/LS27	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/LS28	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/LS29	KA/LS2	KA/LS2	*A. castellanii*	Ma
KA/LS30	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/LS31	KA/LS31	KA/LS31	?	—
KA/LS32	KA/LS2	KA/LS2	*A. castellanii*	Ma
KA/LS33	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/LS34	KA/LS4	KA/LS4	?	—
KA/LS35	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/LS36	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/LS37	KA/LS5	KA/LS5	*A. castellanii*	KA/E3, KA/E8
KA/LS38	KA/LS1	KA/LS1	*A. lugdunensis*	KA/E2, KA/E12
KA/LS39	KA/LS31	KA/LS31	?	—

[a)]JAC/E1; pathogenic *Acanthamoeba* isolated from a patient with amebic keratitis in Japan (Yagita and Endo, 1990).

among 7 groups and 23 reference strains, a dendrogram (Fig. 3) was generated by UPGMA (PHYLIP ver 3.5).

On the basis of rDNA PCR-RFLP, 33 out of 39 isolates were assigned to *A. castellanii* species complex, and two isolates to *A. palestinensis*. Remaining four isolates showed peculiar riboprint patterns. Twenty (KA/LS1 type) out of 33 isolates assigned to *A. castellanii* complex showed identical rDNA PCR-RFLP patterns to *A. lugdunensis* L3a strain. Eight isolates (KA/LS2 type) shared

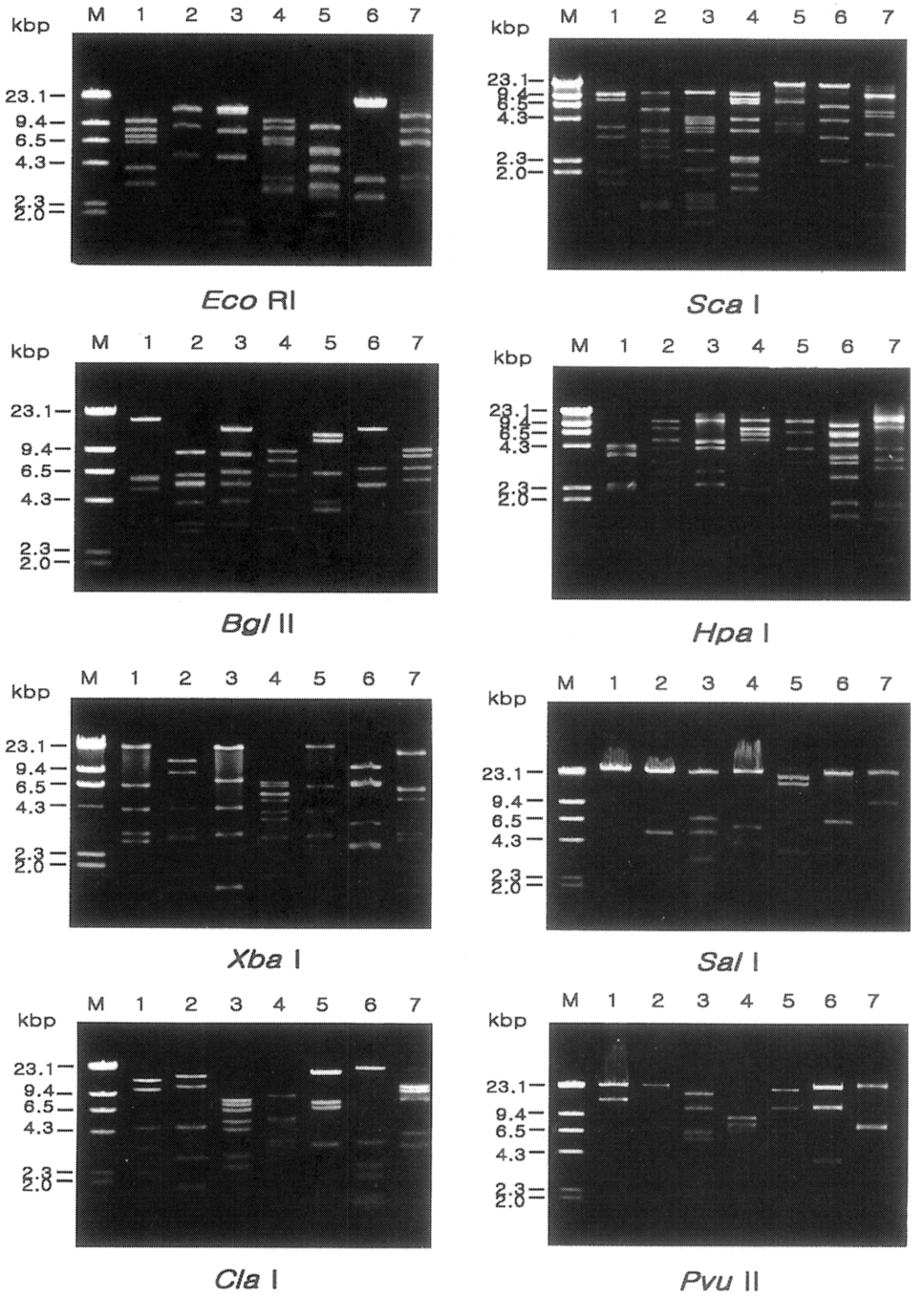

Fig. 2. Agarose gel eletrophoretic restriction fragment patterns of mitochondrial DNA of seven different types of *Acanthamoeba* by eight kinds of restriction enzymes. M, *Hind* III digested λ phage DNA as a DNA size standard. Lane 1, KA/LS1; 2, KA/LS2; 3, KA/LS5; 4, KA/LS4; 5, KA/LS18; 6, KA/LS7; 7, KA/LS31.

identical riboprint pattern with *A. castellanii* Ma strain. Five isolates (KA/LS5 and KA/LS18) revealed very similar riboprint patterns with *A. castellanii* Castellani strain.

DISCUSSION

Ribosomal RNA gene analysis has been used

Table 3. Comparison of mtDNA RFLP type of *Acanthamoeba* isolates from contact lens paraphernalias of different areas of Korea

Type	Seoul	Southeast	Southwest	Total
KA/E2	20 (51.3%)	18 (64.3%)	8 (19.0%)	46 (43.8%)
KA/E3	3 (7.7%)	1 (3.6%)	21 (50.0%)	25 (23.8%)
A. castellanii Ma	8 (20.5%)	0 (0.0%)	9 (21.4%)	17 (16.2%)
Others	8 (20.5%)	9 (32.1%)	4 (9.5%)	17 (16.2%)
Total	39	28	42	105

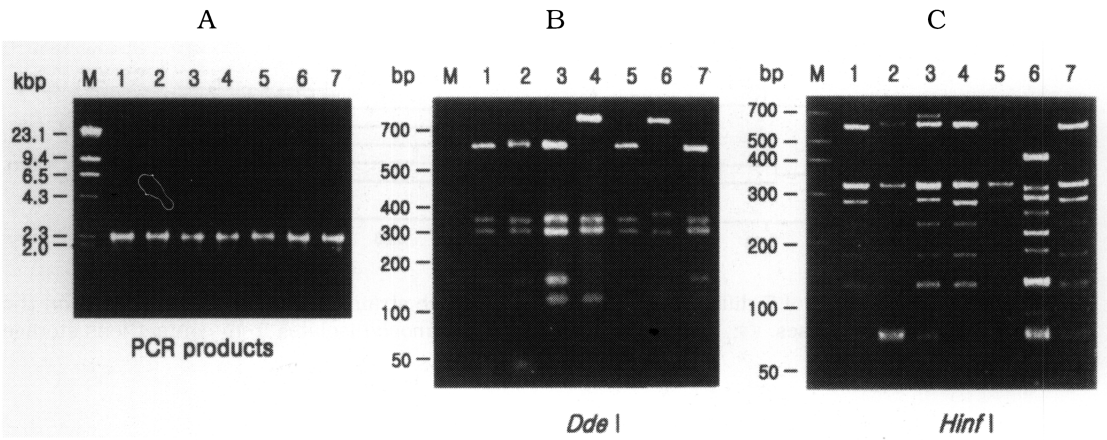

Fig. 3. (A) Agarose gel electrophoretic pattern of PCR products of SSU rDNA of 7 types of *Acanthamoeba* isolates; M, *Hind* III digested λ phage DNA. (B)(C) Electrophoretic patterns of restriction fragments of PCR product from 7 types of *Acanthamoeba* isolates by Dde I and *Hinf* I; B, Agarose gel; C, Polyacrylamide gel; M, 50 bp ladder (BM, Germany); Lane 1, KA/LS1; 2, KA/LS2; 3, KA/LS5; 4, KA/LS4; 5, KA/LS18; 6. KA/LS7; 7, KA/LS31.

in phylogenetic studies of unculturable microbes (Weidner et al., 1996) and for the classification of morphologically indistinguishable protozoa (Clark et al., 1995; Clark and Diamond, 1997; Chung et al., 1998), and it has been generally accepted. The sequence of SSU rDNA is very useful as molecular data for phylogeny and taxonomy; however, the generation of sequence data is labor intensive and expensive, therefore, more than one isolate of a species is rarely analysed. Riboprinting, RFLP analysis of a PCR amplified rRNA gene is simple, inexpensive and fast, and can be used for the classification of *Acanthamoeba* spp. (Chung et al., 1998) especially when a large number of new isolates must be analysed for the survey.

Among the thirty-nine *Acanthamoeba* isolates obtained from contact lens storage cases, 37 isolates belonged exclusively to the morphological group II. The high prevalence of morphological group II *Acanthamoeba* was previously reported by Lee et al. (1997a). Almost all amoebic keratitis have been caused by infection with morphological group II *Acanthamoeba* in Korea (Hahn et al., personal communication). Furthermore, 31 isolates (79.5%) shared the identical mtDNA RFLP patterns (Table 2) with corneal isolates from amoebic keratitis patients; 20 isolates shared the identical mtDNA RFLP with KA/E2 or KA/E12, the corneal isolates from Korean patients, three with KA/E3, other corneal isolate, and eight with Ma strain, the corneal isolates from an American patient.

Lee et al. (1997b) reported that KA/E2 type was the most prevalently isolated from contact lens cases in the Southeast region (Pusan and

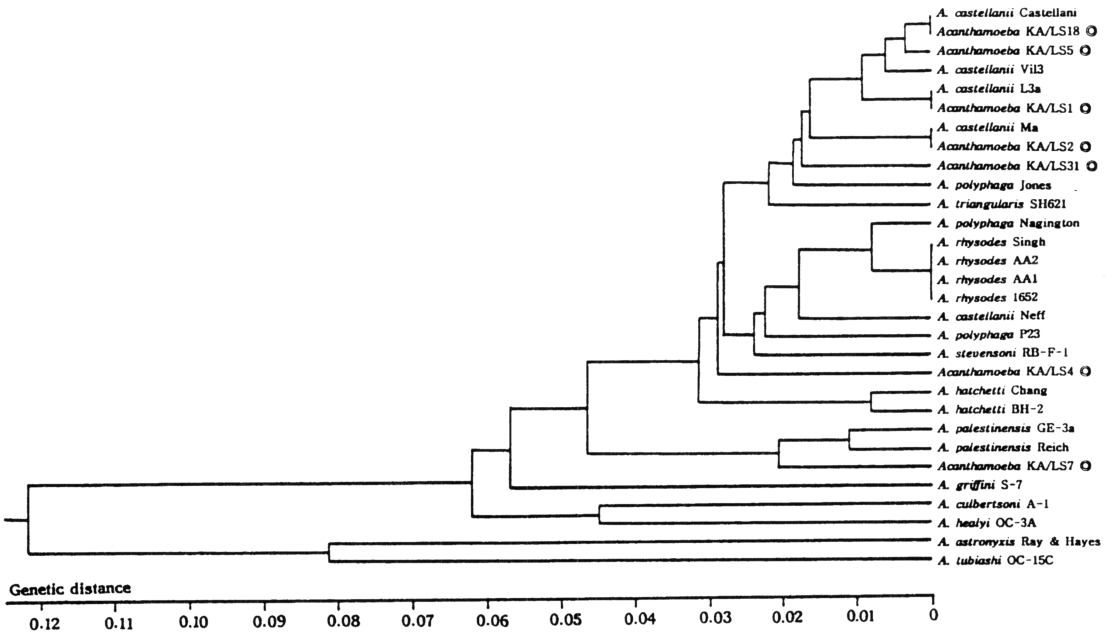

Fig. 4. UPGMA phenogram of 7 different types and 23 reference strains of *Acanthamoeba* based on the SSU rDNA PCR-RFLP analyses. ◎; 7 different types of *Acanthamoeba* isolates from contact lens storage cases.

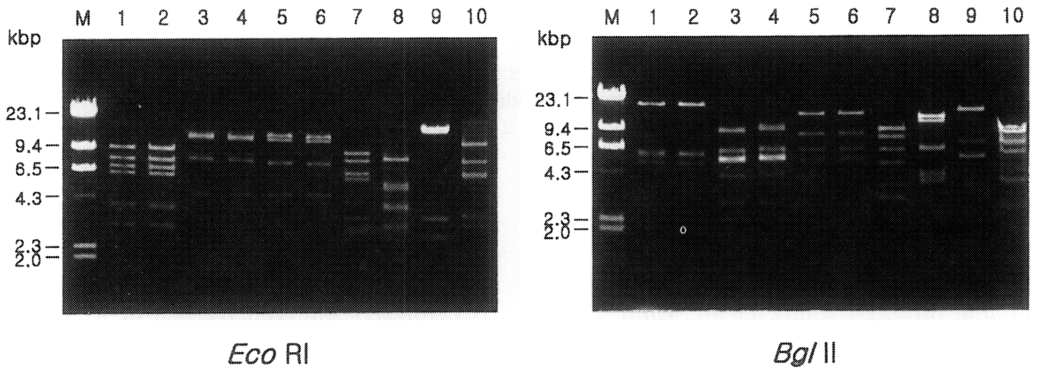

Fig. 5. Comparative mitochondrial DNA RFLP patterns of *Acanthamoeba* isolated from contact lens storage cases with those of reference strains from keratitis. Lane 1, KA/LS1; 2, KA/E2; 3, KA/LS2; 4, Ma; 5, KA/LS5; 6, KA/E3; 7, KA/LS4; 8, KA/LS18; 9, KA/LS7; 10, KA/LS31; M, *Hind* III digested λ phage DNA.

Taegu) in Korea. The results of the present survey were similar: KA/E2 type was the most prevalent (Table 3). However, Ma type, which had not been isolated from the survey of the Southeast regions, was isolated at a rate of 20.5% in this survey. Ma type was the most prevalently isolated type (50.0%) from lens storage cases in the Southwest regions (Kwangju and Chonju) in Korea (unpublished data). Therefore, the results in this survey should be intermediate between those in the Southeast and Southwest regions in Korea.

These results showed that the riboprints and mtDNA RFLP patterns of great majority of *Acanthamoeba* isolates from the lens cases of Korean lens wearers were the same as those of

the clinical isolates which could probably be regarded as keratopathogens. More attention should be paid to the prevention of *Acanthamoeba* contamination and to the disinfection of contact lens storage cases.

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