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Seasonal Fluctuation of *Acanthamoeba* spp. Contamination in Water Containers Placed Indoors and Outdoors

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The medical importance of free living amebas has been increasing as a worldwide problem. In Japan amebic keratitis caused by *Acanthamoeba* has caught the attention of ophthalmologists due to increasing risk of infection for soft-contact lens users. In the present work we examined the process of *Acanthamoeba* contamination in water placed indoors and outdoors under different conditions in Nagasaki city, Japan. The most frequently contaminated water was in coverless outdoor containers in the spring, and the next was in similar conditions, but in the autumn. The highest contamination of indoor water in coverless containers was found in the summer although the frequency was much lower than outdoors. When the containers were covered, complete protection for water bodies from *Acanthamoeba* contamination was obtained indoors, and nearly complete outdoors. Electrophoretic patterns of mitocondrial DNA fragments digested by two restriction enzymes indicated that *Acanthamoeba* contaminants seemed to be season-specific although the number of examined isolates was very small.

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Introduction

Recently the medical importance of free living amebas has attracted the attention of clinicians as well as parasitologists. The first reason for this is their propensity to cause accidental, and potentially, serious infections in humans. For example, the genus *Acanthamoeba* causes chronic granulomatous amebic encephalitis and amebic keratitis, and *Naegleria fowleri* causes primary amebic meningoencephalitis. *Balamuthia mandrillaris* can also cause granulomatous amebic encephalitis. Amebic meningoencephalitis in humans was first reported from Australia and subsequently from the United States;[1,2] both cases were determined later to be *Naegleria fowleri* infections. Acanthamoeba had been reported as a potential cytopathic organism in 1957 and 1958,[3,4] but the first two definitive human cases of *Acanthamoeba*-associated meningoencephalitis were reported by Kenney in 1971,[5] and the first cases of amebic keratitis were reported from England and from the United States in 1974 and 1975, respectively.[6,7] *Balamuthia mandrillaris*-associated encephalitis cases were added at a later date.[8]

The second reason is their role as a vehicle to disseminate pathogenic bacteria such as *Legionella* and *Mycobacterium* spp.,[9-12] that is, the four ameboid genera *Acanthamoeba*, *Hartmannella*, *Naegleria* and *Dictyostelium* were found to support growth of those bacteria, and recently *Balamuthia mandrillaris* has also been found to support it.[12] The bacteria inside an amebic cyst can more easily survive unfavorable conditions.

In Japan, since the first report of *Acanthamoeba*-associated keratitis by Ishibashi et al. in 1988,[13] the number of keratitis cases has increased with an increase of soft-contact lens users, while only a few cases of amebic encephalitis have been reported. These free-living amebae inhabit various water bodies and wet soils as dividing trophozoites, eating microorganisms there. When environmental conditions become unfavorable for them, they alter their shape into cyst-form at resting stage that can survive dry and cold conditions. Cysts are widely distributed, probably with dust in the air. Hence, free-living amebas can grow in any water-containing receptacle. Some of them are assumed to be infectious if they are transported into human eye or nasal cavity.

We have been interested in the manner by which our surrounding environments are contaminated by these amebas, especially by

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those of the *Acanthamoeba* species. In Japan we have four seasons and the meteorological conditions greatly vary among seasons. This changing of seasons may produce different growing environments for free-living amebas and, by the changing wind direction, may affect the sources contributing to the contamination. We conducted the present study to elucidate these points.

Materials and Methods

Test locations

The examination of amebic contamination was done at the Institute of Tropical Medicine, Nagasaki University, Nagasaki city, Japan. Nagasaki city is located in the western edge of Japan at latitude 32°40'N. The average temperature is 17°C with yearly fluctuation between 0°C and 35°C. The rain precipitation is maximum in June with an average precipitation of 360 mm and minimum in December of 57 mm. Water trays were placed on a stand (50 cm high) under the eaves outside the building and on a stand (180 cm high) in a hallway inside the same building.

Meteorological data

The meteorological data for the test period were obtained from the Nagasaki meteorological observatory after the test.

Test periods

The water trays were placed for three weeks from 31 July to 21 August in the summer of 2000, from 3 to 24 November in the autumn of 2000, from 1 to 22 February in the winter of 2001 and from 3 to 24 May in the spring of 2001.

Water containers

A rectangular glass tray with the upper open space of 10.0 cm × 16.7 cm and with the bottom space of 7.3 cm × 14.0 cm and 5.3 cm in depth was used with or without a cover. The trays were washed well, autoclaved and filled with 300 mL of fresh or autoclaved running water, and black lines were marked by oily pens on their sidewalls at the level of water surface. Twelve trays were placed at each location; six trays with covers and six trays without covers. For each set of six trays, three trays contained fresh running water and three contained autoclaved water.

Determination of amebic contamination

Every third day after the beginning of the test, the water in each tray was gently stirred and a 10 mL water sample was collected into a 15 mL plastic graduated centrifuge tube. The trays were refilled with sterilized distilled water to the marked points again, and put back to the same location. The tubes were centrifuged at 3,000 rpm for 10 min, and supernatent water was removed leaving 0.5 mL of bottom water. The tubes were subjected to the vortex, and a drop of suspension was put on a glass slide and observed under microscopy. One hundred micro-liter of suspension was used for ameba culture on an agar plate in a plastic dish. After incubation for a week ameba growth was examined under inverted microscope. Species determination was done according to morphological characteristics, i. e., trophozoites of *Acanthamoeba* have thin, spiny surface extensions termed acanthopodia, and cysts have a double wall structure.

Culture and isolation of ameba

The culture and isolation method described by Endo and Yagita[14] was used. Briefly, 20 mL of autoclaved agar solution (1.5% agar in PBS, pH 7.0) was poured into a plastic laboratory dish (90 mm in diameter and 15 mm in depth) to make an agar plate. At the center of the agar plate, a small hole was made and 100 μL of the suspension described above was added to it. Then, 0.5 mL of heat-killed *E. coli* suspension was poured on the plate. *E. coli* (DH5a strain) was cultured in a LB medium (Bacto tryptone 10 g, Bact yeast 5 g, NaCl 10 g in 1,000 mL distilled water). After 24 hours of incubation at 37°C in a double shaker, the flask was incubated in a water bath at 60°C for an hour to kill bacteria, and kept in a refrigerator until used. After addition of *E. coli* suspension, the cover of the plastic dish was sealed with Scotch tape and incubated at 26°C for a week. The dishes positive for *Acanthamoeba* were kept at room temperature until used. For isolation of ameba the ameba positive dishes were again incubated at 26°C after adding 0.5 mL of *E. coli* suspension. Grown amebas were subjected to the hunger condition to be transformed into cystic forms. Cysts were collected and suspended in PBS (pH 7.0) at proper dilution for isolation of a single cyst. A drop of suspension was placed on a piece of autoclaved cellophane (5 mm × 5 mm) and observed under vertical microscope. If only one cyst was contained in a drop, it was transferred into a culture dish. If ameba growth was observed, they were regarded as a clonal population. Next, cloned amebas were again transformed into cysts, and cysts were treated with 15 times dilution of 7% povidone iodine for 10 min followed by 0.1 N HCl for 12 h at room temperature to kill coexistent bacteria, washed in PBS and finally cultured in a flask containing 10 mL of PYGC medium supplemented with antibiotics and occasionally with 5% fetal bovine serum. When the bottom of the flask was almost completely covered with amebas, the flask was cooled on ice and was shaken rapidly to release amebae. They were collected in a 15 mL centrifuge tube, and subjected to the following analysis of mitochondrial DNAs.[14,15] As the reference strain the isolate from a Japanese keratitis case (JAC/E1) was used.[15]

Analysis of mitochondrial DNAs

A total of about 5×10^6 amebas in the centrifuge tube were washed with cold (kept in ice-water) PBS(-)three times by centrifugation at 1,500-2,000 rpm for 5 min at 4°C and finally suspended in 2 mL of PBS(-). Each 1 mL of suspension was transferred into a 1.5 mL

microtube. The tubes were centrifuged at 5,000 rpm for 1 min, and the supernatent was removed, as much as possible, to leave only precipitate. Onto the precipitate, 100 μL of TEG (50 mM Tris.HCl Buffer, pH 8.0, 0.5 mM glucose, 10 mM EDTA Na) was added, and the amebas were suspended by pumping up and out with the micro-pipette. The suspension was gently mixed with 200 μL of alkaline SDS (0.15 M NaOH, 1% SDS), and was placed on ice. After 5 min the mixture was neutralized with 150 μL of 3 M acetate potassium buffer (pH 5.6), returned to the ice for 30 min and then centrifuged at 15,000 rpm for 5 min at 4°C. About 450 μL of supernatent was transferred into a new microtube, mixed with the same volume of PCI solution (phenol 25 : chloroform 24 : isoamylalcohol 1), shaken strongly, and then centrifuged at 15,000 rpm for 5 min. The upper water layer (about 400 μL) was collected in a new microtube, mixed with 40 μL of 3 M sodium acetate buffer (pH 5.24) and 1 mL of 99.5% ethanol and kept at -80°C in a deep-freezer for 30 min. The tube was taken out and kept on ice until the content melted, and then centrifuged at 15,000 rpm for 5 min. On the bottom of the tube, a small white precipitate (mitochondrial DNA) was found. The precipitate was washed with 70% ethanol and centrifuged 5000 rpm for 10 sec, and then dried after removing alcohol. Finally 50 μL of TE was added in the tube and kept at -20°C until used.

Electrophoretic analysis

(1) Confirmation of extracted mitochondrial DNAs

DNA samples prepared by the above method were subjected to agar-gel elecrophoresis. Thereafter, if some bands with about 42 kb were found, extraction of mitochondrial DNAs was confirmed to be successful.

(2) Digestion of mitochondrial DNAs by restriction enzyme

The mitochondrial DNA samples confirmed above were subjected to DNA restricted enzyme digestion by *Eco* RI and *Bgl* II (Takara Bio Co., Kyoto, Japan). The digestive patterns were compared to the reference strain of *Acanthamoeba casteranii*-like clone derived from a Japanese keratitis patient after agar-gel electrophoresis.[15]

Results

Detection of water contamination with Acanthamoeba spp. in containers

We examined directly centrifuged water samples under light microscope and culture samples under inverted phase-contrast microscope. If *Acanthamoeba* trophozoites or cysts were detected in either examination, water was determined to be contaminated. In Figure 1 the number of contaminated containers with *Acanthamoeba* out of

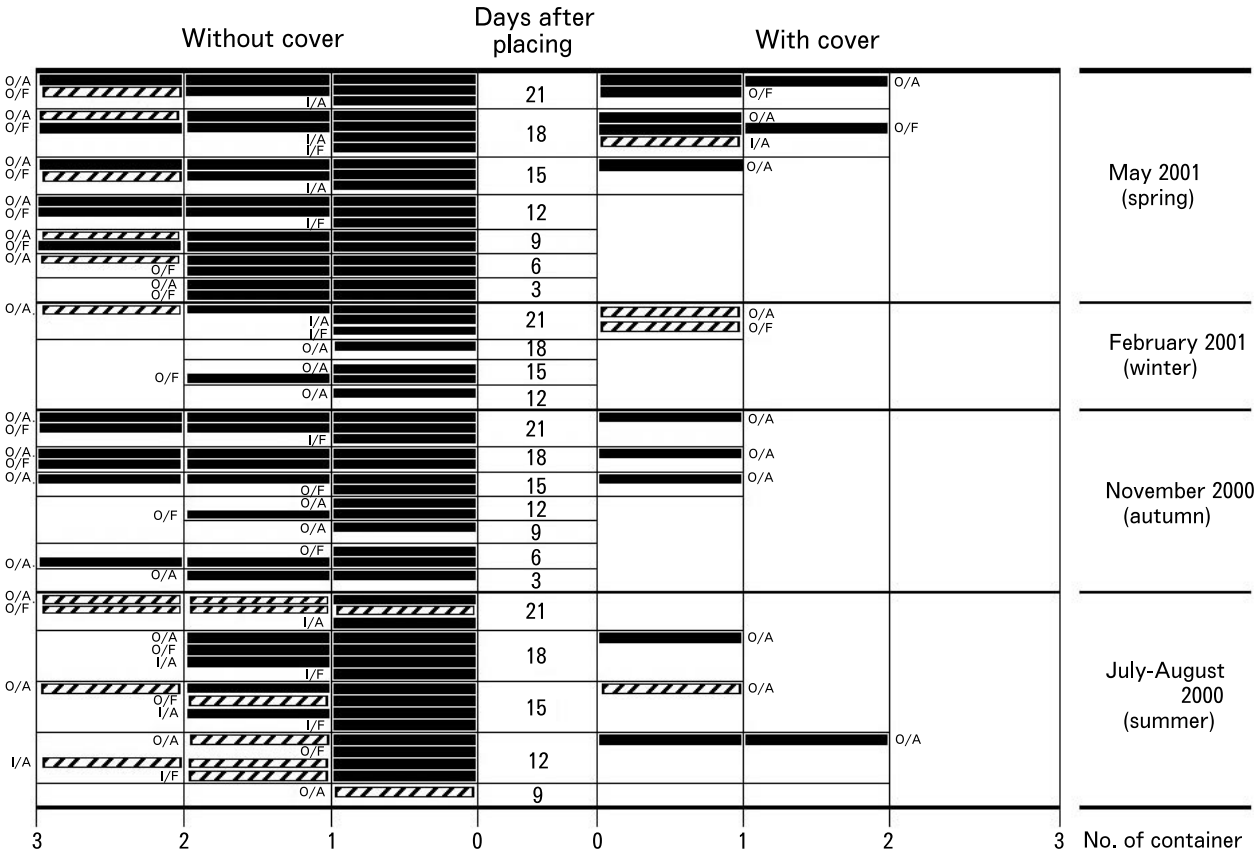

Figure 1. Seasonal fluctuation of amebic contamination of containers' water at various conditions. O/A: outdoors/autoclaved running water; O/F: outdoors/fresh running water; I/A: indoors/autoclaved running water; I/F: indoors/fresh running water. ■ : *Acanthamoeba* contamination; ▨ : Only other protozoan contamination.

three containers placed under each of different conditions were shown every third day for three weeks after placing them. When the containers with covers were placed indoors, water was stayed clear in appearance throughout the experimental period and there was no contamination with amebas although one container was contaminated with some small shapes of protozoas. Initially we were afraid that original running water might contain amebas but the result showed that running water is rather more resistant to amebic contamination than autoclaved water, maybe due to remaining chlorine. When we placed containers with covers outdoors, rain water permeated into containers through small spaces under covers. We also found dust, sometimes ants, and other small insects, but only in the summer. Amebic contamination rarely occurred in outdoor containers with covers, that is, only three samples of running water in the spring, and three samples of autoclaved water in the summer and the autumn and four samples of autoclaved water in the spring were found to be contaminated. In containers without covers, much more contamination was found outdoors than indoors. Although indoor water containers were much less frequently contaminated with *Acanthamoeba* than outdoor containers, they were contaminated the most often in the summer followed by the spring. Besides they were contaminated with many fungus colonies in the summer and with dusts floating in all seasons (data, not shown). Outdoor water containers were easily contaminated; the most often in the spring followed by autumn, summer and winter in this order. In the summer, water quality became dirtiest by contamination with various dust, insects, mosquito larvae and small organisms including protozoans.

Relationship between Acanthamoeba contamination and meteorological data

It is quite natural that only a small number of samples in winter showed *Acanthamoeba* positive because the temperature controlling the growth of microorganisms was low. However, this finding of *Acanthamoeba* contamination suggests that amebas can grow below 10°C (Figure 2). Temperatures from 15°C to 23°C in the spring and from 10°C to 20°C in the autumn seemed to be more favorable than temperatures from 25°C to 30°C in the summer and from 4°C to 14°C in the winter. Unexpectedly there was little difference in the humidity between different seasons. This indicated the humidity was not related to the condition of contamination. Surprisingly, four samples out of six in spring and two in autumn were contaminated with *Acanthamoeba* only three days after starting the test.

Isolation of Acanthamoeba clones

We tried to isolate clones from samples in outdoor containers without covers. We succeeded in establishing four clones from summer

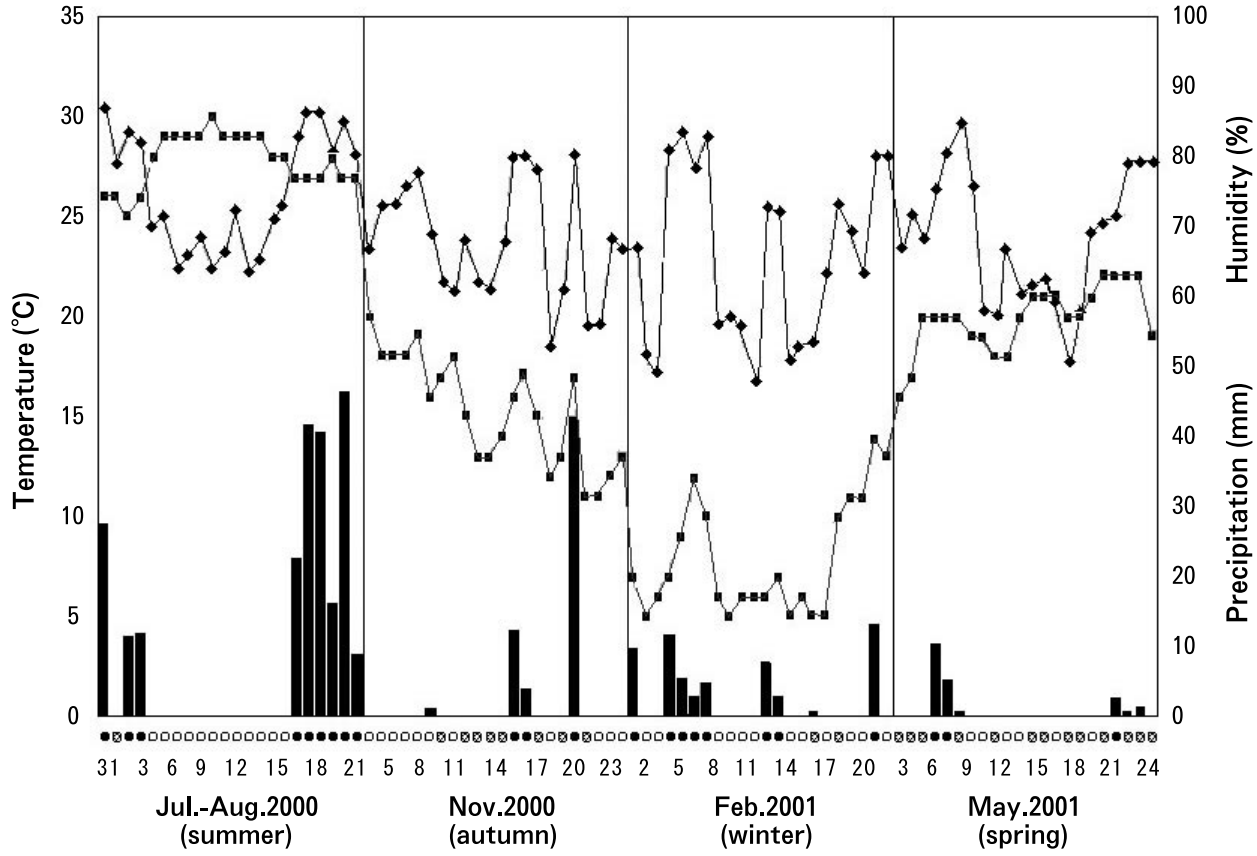

Figure 2. Meteorological data in Nagasaki city during the study periods. —■— : Temperature; —◆— : Humidity; ■: Precipitation; ○: Fine; ◎: Cloudy; ●: Rainy.

samples, six from winter samples and two from spring. Regrettably no clones were isolated from the autumn samples and only two from the spring samples, even though more positive samples were obtained in those two seasons.

Electrophoretic patterns of mitochondria DNA digested by restriction enzyme Eco RI and Bgl II

Three electrophoretic patterns of digestive fragments were obtained by both restriction enzymes and they were different from the reference strain from a Japanese keratitis patient (*Acanthamoeba casterani* type) (Table 1 and Figure 3). The four clones obtained from summer samples showed the same electrophoretic patterns of DNA fragments digested both by *Eco* RI and by *Bgl* II. The other clones from winter samples and spring samples also showed the same tendency of the season-specific character. Therefore, the base pair size of each fragment was expressed as the average of base pair sizes of corresponding fragments of all isolated clones in each season. All the isolated clones from one season belong to the one pattern, and the patterns in different seasons mutually differed. This indicates that a very limited number of clones may be carried in dust and contaminate water in a certain period of a season.

Table 1. Fragment-size (kb)[a] estimates for four digestion phenotypes

Endonuclease fragments		Digestion phenotypeb			
		JAC/E1	Summer isolates	Winter isolates	Spring isolates
Eco RI	1	13.9±1.0[c]	10.0±0.7	12.6±1.4	14.6±1.4
	2	12.2±0.4	8.2±0.5	8.9±0.6	11.3±0.8
	3	7.9±0.2	7.3±0.5	6.0±0.4	5.7±0.3
	4	5.0±0.2	6.6±0.4	5.3±0.4	3.6±0.2
	5	1.8±0.1	4.3±0.3	3.4±0.2	
	6		3.4±0.2	2.8±0.1	
Bgl II	1	13.5±1.1	19.0±2.1	9.9±1.0	13.9±1.3
	2	8.6±0.4	6.3±0.3	8.3±0.7	8.9±0.4
	3	6.5±0.3	5.5±0.2	6.5±0.4	7.8±0.3
	4	5.4±0.2		4.0±0.2	5.0±0.3
	5	4.4±0.2			4.0±0.3
	6	3.3±0.2			

[a]All the isolates in each season showed the same electrophoretic patterns of digested fragments with the two endonucleases, and therefore each fragment size expressed by kb is the average of base pair size of corresponding fragments.
[b]JAC/E1 is the isolate from a Japanese keratitis patient similar to *Acanthamoeba casteranii.* Each isolate was subjected to electrophoretic analyzation more than two times. The reference strain (JAC/E1) was always used for electrophoresis.
[c]Mean±standard deviation.

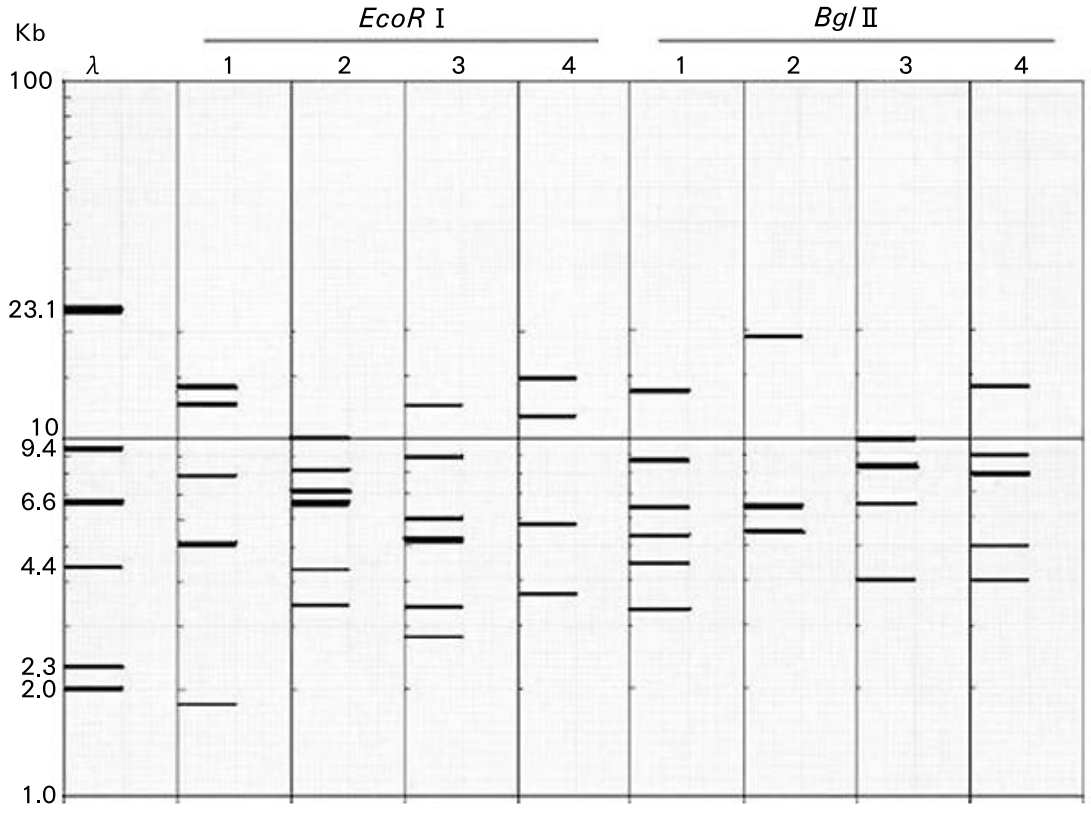

Figure 3. Illustration of 4 electrophoretic patterns of *Acanthamoeba* isolates. Mitochondrial DNA digest of the reference clone JAC/E (lane 1), that of the summer group (lane 2), the winter group (lane 3) and the spring group (lane 4). The size marker (λ) is λ-Hind III digest (Promega Co., Madison, WI).

Discussion

Small free-living amebas belonging to the genera *Acanthamoeba*, *Naegleria* and *Balamuthia* have been increasing their importance in the medical field as accidental agents inducing encephalitis and keratitis and also as vehicles of some pathogenic bacteriae.[1-13] In general, pathogenic species have tendency to survive at temperature over 40°C, especially *Naegleria fowleri* was found mostly in thermally polluted water.[16] *Acanthamoeba* species are commonly found in a variety of habitats such as wet soil and stagnant water and sometimes from nasopharyngeal region of healthy individuals although only limited species are infective to humans.[17] Since amebic keratitis cases have increased with an increase in soft-contact lens users, we examined the frequency of water contamination with *Acanthamoeba* spp. in containers placed at different locations, under different conditions and in different seasons. First, we found that fresh running water was contaminated slightly less frequently than autoclaved running water. This indicates that running water is free from amebas and inhibits growth of microorganisms for ameba food for a while. A cover on water container completely protected water from *Acanthamoeba* contamination indoors and less completely outdoors. The highest contamination occurred in water of the containers placed outdoor without covers. These results revealed that *Acanthamoeba* contamination of contact-lens medium in container should be a very rare event indoors even though the container is left without cap. Of the four seasons, spring and autumn showed higher frequency of contamination in this order. These two seasons had moderate temperature and relatively low humidity. Empirically we know the air in spring is somewhat dustier than other seasons. This may be concerned to the highest contamination in spring. Moderate temperature seems to be more suitable for *Acanthamoeba* growth outdoors, because rapid contamination within 3 days occurred in water without cover outdoors in spring and autumn. High temperature over 25°C and high humidity may accelerate the growth of various organisms but seems to be unsuitable for the growth of *Acanthamoeba*. In the summer other smaller protozoan organisms including a smaller species of ameba, bacteriae, fungi and even mosquito larvae grew better, and this may cause the growth competition among microorganisms and other organisms, which may result in inhibition of the growth of *Acanthamoeba*. A large growth of other microorganisms inhibits *Acanthamoeba* growth in vitro, too.

Interestingly, all the clones isolated from samples in one season showed the same electrophoretic pattern of mitochondrial DNA fragments digested with two restriction enzymes, and the pattern in each season mutually differed. Although the number of clones isolated is very small, it is supposed that only a limited number of *Acanthamoeba* clones were carried with dust in a short period of the season. The three patterns of mitochondrial fragments were compared with those of isolates from Japanese keratitis patients,[15] and were found to be different from those isolates. This indicates that real pathogenic species of *Acanthamoeba* might be seldom carried although we found rapid and frequent contamination with *Acanthamoeba* spp. in the spring and the autumn. Of course, we cannot conclude our isolates are nonpathogenic because we did not examine their virulence experimentally. Since our results revealed that in a short period of the season only a few clones of *Acanthamoeba* are carried in dusts, it is possible to expect that pathogenic *Acanthoamoeba* clone would spread widely in a short period. On the other hand, our results revealed the extremely rare chance of contamination when water is kept in container with cover indoors.

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