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Mitochondrial DNA Fingerprinting of *Acanthamoeba* spp. Isolated from Clinical and Environmental Sources

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Restriction fragment length polymorphism analysis of mitochondrial DNA (mtDNA fingerprinting) was evaluated as an epidemiologic tool for identifying potential reservoirs of *Acanthamoeba* infection. Fingerprints for 15 clinical isolates recovered by our affiliated laboratories were compared with those for 25 environmental isolates from western Washington State and 10 American Type Culture Collection (ATCC) strains. Seven different fingerprint groups emerged from the analysis of clinical isolates with six selected restriction enzymes (*Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Kpn*I, and *Sal*I). Fourteen (56%) environmental and 4 (40%) ATCC isolates displayed fingerprints similar to those of clinical isolates. In all, five of the seven groups contained one or more environmental and/or ATCC isolates. Comparisons with published mtDNA fingerprints for *Acanthamoeba* isolates showed that two groups have counterparts in Europe and Japan and in Europe and Australia. The inclusion of environmental isolates demonstrated that the most common clinical isolates do have counterparts readily recoverable from the surrounding environment and that some of these counterparts appear to be geographically widespread.

Acanthamoeba keratitis is a painful disease characterized by a recalcitrant corneal ulceration and seen most commonly in wearers of soft contact lenses. Transmission is presumed to occur through contamination of contact lenses and lens cases with cysts and/or trophozoites originating from environmental sources (1, 12, 13, 15). While methods used for the recovery and generic identification of *Acanthamoeba* spp. have become standard, identification to the species level is more problematic and reflects uncertainty as to the validity of the 18 or more described species (11, 13, 14). Species identification is essential, however, for epidemiologic studies.

The comparison of restriction endonuclease digest fragment patterns (fingerprints) of mitochondrial DNA (mtDNA) has been examined as an aid in taxonomy (3–5); for its potential in characterizing pathogenic and nonpathogenic *Acanthamoeba* spp. (8, 10, 16); and as a method for tracing possible sources of infection (9). Of the epidemiologic studies conducted to date on *Acanthamoeba* spp., few have included environmental isolates originating from the same geographic area in which clinical cases have occurred. We subsequently undertook an investigation to determine the usefulness of mtDNA fingerprinting as an epidemiologic tool for identifying potential reservoirs of infection.

Isolates studied. The *Acanthamoeba* isolates analyzed included 15 from clinical sources (13 from corneal specimens and 1 each from a contact lens and a contact lens case) in the Seattle, Wash., area; 25 from local environmental substrates (soil, forest detritus, lake and stream sediments, pond water, tree bark, and potting soil) which were unrelated to the affected patients; and 10 from the American Type Culture Collection (ATCC), including *Acanthamoeba culbertsoni* ATCC 30866, *Acanthamoeba castellani* ATCC 30011, *A. castellani* ATCC 30868, *Acanthamoeba rhysodes* ATCC 30973, *Acanthamoeba polyphaga* ATCC 30871, *A. polyphaga* ATCC 30461, *Acanthamoeba astronyxis* ATCC 30137, *Acanthamoeba hatchetti* ATCC 30730, *Acanthamoeba palestinensis* ATCC 30870, and *Acanthamoeba* sp. strain ATCC 30173. Clonal cultures of amoebae, including all ATCC isolates, were

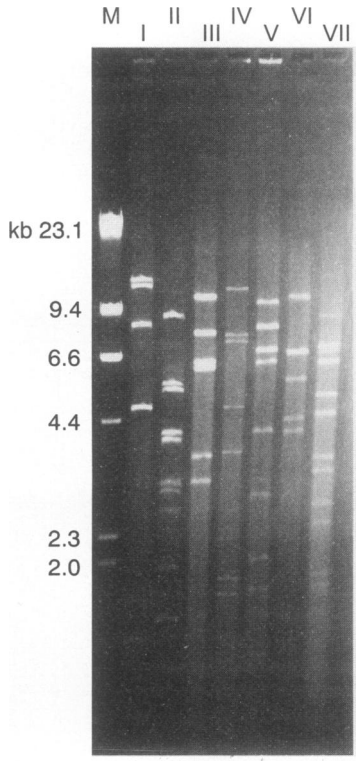

FIG. 1. Agarose gel electrophoresis of *Eco*RI digests of *Acanthamoeba* mtDNA. Lanes I thru VII represent AcUW groups. Lane M is a *Hin*dIII-digested lambda molecular size standard.

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TABLE 1. Estimates of *Acanthamoeba* mtDNA restriction fragment sizes for six endonucleases

AcUW group	Fragment size (kb) for the following restriction endonuclease:					
	*Bam*HI	*Bgl*II	*Eco*RI	*Hin*dIII	*Kpn*I	*Sal*I
I	>23.00	11.92, 8.32, 6.55, 5.50, 4.28, 3.07	12.37, 11.49, 8.22, 4.96, 1.48, 1.20	6.84, 5.72, 5.32, 4.12, 3.15, 2.32, 1.90, 1.57, 1.31, 1.20, 0.89, 0.80	>23.00, 11.49	>23.00, 7.02, 5.47, 3.13
II	>23.00, 15.12	12.66, 11.43, 6.93, 4.46, 3.94, 0.80	8.86, 5.73, 5.57, 4.09, 4.04, 2.94, 2.78, 2.47, 1.75, 1.33	5.60, 5.54, 4.84, 3.76, 3.05, 2.33, 2.27, 2.05, 1.86, 1.54, 1.37, 1.18	>23.00, 13.91	16.68, 15.12, 3.90, 1.72, 0.97
III	>23.00	9.15, 8.46, 7.00, 5.86, 3.00, 2.51, 1.23	11.40, 8.51, 6.58, 6.48, 3.57, 3.08	5.19, 5.00, 4.91, 3.30, 2.32[a], 2.26, 2.03, 1.89, 1.82, 1.52, 1.44[a], 1.37, 1.28[a]	>23.00, 11.49, 3.21	>23.00, 10.59
IV	>23.00, 6.31, 5.93	13.22, 12.80, 6.28, 5.42, 4.30	12.02, 8.50, 7.85, 4.77, 3.62, 1.90, 1.73, 1.46	7.45, 4.85, 4.04, 3.73, 3.57, 2.99, 2.44, 1.83[a], 1.67[a], 1.61, 1.31, 1.81, 1.11	>23.00, 11.73, 1.67	>23.00, 10.23, 5.20
V	17.10, 11.10, 8.72	9.30, 8.24, 6.72, 5.09, 2.79, 2.49, 2.34, 2.17	10.24, 8.54, 7.21, 6.25, 3.93, 2.80, 1.99, 1.69, 1.24	5.78, 4.32, 3.70, 3.22, 3.09, 3.04[a], 2.83, 2.66, 2.58, 2.43, 2.39, 1.35	≥23.00, 15.83, 4.35	18.07, 12.23, 9.86, 5.52
VI	>23.00	11.98, 7.71, 5.57, 4.57, 3.97[a], 1.99, 1.70, 0.74, 0.59	12.32, 7.50, 6.02, 4.42, 4.07, 1.12, 0.64	4.36[a], 4.18, 3.55, 3.37, 2.92[a], 2.34[a], 1.97, 1.65, 1.05, 0.97, 0.70, 0.54, 0.47	19.98, 8.68, 5.72, 3.11, 1.14	19.96, 10.97, 8.84
VII	≥23.00, 11.29, 6.67, 3.42, 2.00	9.58, 6.67, 5.56, 5.17, 4.64, 4.45, 4.28, 2.90, 2.28	7.72, 6.78, 5.36, 4.73, 3.52, 3.27, 2.71, 2.45, 1.88, 1.77, 1.59	7.07, 3.27[a], 2.99, 2.82, 2.64, 2.43[a], 2.28, 2.10, 1.93[a], 1.72, 1.49, 1.41, 1.17	>23.00, 11.62	>23.00, 11.62, 3.25

[a] Possible doublets.

adapted to and maintained in axenic growth at 27°C in Trypticase soy-yeast extract broth.

mtDNA extraction. The method described by Yagita and Endo (16) was adapted for the purification of mtDNA and is a modification of the alkaline lysis technique used for the recovery of plasmids (2). All isolates were grown for 4 to 5 days in Trypticase soy-yeast extract medium at 27°C and harvested at mid-log phase by centrifugation at 300 × *g* for 10 min. Pellets of amoebae (approximately 5×10^6 to 7×10^6 organisms) were washed three times in phosphate-buffered saline (pH 7.4) and resuspended in 100 μl of TES buffer (25 mM Tris-HCl, 10 mM EDTA, 50 mM sucrose; pH 8.0). Amoebae were lysed by the addition of 200 μl of freshly prepared 1% sodium dodecyl sulfate in 0.2 N NaOH, with gentle mixing by inversion, and subsequent incubation on ice for 3 min. Following lysis, 150 μl of chilled 3 M potassium acetate buffer (60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, 28.5 ml of water; pH 6.0) was added, and the suspensions were mixed by inversion and then incubated for 45 to 60 min on ice. The mixtures were centrifuged at 14,000 × *g* for 10 min at 4°C, and the supernatants were extracted with an equal volume of a phenol-chloroform solution saturated with 10 mM Tris–1 mM EDTA (pH 8.0). The mtDNA was precipitated by the addition of a 0.1 volume of 3 M sodium acetate (pH 5.4) and 2 volumes of cold absolute ethanol and kept at −20°C overnight. Samples were centrifuged at 14,000 × g for 20 min at 4°C. The resulting pellets were washed with 70% ethanol, dried with a centifugal evaporator for 5 to 10 min at room temperature, and resuspended in 20 to 30 μl of TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0). Samples were passed through spin columns of Sephadex G-50 before being stored at −20°C.

Endonuclease digestion and agarose gel electrophoresis. Approximately 1 to 2 μg of mtDNA was digested overnight at 37°C with different restriction enzymes in 20-μl reaction volumes with the corresponding restriction enzyme buffers in accordance with manufacturer recommendations. Six different restriction enzymes, *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Kpn*I, and *Sal*I (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), were included in this study. Digests and the standard (*Hin*dIII-digested lambda DNA size marker) were electrophoresed at 4 V/cm in 0.8% horizontal agarose gels (11 by 14 cm) with TAE buffer (40 mM Tris acetate, 1 mM EDTA) for 5 h. Gels were stained with 0.1% ethidium bromide for 1 h, destained for 20 min, and photographed under UV illumination. The molecular weights of the different bands observed

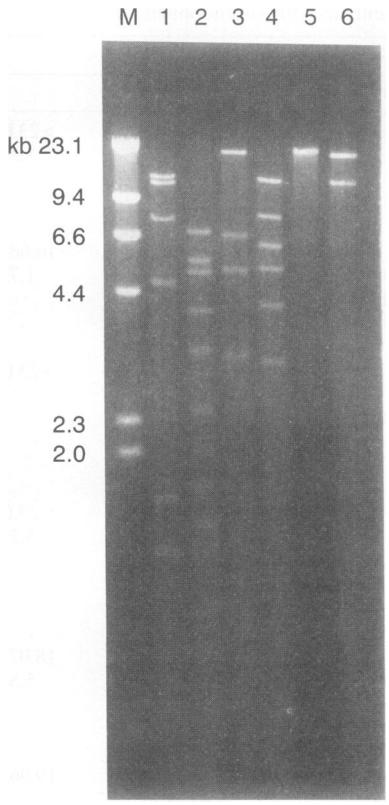

FIG. 2. Agarose gel electrophoresis of *Acanthamoeba* mtDNA representing group AcUW I. Lanes: 1, *Eco*RI; 2, *Hin*dIII; 3, *Sal*I; 4, *Bgl*II; 5, *Bam*HI; 6, *Kpn*I; M, *Hin*dIII-digested lambda molecular size standard.

were estimated with the Sizer Release 5.4 software system from IntelliGenetics, Inc., Mountain View, Calif. Restriction digestion and visualization were repeated three or more times for each isolate.

A total of seven different restriction endonuclease fingerprint groups emerged for the clinical isolates being studied (identified by us as AcUW [*Acanthamoeba* University of Washington] I to VII) (Fig. 1) and are listed in descending order of frequency in Table 1. The number of bands observed varied from 1 to 13, and bands smaller than 0.47 kb were not detected under the conditions used in this study. Fingerprints for all isolates within a group remained consistent for each enzyme tested (Fig. 2). One enzyme, *Bam*HI, produced a single band larger than 23 kb in AcUW I, III, and VI, although no other enzyme yielded a similar pattern among these three groups. Fingerprints for 14 (56%) environmental and 4 (40%) ATCC isolates were found to match those for the clinical isolates (Table 2). Overall, five multiple-isolate groups (AcUW I to V) and two single-isolate groups (AcUW VI and VII) were identified.

AcUW I included three corneal, one contact lens, eight environmental, and two ATCC (*A. castellani* ATCC 30011, the authentic Castellani strain, and *A. castellani* ATCC 30868, the Nagington isolate from a human cornea) isolates. Fingerprints produced with *Eco*RI, *Hin*dIII, and *Bgl*II were also similar to those of group C of Kilvington et al., which included isolates from three keratitis cases in England (8). While our initial analysis failed to detect two *Eco*RI bands of approximately 9.4 and 5.6 kb (data not shown) present in that study, repeated testing with whole-cell DNA rather than the mtDNA fraction revealed the banding patterns to be similar. The presence of these bands may have resulted from partial digestion of whole-cell DNA. The JAC/E1 eye strain described from Japan by Yagita and Endo and found to be similar to the original Castellani strain (16) was also similar to AcUW I isolates when the enzymes tested in common were used (*Eco*RI, *Hin*dIII, and *Bgl*II). Considering the frequency with which clinical and environmental *Acanthamoeba* isolates display this particular group profile, their recovery and description from several continents, and the known association of *A. castellani* with corneal infection, organisms displaying this profile may be considered important pathogens with enhanced virulence potential.

TABLE 2. Distribution of *Acanthamoeba* isolates from clinical and environmental sources by mtDNA fingerprinting

AcUW group	No. (%) of isolates:			ATCC isolates
	Clinical	Environmental	Total	
I	4 (27)	8 (32)	12 (30)	*A. castellani* ATCC 30011 and *A. castellani* ATCC 30868
II	4 (27)	2 (8)	6 (15)	
III	2 (13)	0 (0)	2 (5)	*Acanthamoeba* sp. strain ATCC 30173
IV	2 (14)	1 (4)	3 (8)	
V	1 (7)	3 (12)	4 (10)	*A. hatchetti* ATCC 30730
VI	1 (7)	0 (0)	1 (3)	
VII	1 (7)	0 (0)	1 (3)	
Other	0 (0)	11 (44)	11 (27)	
Total	15 (100)	25 (100)	40 (100)	

AcUW II included four corneal and two environmental isolates and was similar to group G of Kilvington et al., which contained isolates recovered from keratitis cases occurring in France, England, and Australia (8). This group also had an *Eco*RI fingerprint similar to that of the Wang strain of *A. polyphaga* (4). Considering the frequency with which *A. polyphaga* has been implicated as a cause of amoebic keratitis, we found it unusual that the group profile of neither of the *A. polyphaga* ATCC isolates included in this study matched the profile of any other isolate examined.

AcUW III included two corneal isolates and one ATCC isolate (*Acanthamoeba* sp. strain ATCC 30173), the latter having been originally isolated from a human nasal swab and known to harbor an endosymbiont (7). This fingerprint did not match any published fingerprint, however, or have any counterpart among the environmental isolates examined. The occurrence of these isolates in clinical situations alone suggests that other isolates displaying this group profile should be evaluated for inherent pathogenic potential.

Environmental isolates displaying mtDNA fingerprints similar to those of AcUW IV and V were also recovered. The *Eco*RI fingerprint of AcUW IV was also similar to that of the Boyce strain of *A. castellani*, recovered from a human bone infection (4). The AcUW V profile matched that of *A. hatchetti* ATCC 30730. The remaining two, single-isolate groups (AcUW VI and VII) did not have counterparts among either the environmental or the ATCC isolates examined or in the published literature. Overall, no correlation was evident between the habitats from which the environmental isolates originated and the AcUW groups to which they belonged.

The isolates used in this study were previously examined for the presence of bacterial endosymbionts (6). Four of eight

(50%) environmental isolates and one ATCC isolate (ATCC 30868) displaying the AcUW I profile harbored such endosymbionts. Three of four (75%) clinical isolates displaying the AcUW II profile also harbored endosymbionts, as did ATCC 30173, representing AcUW III, and the one clinical isolate representing AcUW VII. While bacterial-amoebic interactions would appear to be restricted to certain specific taxonomic combinations, conclusions regarding the potential for such combinations to display enhanced virulence must await further investigations.

This study confirms the usefulness of mtDNA fingerprinting in the analysis of *Acanthamoeba* epidemiology and possibly systematics as well. The inclusion of a number of environmental isolates demonstrated that the most common clinical isolates do have counterparts readily recoverable from the surrounding environment. Furthermore, the diversity of mtDNA fingerprints detected indicates that the development of specific gene probes is possible and may augment studies of the amoebic genome in determining taxonomic relationships of members of the genus. While the use of this technique in the typing of pathogenic and nonpathogenic *Acanthamoeba* isolates appears promising, future studies correlating such typing with traditional in vivo and in vitro pathogenicity assays will need to be performed.

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