

Contact Lens-Associated *Acanthamoeba* Keratitis in Iran

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Abstract

Acanthamoeba keratitis is a vision-threatening infection caused by pathogenic species of the genus *Acanthamoeba*. In this study, 13 *Acanthamoeba* keratitis cases were diagnosed among 52 keratitis patients. To confirm the identity of *Acanthamoeba* at the genus level, a PCR-based method was used, and their pathogenic potential was determined using in vitro cytotoxicity assays on human corneal epithelial cells. Twelve (92.3%) of *Acanthamoeba* keratitis patients were contact lens wearers; among them eleven (91.7%) wore soft contact lenses. 11/13 (84.6%) isolates were axenised in liquid culture medium, of which 10 (90.9%) isolates disrupted corneal cells. Nine (69.2%) isolates showed *Acanthamoeba* sp. group II, and four (30.8%) showed group III morphology. To our knowledge this is the first report of determination of *Acanthamoeba* pathogenicity in Iran. This study confirms the importance of determination of pathogenic potential of *Acanthamoeba* isolates for clinical purposes.

Keywords: *Acanthamoeba*, *Amoebic keratitis*, *Contact lens*, *Iran*

Introduction

During the last decade, free-living amoebae belonging to the genus *Acanthamoeba* have got increasing clinical significance mainly as causative agents of a seriously progressing keratitis. The first amoebic infection of the eye was diagnosed in 1974 (1). The occurrence of *Acanthamoeba* keratitis has been rising since 1990 in correlation to the growing number of contact lens wearers. To date more than 2000 cases have been published around the world (2). The annualized incidence of *Acanthamoeba* keratitis is estimated as 0.27-0.33 per 10000 contact lens wearers (3, 4). Contaminated contact lens care systems usually are the first step in *Acanthamoeba* keratitis pathogenesis. The most prevalent risk factors are contact lens wear, poor

hygiene, and a compromised corneal barrier. Users of extended-wear lenses are at special risk. Nevertheless, about 10 to 15% of cases of *Acanthamoeba* keratitis occur in persons who do not wear contact lenses (5). Unfortunately, due to the very often misdiagnosis and the complicated treatment, *Acanthamoeba* keratitis frequently gets a serious progression that may cause serious visual loss and perforating keratoplasty. Therefore, fast and reliable diagnosis is of crucial importance. *Acanthamoeba* keratitis is easily confused with atypical herpes simplex keratitis or fungal keratitis. Clinical diagnosis should be based on the presence of keratitis with severe pain and photophobia, ring-like stromal infiltrates, radial keratoneuritis, and sometimes pseudodendriiform epithelial lesions.

Cysts or trophozoites, found in corneal scrapings, on contact lenses, and inside of lens storage cases, are confirmatory. Agar culture is the mainstay for laboratory detection of *Acanthamoeba* (6).

Various *Acanthamoeba* species have been reported to be able to cause keratitis: *A. castellanii*, *A. polyphaga*, *A. hatchetti*, *A. culbertsoni*, *A. rhyodes*, *A. lugdunensis*, *A. quina*, and *A. griffini* (6). Although isolates can easily be recognized as belonging to the genus *Acanthamoeba* by their polygonal cysts, accurate species determination is still problematic. An important step forward in the differentiation of *Acanthamoeba* was the division of the genus into three morphological groups by Pussard and Pons (7).

In this study, we tested 62 clinical samples for the identification of *Acanthamoeba* using plating assays as well as polymerase chain reaction and their pathogenic potential determined with in vitro cytotoxicity assays using human corneal epithelial cells.

Materials and Methods

Specimens During 1998 to 2003, about 62 clinical specimens including corneal scrapings and contact lenses of 52 keratitis patients were examined in Dept. of Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Iran, for investigation of *Acanthamoeba*. Corneal scrapings were obtained by surgical procedure in ophthalmology sections of different university hospitals (generally Farabi and Labbafinejad) in Tehran and transported in a low osmolar transport solution to the laboratory.

Isolation The clinical specimens were inoculated at neighbourhood of the old culture of *Escherichia coli* or *Enterobacter aerogenes* (formerly known as *Klebsiella aerogenes*) onto non-nutrient agar plates [10 g purified agar in 1 litre Page's amoeba saline (PAS), (2.5 mM NaCl, 1 mM KH₂PO₄, 0.5 mM Na₂HPO₄, 40 mM CaCl₂.6H₂O, and 20 mM MgSO₄.7H₂O)].

The plates were sealed and incubated at 30°C for 14 days and observed daily for amoeba growth by using an inverted microscope.

Positive initial cultures were diluted in order to eliminate co-contaminants by harvesting amoebae at a noncontaminated site of the plate with a sterile spatula and transferring the amoebae to a fresh plate overlaid with bacteria.

Morphological classification Amoebae were identified as belonging to one of the cyst morphological groups (*Acanthamoeba* groups I to III) established by Pussard and Pons (7). Differentiation was achieved mainly on the basis of cyst size and number of opercula.

Axenization Amoebae were axenised by removing single cells (using an inverted microscope) and transferred into fresh non-nutrient agar plates seeded with UV killed *K. aerogenes*. After 48 h a stamp sized agar piece (~10 cm²) was removed and inoculated in 10 ml of PYG medium [Proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v) and glucose 1.5% (w/v) (Difco Laboratories, Detroit, Mich.)] in 15 ml universal containers. To eliminate bacteria from the liquid growth media, they were supplemented with penicillin G (100 U/ml) and streptomycin (100 µg/ml) and incubated at 30°C. Samples were examined daily for growth of *Acanthamoeba*. For further assays, *Acanthamoeba* isolates were grown in 150-cm² tissue culture flasks at 30°C without shaking.

Cytopathic effect (CPE) assays Pathogenicity of all axenically grown isolates was determined by CPE assays as essentially described by Cao et al. (8). Briefly, immortalised human corneal epithelial cells (HCEC) were grown to monolayers in 24-well plates. Mid log phase *Acanthamoeba* cells (5×10^5 per well) grown in PYG medium were added to the wells, contained monolayers, in duplicate. The plates were incubated at 37°C in a 5% CO₂ incubator for 24 h. Monolayers were observed under inverted microscope and cytotoxicity determined by measuring lactate dehydrogenase (LDH) release.

Additionally, reference *Acanthamoeba* species (*A. castellanii*, ATCC 30234 and *A. astronyxis*, ATCC 30137) obtained from the American Type Culture Collection (ATCC) was included to the test as controls (pathogen and nonpathogen, respectively).

Extraction of the total DNA Briefly, mid log phase *Acanthamoeba* ($\sim 10^3$ cells) in PYG were harvested by centrifugation at $800 \times g$ for 5 min and washed in PAS three times. To lyse the cells, 30 ml of lysis buffer [100 mM KCl, 40 mM Tris, 5 mM MgCl₂, 1% (w/v) Tween and 200 µg/ml Proteinase K] was added, and incubated at 55° C for 1 h followed by incubation at 99.9° C for 10 min. Lysed cells were centrifuged at $2000 \times g$ for 5 min, and the supernatant was used as DNA template.

From isolates which would not grow in axenic culture, DNA was extracted directly from the plate culture. In this method ~ 100 trophozoites directly gathered from the surface of the plate cultures and put in 0.5-ml microfuge tubes. Thirty µl insta-gene matrix (chelex, Sigma Labs., Poole, Dorset, England) was added to each tube and was incubated at 56° C for 20 min and then at 99.9° C for 10 min. Tubes were spun in a microcentrifuge at $2000 \times g$ for 5 min and supernatant was used as DNA template.

The concentration of DNA samples was estimated by measuring optical density at 260 nm using spectrophotometer.

Polymerase chain reaction analyses Genus-specific primers were used for the PCR amplification of 18S rDNA as previously described by Kong and Chung 1996 (9). Primer sequences were 5'-TTTGAATTCGCTCCAATAGCGTATATTAA and 5'-TGAATTCAGAAAGAGCTATCAATCTGT. PCR was performed in 50 µl containing 1.25 U Taq polymerase (Amersham Biosciences, Little Chalfont, Buckinghamshire, England), 0.1-0.5 µg DNA, 200 µM deoxynucleoside triphosphates, 4 mM MgCl₂, and 0.5 µM primer. The amplification profile was 94° C for 1 min, 55° C for 1 min, 72° C for 2 min for 30 cycles followed by a final extension step of

10 min at 72° C. Amplified DNA was electrophoresed on a 1.5% agarose gel prepared in TAE buffer (40 mM Tris, 1 mM EDTA, 0.11% glacial acetic acid, pH 8.0). Gels were stained using 0.5 µg/ml of ethidium bromide, and photographed under shortwave UV transillumination.

Results

Patients The definitive diagnosis of *Acanthamoeba* keratitis on the basis of typical clinical signs, no response to antibacterial or antiviral treatment, and detection of *Acanthamoeba* sp. in the corneal epithelium or contact lenses was verified for 13 (25%) patients. Twelve (92.3%) of them were contact lens wearers (Table 1). Of these, eleven (91.7%) wore soft contact lenses and one (8.3%) wore hard lenses. One patient (7.7%) was not contact lens wearer and had a history of trauma to his eye. Patients' ages ranged from 15 to 54 years (mean, 20.3); 7 (53.9%) were females. Patients were from different cities of Iran and had been referred to the ophthalmology sections of university hospitals (generally Farabi and Labbafinejad) in Tehran with diagnosis of microbial keratitis.

All patients were successfully treated within 6-7 months by a combination of topical propamidine isethionate (Brolene) and Neosporin (neomycin plus polymyxin B sulfates plus bacitracin), except case 64 who wore hard contact lenses and lived in Rasht (the center of Gilan province, northern Iran). While he was riding motorbike, an insect entered his eye. He rubbed vigorously his eye while wearing contact lens, which resulted in corneal abrasion. Three weeks after beginning the clinical signs including severe pain, photophobia and stromal infiltrates, he was referred to Farabi Hospital in Tehran. His contact lens was then sent to our laboratory and based on detection of cysts on his lens and growth of *Acanthamoeba* in culture; he was diagnosed to have *Acanthamoeba* keratitis. The anti-amoebic therapy was initiated but showed no response. Finally due to the

failure of the medical therapy, he successfully underwent corneal transplantation.

Morphological classification Several isolates exhibited rather varied cyst morphologies with respect to size and number of opercula although they were derived from a clone. These isolates were classified according to the average cyst morphology. Nine (69.2%) of the 13 *Acanthamoeba* isolates were identified as belonging to *Acanthamoeba* sp. group II. Four (30.8%) isolates were designated morphological group III, as the cysts being rather small and round and endocysts had 3-5 gentle corners. No isolate exhibited group I morphology.

Pathogenicity In this study, two out of 13 (15.4%) isolates failed to axenise in PYG medium. So in cytopathic effect assays, only 11 (84.6%) isolates examined.

To determine the pathogenicity of *Acanthamoeba* isolates, in vitro cytotoxicity assays

were performed by incubating *Acanthamoeba* with human corneal epithelial cell monolayers. Microscopic examination revealed that 10 out of 11 (90.9%) axenised isolates were able to disrupt host cell monolayers (Table 2). But no effect on host cells was determined in one isolate after 24 h incubations. To determine whether corneal epithelial cell monolayer disruptions represent actual cell death, cytotoxicity assays were performed by measuring LDH release and the results were compared with those of reference strains purchased from ATCC. Lactate dehydrogenase is a stable cytoplasmic enzyme present in all cells and is released into the culture supernatant upon plasma membrane damage. *Acanthamoeba* isolates exhibiting more than 50% cell death were considered pathogens (Table 2).

Table 1: Characteristics of *Acanthamoeba* keratitis patients

No.	Patient code	Sex	Age	Contact lens type	Clinical sample
1	52	F	18	Soft	Contact lens
2	53	F	20	Soft	Contact lens
3	54	M	54	–	Corneal scraping
4	55	F	21	Soft	Contact lens
5	56	M	22	Soft	Contact lens
6	58	M	U	Soft	Contact lens
7	59	M	U	Soft	Contact lens
8	60	F	16	Soft	Contact lens
9	61	F	21	Soft	Contact lens
10	62	F	20	Soft	Contact lens
11	63	M	26	Soft	Contact lens
12	64	M	31	Hard	Contact lens
13	65	F	15	Soft	Contact lens

U: unknown (the complete data of two patients were not available)

Table 2: Cytotoxicity determined using LDH assays

No.	Morphological group	Cytotoxicity (%) ^a (Mean ± SD)	Pathogenicity
1	II	100	Pathogen
2	II	76.56 ± 2.2	Pathogen
3	III	9.1 ± 4.3	Nonpathogen
4	II	78.4 ± 9	Pathogen
5	II	69.3 ± 3.8	Pathogen
6	II	54.2 ± 1.8	Pathogen
7	II	100	Pathogen
8	II	100	Pathogen
9	II	82 ± 1.1	Pathogen
10	II	ND	ND
11	III	ND	ND
12	III	91.1 ± 0.07	Pathogen
13	III	100	Pathogen
Ctrl + ^b	II	51.5 ± 4.5	Pathogen
Ctrl - ^c	I	5.4 ± 1.1	Nonpathogen

^a Each assay was performed in duplicate

^b *A. castellanii*, ATCC 30234

^c *A. astronyxis*, ATCC 30137

ND: not determined

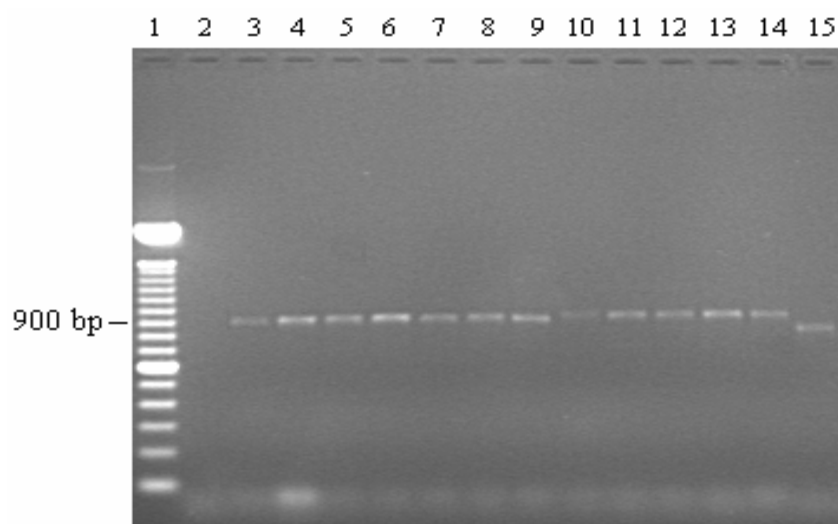


Fig. 1: Amplification of 18S rDNA for genus identification of the isolates. Lanes: 1, 100 bp DNA ladder; 2, control negative; 3, *A. sp.* (52); 4, *A. sp.* (53); 5, *A. sp.* (54); 6, *A. sp.* (55); 7, *A. sp.* (56); 8, *A. sp.* (58); 9, *A. sp.* (59); 10, *A. sp.* (60); 11, *A. sp.* (61); 12, *A. sp.* (62); 13, *A. sp.* (64); 14, *A. sp.* (65) and 15, *A. sp.* (63)

Discussion

In this study, 13 *Acanthamoeba* keratitis cases were diagnosed among 52 keratitis patients. But the number of the amoebic keratitis might be more than that, because most of the patients had already been treated empirically with different antibiotics such as chloramphenicol, gentamicin, and econazole, and antibacterial and antifungal treatment is at least partly effective against free-living amoebae. On the other hand, contact lens disinfectants, even if not used properly, compromise amoebal viability. Moreover, amoebae penetrate the cornea during the course of infection, protruding up to Descemet's membrane. It might therefore in some cases be impossible to isolate viable amoebae by scraping. Several studies report on unsuccessful attempts to culture amoebae from clinical specimens (5, 10).

Eleven out of 12 (91.7%) contact lens wearer patients discussed here wore soft contact lenses, consistent with other studies (3, 5, 11). Wearers of soft lenses are more likely to acquire *Acanthamoeba* keratitis, as the hydrophilic material seems to support attachment and survival of cysts. In the only patient who wore rigid gas-permeable contact lenses, there was a history of corneal trauma due to vigorously eye-rubbing following the entrance of an insect in his eye.

Morphological determination was rather difficult in some cases as cysts, although all deriving from one clone, had varied morphologies, and generally intraspecific polymorphism is rather common among *Acanthamoeba* (12). Especially, *A. hatchetti*, *A. castellanii*, *A. culbertsoni*, *A. palestinensis*, and *A. polyphaga* have been described to be polyphyletic (13). So, it is obvious that a thorough molecular analysis will be a prerequisite for accurate classification of the isolates in the genus *Acanthamoeba*. Nevertheless, using morphological characteristics, about 70% of our isolates were designated in group II and the rest exhibited group III morphology. Although most of the pathogenic species of *Acanthamoeba* settle in group II, but a

species in group III, *A. culbertsoni*, has also been reported to be cause of keratitis (13, 14).

We used fast and simple DNA extraction methods that can be applied in clinical use to detect a few cells. Because there are no precipitation or wash steps, loss of DNA is eliminated.

Vodkin et al. (15) were the first to use PCR for the genus-specific detection of *Acanthamoeba*, using primer pair ACARNA-for.1383 and ACARNA-rev.1655, which amplifies 272 bp of 18S rDNA. This primer pair was also tested by Lehmann et al.(16) in their clinical study, along with a second 18S rDNA-based primer pair, P1GP-for. 2379 and P1GP-Rev. 2632, which amplifies a 253-bp amplicon. But Schroeder et al. (17) have shown that the above two primer pairs could also amplify rDNA of related amoebae, i.e., *Balamuthia* and *Hartmanella* spp. The primers used in our study (NA1L-R) were used by Kong and Chung 1996 (9) from a large database of 18S rDNA sequences and were shown to be highly genus specific for *Acanthamoeba*. These primers had failed to amplify DNA from closely related amoebae and from several bacterial, fungal, and human DNAs. In the present study we tested different PCR conditions for achieving high sensitivity and specificity (data not shown) and finally employed the best set of PCR conditions that provide both high sensitivity and specificity.

Adaptation of isolates to axenic culture was one of the most time-consuming parts of this study. Some of the *Acanthamoeba* species are naturally fastidious and needs the bacteria as a source of food, so they are not easily axenised. In the present study we tried to axenise all of the isolates, but in spite of lots of efforts and several repetition of the assay, two of the isolates were not adapted to axenic culture.

In CPE assays, all axenised isolates, except one, exhibited significant cytotoxicity to a monolayer culture of corneal epithelium. It has been shown that cell culture pathogenicity is correlated with in vivo pathogenicity (18). In our study one isolate showed very slight damage of

epithelial cells and might be nonpathogen. The occurrence of nonpathogenic *Acanthamoeba* on the eye is still of medical interest, as they can harbor bacteria inside their cysts, protecting them from disinfectants, and thus function as vectors. It has been shown that viable *Pseudomonas aeruginosa*, one of the major ocular pathogens, can survive in and be reisolated from cysts of *Acanthamoeba* (19).

It has been reported that passaging amoebae through cell culture increases the virulence of the amoebae and that the virulence of *Acanthamoeba* attenuates during axenic culture (20). Our reference species had maintained in axenic culture for a long time, so the cytotoxicity percentage of *A. castellanii*, ATCC 30234 has slightly reduced.

In conclusion, while awareness regarding viral and bacterial keratitis is relatively high in a majority of the eye hospitals in Iran, as reflected in several publications, information regarding *Acanthamoeba* keratitis is grossly inadequate. Through this report, we would like to emphasize that with appropriate tests a greater number of cases of *Acanthamoeba* keratitis can be differentiated from bacterial, fungal, or viral keratitis and treated appropriately before it is too late.

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Footnotes

The abbreviations used are: PAS, Page's amoeba saline; PYG, proteose peptone/yeast extract/glucose; CPE, cytopathic effect; HCEC, human corneal epithelial cells; LDH, lactate dehydrogenase; ATCC, American Type Culture Collection.

References

1. Nagington F, Watson PG, Playfair TJ et al. (1974). Amoebic infection of the eye. *Lancet*, 2: 1537-40.
2. Walochnik J, Duchene M, Eibl H, Aspöck H (2003). Treatment of *Acanthamoeba* keratitis: standard, problems, and new approaches. *Wien Klin Wochenschr*, 115 (Suppl 3):10-7.
3. Seal DV (2003). *Acanthamoeba* keratitis update-incidence, molecular epidemiology and new drugs for treatment. *Eye*, 17(8): 893-905.
4. Seal DV, Beattie TK, Tomlinson A et al. (2003). *Acanthamoeba* keratitis. *Brit J Ophthalmol*, 87: 516-17.
5. Illingworth CD, Cook SD (1998). *Acanthamoeba* keratitis. *Surv Ophthalmol*, 42: 493-508.
6. Marciano-Cabral F, Cabral G (2003). *Acanthamoeba* spp. as agents of disease in humans. *Clin Microbiol Rev*, 16(2):273-307.
7. Pussard M, Pons R (1977). Morphologie de la paroi kystique et taxonomie du genre *Acanthamoeba* (Protozoa, Amoebida). *Protistologica*, 8: 557-98.
8. Cao Z, Jefferson MD, Panjwani N (1998). Role of carbohydrate-mediated adherence in cytopathogenic mechanisms of *Acanthamoeba*. *J Biol Chem*, 273(25): 15838-45.
9. Kong HH, Chung DI (1996). PCR and RFLP variation of conserved region of small subunit ribosomal DNA among *Acanthamoeba* isolates assigned to either *A. castellanii* or *A. polyphaga*. *Korean J Parasitol*, 34:127-34.
10. Schaumberg DA, Snow KK, Dana MR (1998). The epidemic of *Acanthamoeba* keratitis: where do we stand? *Cornea*, 17:3-10.
11. Radford CF, Lehmann OJ, Dart JKG (1998). *Acanthamoeba* keratitis: multi-

- centre survey in England 1992-6. *Br J Ophthalmol*, 82:1387-92.
12. Badenoch PR, Adams M, Coster DJ (1995). Corneal virulence, cytopathic effect on human keratocytes and genetic characterisation of *Acanthamoeba*. *Int J Parasitol*, 25: 229-39.
 13. Stothard DR, Schroeder-Dietrich JM, Awwad MH et al. (1998). The evolutionary history of the genus *Acanthamoeba* and the identification of eight new 18S rDNA gene sequence types. *J Eukaryot Microbiol*, 45: 45-4.
 14. Yu HS, Hwang MY, Kim TO et al. (1999). Phylogenetic relationships among *Acanthamoeba* spp. based on PCR-RFLP analyses of mitochondrial small subunit rRNA gene. *Korean J Parasitol*, 37:181-88.
 15. Vodkin MH, Howe DK, Visvesvara GS, McLaughlin GL (1992). Identification of *Acanthamoeba* at the generic and specific levels using the polymerase chain reaction. *J Protozool*, 39:378-85.
 16. Lehmann JO, Green SM, Morlet N et al (1998). Polymerase chain reaction analysis of corneal epithelial and tear samples in the diagnosis of *Acanthamoeba* keratitis. *Investig Ophthalmol Vis Sci*, 39: 1261-65.
 17. Schroeder JM, Booton GC, Hay J Niszl IA et al (2001). Use of subgenetic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *Acanthamoeba* from humans with keratitis and from sewage sludge. *J Clin Microbiol*, 39:1903-11.
 18. Cursons RTM, Brown TJ (1978). Use of cell cultures as an indicator of pathogenicity of free-living amoebae. *J Clin Pathol*, 31: 1-11
 19. Walochnik J, Picher O, Aspöck C et al. (1999). Interactions of "Limax amoebae" and gram-negative bacteria: experimental studies and review of current problems. *Tokai J Exp Clin Med*, 23:273-78.
 20. Stevens AR, O'Dell W (1974). In vitro growth and virulence of *Acanthamoeba*. *J Parasitol*, 60:884-85.