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Original Article

Potentially Pathogenic Free-Living Amoebae in Contact Lenses of the Asymptomatic Contact Lens Wearers

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Abstract

Background: Free-living amoebae (FLA) including *Acanthamoeba* spp. and *Hartmannella* spp. are the causative agents of serious corneal infection especially within contact lens wearers. Thus contact lenses and their storage case could be a suitable niche for potentially pathogenic amoebae. The main objective of the present study was to evaluate the contamination of contact lenses to free living amoebae using morphological and sequencing based methods.

Methods: Overall, 90 volunteers provided their contact lenses. All volunteers wore soft contact lenses. Both lenses were cultured in the same plate. Forty-eight of the volunteers were medical and dentistry student and 42 were ophthalmology attendees of hospitals in Tehran, Iran. All of the samples were inoculated to non-nutrient medium and monitored daily for the outgrowth of the amoebae. PCR and sequencing were performed using various primer pairs.

Results: Of the 90 volunteers, 9 (10%) were positive for free-living amoebae outgrowth. Morphological analysis revealed that 3 isolates were belonged to *Hartmannella* genus according to small round cysts and 6 isolates were belonged to *Acanthamoeba* genus based on the star shape of endocysts. Sequencing revealed that *Acanthamoeba* belonged to T4, T3 and T5 genotype. *Hartmannella* were also belonged to *vermiformis* species.

Discussion: The presence of potentially pathogenic free living amoebae including *Acanthamoeba* and *Hartmannella* could be a high risk for people using soft contact lenses. These results revealed that improved clarification and professional recommendations for contact lens wearers is of utmost importance.

Introduction

Free-living amoebae (FLA) include various genera such as *Acanthamoeba*, *Hartmannella* and *Vannella*. The ubiquitous nature of these amoebae leads to their cosmopolitan distribution around the world from hot springs to sea water (1-3). *Hartmannella* keratitis has been reported during last years as the causative agent of human keratitis. Among many genera of *Hartmannella* genus, *H. vermiformis* is the most common species of this genus which can lead to corneal ulcers (4).

Acanthamoeba spp. is the ubiquitous free-living amoebae (FLA) which classified to 17 different genotype based on the sequence differences in Diagnostic fragment 3 (DF3) of 18S rRNA gene (5). Out of 17 genotypes some are potentially pathogens to human and can lead to severe human disease including amoebic keratitis (AK), Granulomatose amoebic encephalitis (GAE) and cutaneous ulcers (6). Several genotypes have been attributed as corneal pathogens such as T2, T3 and T4 and some types including T10 or T11 are still non pathogen (6). Among *Acanthamoeba* related disease amoebic keratitis continues to rise in Iran and worldwide according to the various reports of researchers (7, 8). Researches in Iran showed that *Acanthamoeba* belonging to T4 genotype is the predominant genotype causing AK with poor prognosis (9). The most important risk factor for developing such disease is the improper usage of contact lenses specially the soft contact lenses. Indeed, soft contact lenses are now the most important risk factor for new cases of AK in Iran and around the world (10). To date contact lens cases introduced as the suitable source of harboring *Acanthamoeba* spp. (11). These cases can retain biofilms containing protein molecules, microorganisms such as bacteria, fungi and FLA. The presence of potentially pathogenic free living amoebae such as *Acanthamoeba* can be a threat for developing corneal diseases. Indeed *Acanthamoeba* could readily transfer to the cornea by contaminated contact lenses (10, 11).

To date, *Hartmannella* is also introduced as the probable cause of corneal ulcers. Lorenzo et al. showed that *Hartmannella* could lead to amoebic keratitis in a patient with usage of soft contact lens. It is important to note that keratitis due to other FLA could lead to the poor response to anti-amoebic treatment (12).

The main aim of the present research was to address the presence of *Acanthamoeba* in contact lenses of asymptomatic soft contact lens wearers using culture and molecular based approaches and also to determine the genotypes of the isolated *Acanthamoeba* spp using sequencing of DF3 region.

Material and Methods

Subjects

Overall, 90 contact lenses were obtained from students and client attending to different ophthalmology hospitals and private clinics in Tehran, Iran. There were no participants with a history of eye disease and they were all cosmetic or corrective contact lens wearers. Information regarding age, sex, occupation, type of contact lens, lenses caring solution and the way of cleaning methods of the participants were recorded for each patient.

Isolation of the amoebae

Contact lenses of each attendee were opened aseptically and were placed in non-nutrient agar plate along with a layer of heat killed *Escherichia coli*. Each plate was sealed and incubation was done at 25-30 °C for one months. Positive plates including any type of free living amoebae were subjected to cloning for further evaluation. All cloned amoebae were evaluated with morphological criteria according to page key (13).

DNA extraction, Polymerase Chain Reaction and Sequencing

Cloned plates were washed with sterile PBS buffer. The amoebae were then centrifuged at 250 xg for 5-10 min. DNA extraction of the amoebae were done using two different methods, Chelex kit and modified phenol- chloroform extraction procedures. DNA extraction using Chelex kit was performed according to manufacturer's instruction. DNA extractions for cysts were performed using a modified phenol-chloroform method according to our previous study (14).

The PCR reaction was done using two different primer pair for *Acanthamoeba* and *Hartmannella*. Presence of *Acanthamoeba* was confirmed by genus specific primer pairs JDP1-2 (14) and NA primers were used for identification of *Hartmannella* spp. (14). PCR was performed in 30 µl Ampliqone (Taq DNA Polymerase Master Mix Red, Denmark) as a ready-made solution. Briefly, 25 µl of master mix with 5-10ng DNA templates and 20 pmol primers were mixed to achieve a volume of 30 µl. PCR condition adjusted for 35 cycles of denaturation at 94°C for 1 min, followed by 35 repetition cycles at 94°C for 35 s, annealing at 56°C and 58 °C for 45 s, and extension at 72°C for 1 min. Electrophoresis of PCR products were done using 1.5% agarose gel stained with a solution of ethidium bromide

(25 mg ml⁻¹) and examined under UV illumination.

Sequencing and genotype identification

PCR-products were submitted to sequencing using an ABI 3130X automatic sequencer. Homology analyses of the nucleotide sequences were done using BLAST (Basic Local Alignment Search Tool) software from the National Center for Biotechnology Information (NCBI) webpage. The highest homology and query overage of the obtained sequence with other sequences available in the gene data bank was the base of genotype identification.

Result

Out of 90 clients in the present study 81 (90 %) were female and 9 (10 %) were male. The average age of the participant was 25 yr. Eighty nine of the clients wore soft contact lenses and only one male wore hard lenses. Sixty nine (69 %) clients used appropriate disinfection solution such as Opitfree, ReNew and the remaining used no commercially solution and they reported that they were used homemade saline or tap water for cleaning their lenses. These participants also did not maintain a proper lens care. Interestingly, 8 of our positive sample were from those who did not use the commercial cleaners (Table 1).

Table 1: Data of positive contact lens wearers and isolated amoebae from lenses

Isolates	Patients age	Type of lens	Culture	PCR for <i>Acanthamoeba</i>	PCR for <i>Hartmannella</i>	Genus	Species /genotype
CL13	32	Soft	+	+		<i>Acanthamoeba</i>	T4
CL21	17	Soft	+	+		<i>Acanthamoeba</i>	T4
CL27	26	Soft	+	+		<i>Acanthamoeba</i>	T3
CL33	33	Soft	+		+	<i>Hartmannella</i>	<i>vermiformis</i>
CL42	24	Soft	+	+		<i>Acanthamoeba</i>	T5
CL45	32	Hard	+	+		<i>Acanthamoeba</i>	T4
CL50	20	Soft	+		+	<i>Hartmannella</i>	<i>vermiformis</i>
CL59	18	Soft	+		+	<i>Hartmannella</i>	<i>vermiformis</i>
CL73	19	Soft	+	+		<i>Acanthamoeba</i>	T4

Overall, 9 samples (10%) were positive for free living amoebae. Morphological identification revealed that 3 isolate (CL33, CL50, CL59) contains round small cysts with wormy shaped amoebae (Fig. 1). Sequencing analysis and homology search confirmed that these three strains were belonged to *Hartmannella vermiformis*. The PCR product revealed a 800 bp band on the agarose gel (Fig. 2).

Six plates showed the outgrowth of *Acanthamoeba* cysts with round ectocysts and star

shape or triangular endocysts (Fig. 1). PCR analysis showed a specific band of the genus *Acanthamoeba* which is 500 bp bands on the agarose gel (Fig 2). Homology analysis showed that *Acanthamoeba* isolates were belonged to T3 corresponding to *A. griffinii* (isolate: CL27), T4 (isolate: CL13, CL21, CL73, CL45) and T5 (corresponding to *A. lenticulata*) (isolate: CL42). This is an interesting finding as all of the isolated amoebae are potentially pathogenic amoebae for human.

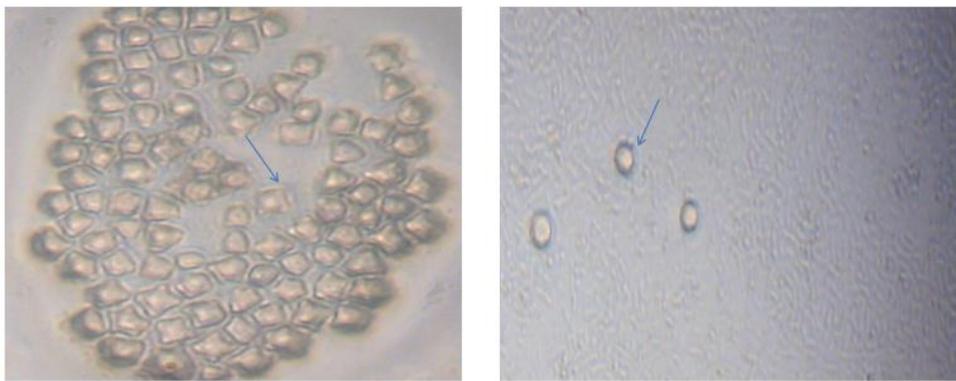


Fig. 1: *Acanthamoeba* spp. double wall cyst. X 400 (left). *Hartmannella* spp. round cyst. X 400 (right)

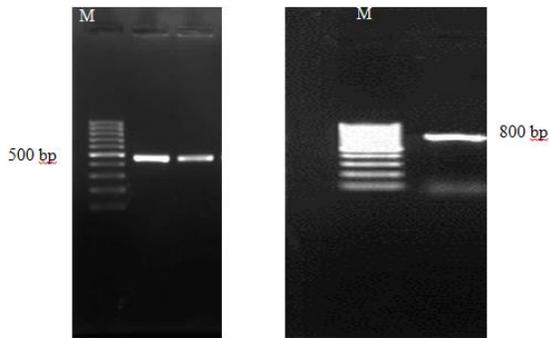


Fig. 2: PCR product of *Acanthamoeba* spp. (500 bp, Left) and *Hartmannella* spp. (800 bp, Right), M= 100bp marker

Discussion

This is the first study regarding the presence of free-living amoebae in the contact lenses of asymptomatic lens wearers in Iran. The find-

ing of the present research showed that potentially pathogenic free-living amoebae could colonize on the surface of contact lenses or their storage cases. However, this is mainly occurring in people who do not have sufficient care and maintenance for their cosmetic or corrective lenses (10, 11, 16). The present study confirmed that soft lenses have the potential to attract free living amoebae. This is may be due to their plastic surface as previous researches reported that plastic surfaces such as soft lenses are relatively resistant to antibiotic and antiseptics and accumulation of microorganisms could lead to biofilm formation. Biofilm can act as an attractive niche for most microorganisms including free living amoebae (17). The presence of free living amoebae in contact lenses or storage cases is in concordance with others studies worldwide (16, 17). Out of 81 lens samples who Pen et al. ana-

lyzed, 7 (9%) were positive for *Acanthamoeba* spp. Confirmation for the presence of amoebae was based on morphological and PCR approaches (10). In addition, Korean researchers demonstrated that contact lens storage cases were contaminated to *Acanthamoeba* in 10% of their samples (15, 16). In contrast Boost et al. reported that out of 100 contact lens cases only one were positive for *Acanthamoeba* spp. All of researches showed that subjects had a poor compliance with lens care cleanings (18, 19).

All of the isolated amoebae in the present study were potentially pathogens of the cornea. This finding could confirm that addition to wearing improper contact lens, corneal ulcer is the important risk for developing threatening vision disease such as amoebic keratitis (1, 10). However, the contaminations of contact lenses in asymptomatic wearers are of utmost importance and make the possibility of their transmission to cornea via contaminated lenses. Therefore, improved clarification and professional recommendations for contact lens wearers is necessary.

The presence of the most prevalent genotype of *Acanthamoeba* (T4 type) in asymptomatic lens wearers is interesting. Indeed, the occurrence of *Acanthamoeba* T4 genotype in asymptomatic contact lens wearers can be explained by different pathogenicity of the T4 type and the level of sIgA in the tear of the participant (1, 3). Indeed, T4 genotypes have different pathogenic abilities, some are highly pathogen and some are less pathogen according to several researches (20).

Conclusion

The presence of potentially pathogenic free-living amoebae including *Acanthamoeba* and *Hartmannella* could be a high risk for people using soft contact lenses. These results revealed that improved clarification and professional recommendations for contact lens wearers is of utmost importance.

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