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Original Article

Species Identification of *Acanthamoeba* Strains Isolated from Patients Referring to Farabi Eye Reference Center Using PCR-RFLP Method

Mir Mostafa Ghamilouie[1], *Zarrintaj Valadkhani[1], Fariba Khoshzaban[2]

[1]Department of Parasitology, Pasteur Institute of Iran, Tehran, Iran;
[2]Department of Parasitology, Shahed University of Medical Science, Tehran, Iran.

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Introduction: *Acanthamoeba* is an opportunistic protist, which is ubiquitously distributed in the environment. Infection with *Acanthamoeba* spp. poses threat to human health, such as, *Acanthamoeba* keratitis (AK) that is a vision-threatening infection of the cornea. This study aimed to identify the species of *Acanthamoeba* strains isolated from cornea of keratitis patients by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. **Methods:** *Acanthamoeba* isolates investigated in this cross-sectional study, were collected from patients referring to Farabi Eye Reference Center. All 10 isolates were subjected to species identification using DNA based method. BspLI (NlaIV) and HpyCH4IV restriction enzymes were used to categorize the PCR amplified DNA by PCR-RFLP method. **Results:** Six samples were identified as *Acanthamoeba palestinensis* and 4 isolates as *Acanthamoeba culbertsoni*, which implies that all the isolates belong to pathogenic strains of *Acanthamoeba*. **Conclusion:** *Acanthamoeba* can enter the corneal tissue and survive in the eye, which results in AK. To the authors' knowledge, no study is available on species identification of this genus using these enzymes and technique. This is the first time in Iran that *Acanthamoeba* isolates are subjected to species identification using PCR-RFLP method. *J Med Microbiol Infec Dis, 2014, 2 (3): 125-129.*

Keywords: *Acanthamoeba* Keratitis, Cornea, DNA Restriction Enzymes, PCR-RFLP.

INTRODUCTION

Pathogenic free-living amoebas (FLAs) can complete their life cycles in environment without entering a human or animal host. Of many FLAs, three genera and spp. are known to be associated with human disease. These microorganisms have been detected in different sources, such as soil, mud, and water. Some members of FLAs cause *Acanthamoeba* keratitis (AK), an infection of the eye that can lead to permanent visual impairment or blindness. However, if the infection is diagnosed at early stages, it can be treated easily without sequelae. There is a significant emphasis on *Acanthamoeba* as a Trojan horse of other microorganisms, including viral, bacterial, protist, and yeast pathogens [1]. Contact lenses wearers are more susceptible to eye infections due to tiny scrapes of the cornea. Due to improper disinfection of the lenses and the presence of parasite, it survives in the space between the lens and the eye and invades the corneal tissue, leading to infection.

Symptoms of AK include foreign body sensation, photophobia, decreased visual acuity, tearing, pain, and redness of the eye. Genotyping and species identification of *Acanthamoeba* has been reported from different geographical areas. Cysts are resistant and infectious form of *Acanthamoeba*. Species identification based on cyst morphology, following cultivation of isolates on non-nutrient agar (NNA), has been reported previously [2]; however, it should be considered that the morphological characteristics of the cyst can change with culture conditions [3].

With the advent of molecular techniques, various genetic markers, such as Aca 16S, 18S, Ac6/210, GP, JDP, and Nelson have been used for species identification of *Acanthamoeba* [4]. *Acanthamoeba jacobsi* (a thermophilic species of this genus) was investigated by PCR and subjected to sequence type classification by sequencing part of the 18S rRNA gene and allocated to the sequence type T15 [5].

Among different species of *Acanthamoeba* isolated from infected eyes, such as *Acanthamoeba castellani, Acanthamoeba palestinensis, Acanthamoeba griffini, Acanthamoeba culbertsoni, Acanthamoeba polyphaga, Acanthamoeba lugdunensis, Acanthamoeba hatchetti, Acanthamoeba rhysodes, and Acanthamoeba quina,* three has been reported as the predominant ones, *i.e., A. castellani, A. polyphaga,* and *A. griffini*. Due to the increased use of contact lenses (more than 120 million individuals worldwide), AK has been rising among young individuals within the age range of 14-36 years [6]. Our previous study showed that contact lenses wearers within age group of 18-30 years were most infected with AK, which in turn indicates the correlation between age group and risk of infection [7].

***Correspondence:** Zarrintaj Valadkhani
Department of Parasitology, Pasteur Institute of Iran, No. 69, Pasteur Ave, Tehran, Iran, 1316943551.
Email: valad.zarrin@gmail.com

Tel/Fax: +98 (21) 66968855

Determination of the pathogenic potential of isolates are important for clinical purposes. *In vitro* cytotoxicity assays were used to determine the pathogenic potential of clinical samples isolated from keratities patients [8]. Considering the lack of use of species identification by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method in Iran, this study aimed to identify the prevalence of *Acanthamoeba* species among AK patients referring to Farabi Eye Reference Center, Tehran, Iran.

MATERIAL AND METHODS

Samples were obtained from patients referring to Farabi Eye Reference Center affiliated to Tehran University of Medical Sciences, Tehran, Iran. The patients presented with eye pain, redness, tearing, and sensitivity to light, and sensation of foreign body in the eye. In most patients, only one eye was affected, and five patients had been using contact lenses. Using a surgical blade, a corneal scraping was obtained from the infected eye of each patient by an ophthalmologist, and transferred to phosphate buffer saline (PBS), as described previously [7]. One hundred microliters of each sample was inoculated into Petri dishes containing 1.5% NNA in page's saline, seeded with *Escherichia coli*, and incubated at 30°C. The plates were checked daily under an inverted microscope for the presence of *Acanthamoeba* cyst for two weeks. The positive samples were transferred from NNA media into tubes containing PYG medium (0.75% proteose peptone, 0.75% yeast extract, and 1.5% glucose) and penicillin (100 U/ml), streptomycin (100 µg/ml), and gentamicin (40 µg/ml) and then kept at 30°C.

PCR and RFLP analysis. The amoeba DNA was extracted using a commercial DNA extraction kit (HiPurA Multi-Sample DNA Purification Kit, HiMedia, India) according to the manufacturer's instruction. Partial sequence of 18S rRNA gene was targeted using primers JDP1 (5′-GGCCCAGATCGTTTACCGTGAA-3′) and JDP2 (5′-TCTCACAAGCTGCTAGGGGAGTCA-3′), as reported previously [9]. The amplifications were performed in 20 µl reaction mixtures containing 1 U of Taq polymerase, 10 pM of each primer, 2.5 mM $MgCl_2$, and 200 mM dNTPs. All the reagents were provided by CinnaGen Co., Iran. The thermocycler was programmed for an initial denaturation step at 95°C for 5 min, followed by 35 amplification cycles of denaturation for 45 s at 95°C, annealing for 45 s at 57.7°C, extension for 50 s at 72°C, and a final elongation step at 72°C for 8 min [10]. The amplicons (<500 bp) were subjected to digestion with restriction enzymes HpyCH4IV (New England BioLab, Lithuania) and BspL I (NlaIV) (thermoscience, Lithuania). An amount of 10 µl of amplicons was digested with 10 units of the restriction enzymes BspL I (NlaIV) and HpyCH4IV at 37°C for 3 h and 15 min, respectively. The digested samples were electrophoresed on a 2.5% agarose gel along with the 100 bp DNA Ladder (GeneRuler™, Fermentas, Lithuania), and then stained with DNA safe stain and visualized under a UV transilluminator [9]. The restriction fragments of the isolates were compared with the fragment sizes predicted by restriction map and CLC Genomics Workbench 3 software (Table 1).

Although, the BseDI enzyme was also used during the prediction of fragment sizes by restriction map and CLC Genomics Workbench 3 software (Table 1), the species were identified using the other two enzymes, *i.e.*, BspL I (NlaIV) and HpyCH4IV, and there was no need to use BseDI anymore. This enzyme may also be used for the verification of the tests.

Ethical consideration. This study was approved by the Ethics-in-Research Commission of Pasteur Institute of Iran, and a written informed consent was obtained from each participant.

Table 1. Predicted fragment sizes (bp) of *Acanthamoeba* species, after digestion with HpyCH4IV, NlaIV, and BseDI

Acanthamoeba Species	Restriction enzyme	Predicted fragment sizes (bp)
Castellani	HpyCH4IV	269,122,73
	NlaIV	303,97,32,31
	BseDI	240,138,61,24
Lenticulata	HpyCH4IV	346,77
	NlaIV	326,96
	BseDI	244,178
Palestinensis strain 1	HpyCH4IV	342,125
	NlaIV	305,97,64
	BseDI	240,138,62,26
Palestinensis strain 2	HpyCH4IV	340,108
	NlaIV	318,97,32
	BseDI	238,209
Polyphaga	HpyCH4IV	343,105
	NlaIV	350,97
	BseDI	241,206
Culbertsoni Diamond	HpyCH4IV	342,120
	NlaIV	332,97,32
	BseDI	240,139,62,20
Culbertsoni Lilly	HpyCH4IV	222,123,115
	NlaIV	361,98
	BseDI	224,131,104

RESULTS

Ten corneal scraping samples were successfully grown on NNA medium. PCR amplification of DNA extracted from the cultured samples yielded the expected bands based on the identified species. Comparison of the restricted fragments with those predicted by the restriction map and CLC Genomics Workbench 3 software revealed that 6 isolates were belonged to *A. Palestinensis* (Figure 1) and 4 isolates belonged to *A. culbertsoni* species (Figure 2).

The two bands sized 97 bp and 125 bp are weakly observed. However, according to the bands sized 305 bp and 342 bp, which have only been detected in *A. Palestinensis*, the sample should be *A. Palestinensis*. The smallest band size with NlaIV enzyme, *i.e.*, 64 bp, were not supposed to be visible on the agarose gel due to its very small size.

The Figure 2 shows the restriction enzymes' pattern of *A. culbertsoni* with estimated bands using BspL I (NlaIV) and HpyCH4V restriction enzymes. Although some bands were seen weakly, as there were no extra-suspected bands, the species were recognized correctly.

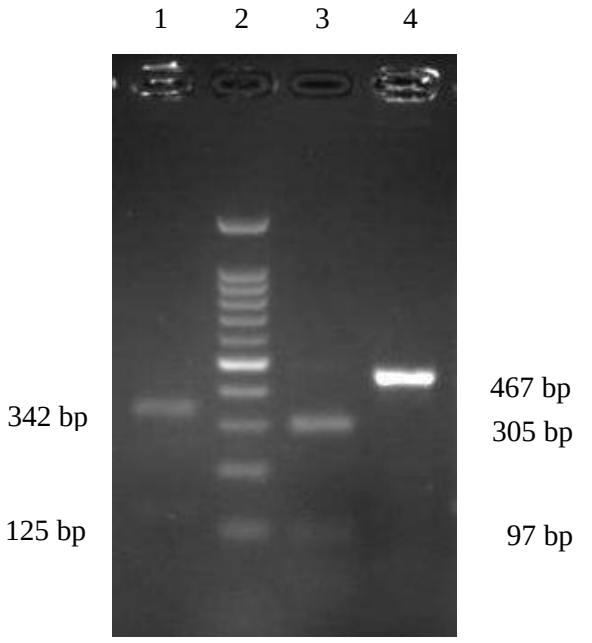

Fig. 1. Digestion of 18S rRNA of *A. Palestinensis* with HpyCH4V and BspL I (NlaIV) restriction enzymes Bands' pattern: 1, HpyCH4V Restriction enzyme (342 bp, 125 bp); 2, 100 bp DNA Ladder; 3, BspL I (NlaIV) restriction enzyme (305 bp, 97 bp); and 4, undigested PCR product.

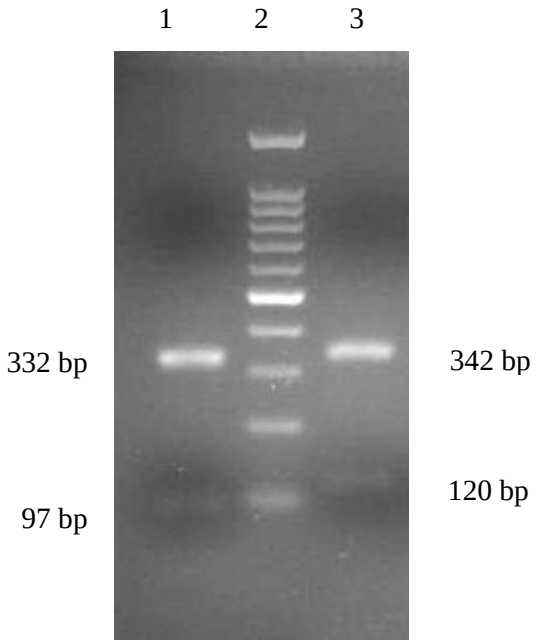

Fig. 2. Digestion of 18S rRNA of *A. culbertsoni* with HpyCH4V and BspL I (NlaIV) restriction enzymes Bands' pattern: 1, BspL I (NlaIV) restriction enzyme (332 bp, 97 bp); 2, 100 bp DNA ladder; and 3, HpyCH4V restriction enzyme (342 bp, 120 bp).

DISCUSSION

Acanthamoeba was suggested to be pathogenic in human for the first time in 1958 [11]. Some members of this genus can cause vision-threatening keratitis [1]. Immuno-compromised patients, including AIDS patients and immunosuppressive drug users are particularly susceptible to *Acanthamoeba* infections. [12].

In the present study, based on PCR-RFLP-based-species identification of 10 *Acanthamoeba* isolates from corneal scrapings, 6 isolates were identified as *A. Palestinensis* and 4 isolates as *A. culbertsoni*. All the isolates belonged to the genotype T4, which has been shown to be the predominant genotype in Iran [13]. Therefore, it can be implied that all the isolates of our study belonged to pathogenic species of *Acanthamoeba*. The results of this study are similar to those of Niyyati *et al.*, who based on the genotypic identification of *Acanthamoeba* species., demonstrated that most of the *Acanthamoeba* strains belonged to the genotype T4 both in clinical and environmental samples; however, genotype T11 was also reported for the first time in their clinical samples [7].

In a study in Taiwan, *Acanthamoeba* spp. concentrations were determined according to TaqMan real-time qPCR method, through which 10 different genotypes of *Acanthamoeba* could be detected simultaneously. *Acanthamoeba* genotypes of all isolates were determined as T4 and the species were categorized to *A. culbertsoni*, *A. polyphaga*, *A. castellanii*, and *A. hatchetti* [14]. Most of the human *Acanthamoeba* infections have been associated with the isolates of the genotype T4. For example, more than 90% of AK cases belong to this genotype, suggesting that the abundance of T4 isolates in human infections can be due to their greater virulence and/or other properties that increase their transmissibility as well as their low susceptibility to chemotherapeutic agents [1].

Michalek *et al.* reported acanthaporin as the first pore-forming toxin to be described from *Acanthamoebae* that

causes severe and often fatal diseases [15]. Contact lenses wearers are more susceptible to AK, so that about 80% of this infection occurs in young and healthy people who use medical or colored contact lenses; however, eye trauma and poor water supply are other major risk factors associated with AK [16]. Our previous study also showed that contact lenses wearers within age group of 18-30 years were most infected with AK [7]. Rezaeian *et al.* [17] and Maghsood *et al.* [18], reported that most of the AK isolates were from contact lenses wearers. De Jonckheere reported that 15 strains of *Acanthamoeba* were isolated from contact lenses, boxes, and saline solutions of 9 keratitis patients. All their isolates belonged to T4 that has different subtypes. They also found a strain of *Hartmannella* from a cornea and two *vahlkampfiids* isolates from contact lenses, which had no connection with keratitis. They concluded that only *Acanthamoeba* genotype T4 is responsible for keratitis in Belgium [19].

The assignment of *Acanthamoeba* species to a series of 18S rRNA gene sequence types is being widely accepted as a genetic classification system [5]. Booton *et al.* examined partial 18S ribosomal DNA (Rns) sequences of *Acanthamoeba* isolates in Hong Kong. They examined the links between strains isolated from corneal scrape specimens, contact lenses, lens cases, lens case solutions, and home water-supply faucets of patients with *Acanthamoeba* and also looked for evidence of mixed infections. They found 12 Rns genotype T4 and one Rns genotype T3 [10]. In a study in Malaysia, 21 cloned *Acanthamoeba* isolates, designated as IMU1 to IMU21, were established from positive primary cultures. Five species were identified based on the morphological criteria of Pussard and Pons, *i.e.*, *A. castellanii*, *A. culbertsoni*, *A. griffini*, *A. hatchetti*, and *A. polyphaga*, however, 5 species could not be determined morphologically [20]. It should be considered that the morphological characteristics of the cyst can change with the culture conditions, so it is not an appropriate criteria for species identification [3]. In another study from southwestern Korea, ribosomal DNA PCR-RFLP and mitochondrial DNA-RFLP analysis were performed for 43 *Acanthamoeba* environmental isolates from contact lens storage cases. These isolates were compared to American Type Culture Collection (ATCC) strains and clinical isolates from patients with keratitis. Their results showed that the most predominant (48.8%) species was *A. castellanii*. They also found that 38 (88.4%) out of 43 isolates from contact lens storage cases were identical to those of clinical isolates, and were considered as potential keratopathogens [21]. Also, the results of our study indicated that contact lenses could increase the susceptibility of individuals to infection with *Acanthamoeba* species, which is consistent with the results of the above mentioned studies. Although, the results of the present study have similarity with those of other studies, geographical conditions is also a factor that can affect the results.

To the authors' knowledge, no report is available on the species identification of *Acanthamoeba* by PCR-RFLP method. From the results of this study, it can be concluded that predominant species of the *Acanthamoeba* vary with geographical locations and the methods used in different studies. Given that this pathogen is emerging as a risk for human health in Iran and worldwide, identification of pathogenic species could be important for clinical purpose, such as diagnosis and treatment.

The limitation of this study was the cost effectiveness of the method used.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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