

Original Article

Genotyping of *Acanthamoeba* Species Isolated from Keratitis Patients by PCR Sequencing Methods in Tehran, Iran

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ABSTRACT

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Key words

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Background and Aims: Amoebae of the genus *Acanthamoeba* are unicellular amphizoic opportunistic pathogens that may cause fatal granulomatous encephalitis, eye keratitis, amebic pneumonitis and skin nodules as well as abscesses in humans and animals. *Acanthamoeba keratitis* is caused by trauma to the eye, contaminated cleaning solutions and the use of contact lenses. The aim of the present study was to identify the genotypes of *Acanthamoeba* in all patients with a clinical diagnosis of *Acanthamoeba keratitis* referring to eye clinic in Tehran using polymerase chain reaction (PCR).

Materials and Methods: In this study, samples were collected from 35 patients who had referred to the eye clinic and were cultured on 1.5% non-nutrient agar. DNA was extracted, and then PCR amplification was performed using genus specific primers. Sequencing analysis and basic local alignment search tool search were conducted to determine the genotypes. Phylogenetic tree was generated using maximum likely algorithm in phylogenetic program MEGA version 6.

Results: Eight cases were positive for *Acanthamoeba* using genus specific primer pairs. All specimens were reported as genotype T4.

Conclusions: Determination of genotypes showed all isolates belonging to genotype T4; this abundance may be due to its higher prevalence in the environment or its greater virulence. However, further analysis of clinical and environmental samples is necessary to clarify this property.

Introduction

Acanthamoeba is a unicellular amphizoic opportunistic and pathogenic protozoon inducing keratitis in the eyes and fatal granulomatous encephalitis [1, 2]. *Acanthamoeba* has a broad distribution across the globe and is separated from various environmental sources [3]. The life cycle of *Acanthamoeba* consists of infective trophozoite living on various bacteria and resistant cysts stage and causing adverse conditions [4, 5]. The first signs of *Acanthamoeba keratitis* (AK) are redness with inflammation, photophobia, edema, epithelial defects and loss, pain, ring infiltration of corneal tissue and finally loss of vision [6, 7]. In Iran, the highest number of AK has been reported in contact lens wearers. Rezaian et al. reported that most of AK patients in Iran are the women 15 to 25 years old [8]. Increase of *Acanthamoeba* infections is possibly because of the increase in population of the contact lens users and the human immunodeficiency viruses patients [9]. AK is detected by several methods such as microscopic observation, culture and DNA-based methods [10, 11]. Microscopic observation and DNA-based methods can provide early diagnosis of *Acanthamoeba* [10, 12, 13]. Diagnosis is difficult and the disease can be confused with herpes simplex, adenovirus or fungal keratitis [14]. Successful treatments of ocular infections include cationic antiseptic that inhibits the membrane functions, aromatic diamidines inhibiting DNA synthesis, and polyenes such as amphotericin B [15]. Studies conducted in Iran indicate three species

of *Acanthamoeba* related to keratitis including *Acanthamoeba castellanii* (T4), *Acanthamoeba griffini* (T3) and *Acanthamoeba palestinensis* (T2) [16]. *Acanthamoeba* has 20 genotypes (T1–T20) based on analysis of diagnostic fragment sequence of 18S rRNA gene [17]. T4 is the most common genotype observed in AK [18, 19]. Therefore, the aim of this study was to identify the frequency of the genotypes of *Acanthamoeba* in clinical samples in Tehran.

Materials and Methods

Sample collection and histological study

Thirty-five samples (corneal scraping, corneal biopsy, tear, and contact lens) were collected from patients with amoebic keratitis who had referred to Bina Afarin eye clinic during 4 years in Tehran, Iran. All samples in page's saline were transferred to the department of parasitology, faculty of medical sciences, Tarbiat Modares University, Tehran, Iran. The study was approved by the Ethics Committee of Tarbiat Modares University, Tehran, Iran.

DNA extraction and polymerase chain reaction (PCR)

Samples were cultured on non-nutrient agar (NNA) plates enriched with *Escherichia coli* bacteria strain K12. The plates were sealed and incubated at 30°C for one month. Amoebae in plates were washed using phosphate-buffered saline (PBS). The resulting solution was then transferred into a microtube of 1.5 ml and centrifuged at 4000×rpm for 10 min. Subsequently, the

supernatant was removed and PBS was added and centrifugation was repeated. Finally, DNA extraction was performed using a DNGTM-PLUS (Cinna colon, Iran) according to the manufacturer's instructions, and DNA stored at -20°C. A 461 bp region of the 18S rRNA gene was amplified using the genus-specific primers JDP1 5'-GGCCCAGATCGTTTACCGTGAA-3' and JDP2 5'-TCTCACAAGCTGCTAGGGGAGTCA-3' (Takapozist, Iran) [20-22]. The PCR cycle profile was as the following: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 61°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. Instead of the DNA, distilled water was used in a negative control.

Sequencing and phylogenetic analysis

PCR products were electrophoresed on 1.5% agarose gels containing SYBR safe DNA gel stain (Cinna colon, Iran) and were visualized under UV illumination. A 100-bp DNA ladder was used as a DNA size marker. PCR products of eight isolates were purified using Gent Bio purification kit (Tabasmed, Iran) and sequenced by Pishgam company (Iran). The obtained sequences were aligned using Chromas software (version 2.33). The genus and genotype of the species were identified according to the highest similarity to the genes found in the GenBank database [23, 24]. Identity levels of the sequences were compared with other *Acanthamoeba* species in GenBank using CLUSTAL OMEGA. Phylogenetic maximum likelihood reconstructions were performed using the phylogenetic program

MEGA version 6. Evolutionary distances were computed using the Kimura two-parameter distance algorithm, and the bootstrap consensus tree was inferred from 1000 replicates. Phylogenetic trees were generated using maximum likelihood methods in MEGA6. The DNA sequences for the new isolates were deposited in the GenBank genetic sequence database at the National Center for Biotechnical Information (NCBI) under accession numbers KU877548, KU877549, KU877550, KU877551, KU877552, KU877553, KU877554, KU877555.

Results

Eight clinical specimens were positive for *Acanthamoeba* using genus specific primers. The samples collected from keratitis patients included 3 men (37.5%) and 5 women (62.5%) with the age range of 20 to 72 years (mean 36 years). Five patients (62.5%) had soft contact lenses or used self-made solutions and three patients (37.5%) had a history of trauma with the eye. All specimens were reported as genotype T4. PCR amplification revealed a fragment about 461 base pair (Fig. 1).

Sequencing and homology analysis of the obtained sequences in Basic Local Alignment Search Tool (BLAST) showed all *Acanthamoeba* strains belonging to the potentially pathogenic T4 genotype (Identity 99-100%) (Table 1) which is most common in human infections. The phylogeny tree was drawn with samples recorded in the Gene Bank (T1, T2, T3, T4, T5, T6, T10, T11, T13, T15, T16 and T17) sequenced samples in this study (n=8) thus showing the similarity of samples with those recorded in the Gene Bank (Fig. 2).

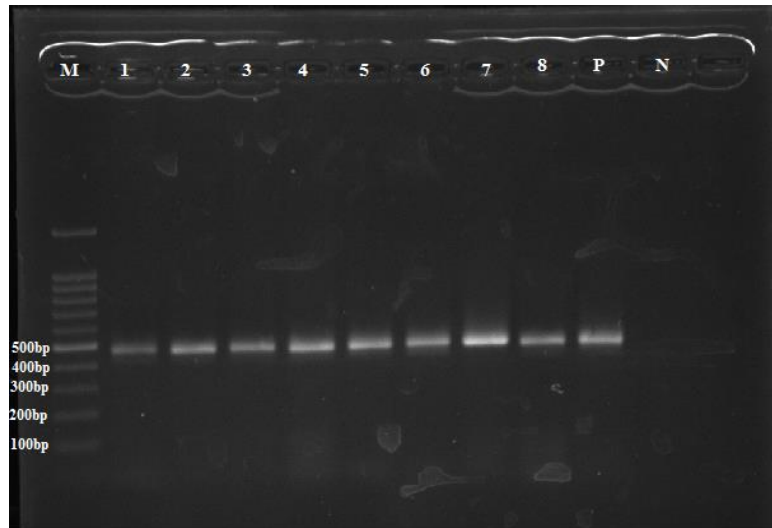


Fig. 1. Gel electrophoresis of PCR products of *Acanthamoeba* strains isolated from keratitis patients; M= Molecular size marker; 1-8 lanes= The samples; P= Positive control; N= Negative control

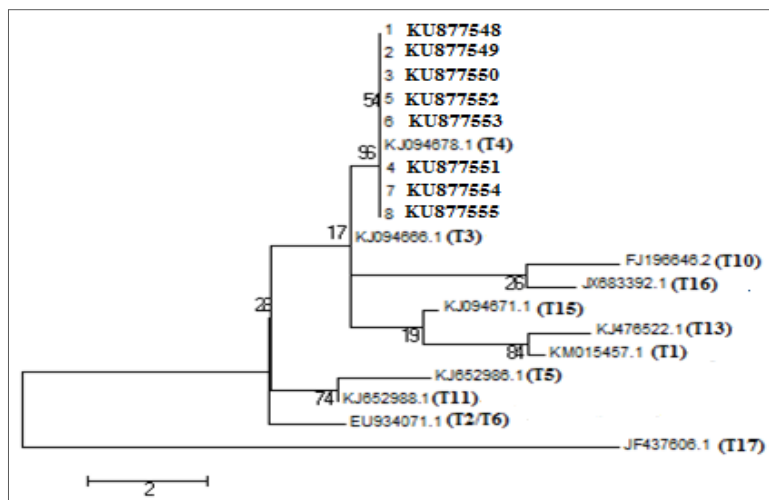


Fig. 2. Identification of the genotypes of the isolates examined in this study by phylogenetic analysis. A tree was constructed using the Maximum likelihood algorithm based on evolutionary distances calculated from Kimura two-parameter with 1000 bootstrap sampling

Table 1. Genotypes of *Acanthamoeba* isolates obtained from keratitis patients

Isolates code	Culture	PCR	Genotype	Accession number
1	+	+	T4	KU877548
2	+	+	T4	KU877549
3	+	+	T4	KU877550
4	+	+	T4	KU877551
5	+	+	T4	KU877552
6	+	+	T4	KU877553
7	+	+	T4	KU877554
8	+	+	T4	KU877555

Discussion

In the previous studies, different genotypes such as T2, T3, T4 and T6 were separated from environmental samples, amoebic keratitis, granulomatous encephalitis, and skin wounds whereas some other genotypes such as T10 were not separated from clinical specimens [25].

In this study, all positive samples belonged to T4 genotype the results of which are consistent with the findings of previous studies [11, 26-28]. T4 has been confirmed as a predominant *Acanthamoeba* genotype in AK. The main reason of abundant cases due to T4 genotype is unclear yet and some researchers have noted that one of the reasons of its global distribution and high transmission is its dominant genotype [29]. The present study revealed that eight specimens of patients referring to Bina Afarin eye clinic in Tehran were infected with *Acanthamoeba* spp. In a study by Ghamilouie et al. (2014), five clinical samples (5.6%) of 89 specimens collected from keratitis patients and sequence analysis demonstrated that T4 is the dominant genotype in these isolates and the primary genotype in AK [30]. Another study by Maghsood et al. (2005) reported 13 clinical cases with acanthamoebiasis in which eight out of 13 cases were T4, two out of 13 cases were T3, and three out of 13 were T2 genotype [18]. The T4 genotype is also most prevalent in environmental samples; a study had shown the contamination of geothermal water samples with an average temperature of 30-50°C by *Acanthamoeba* in southwestern Iran. The contamination was estimated to be up to 50%.

Genotyping of the diagnostic fragment showed 15 isolates (93.75%), which belonged to the T4 genotype and a single strain labeled as T2 genotype [31]. Another research in raw wastewater treatment facilities in west and south of Tehran detected *Acanthamoeba* belonging to T4 (83%) and T11 (17%) genotypes [32].

Conclusion

T4 is the most prevalent genotype related to parasitic infection of cornea by *Acanthamoeba* in Iran and worldwide. Further molecular studies are needed to specify the distribution of *Acanthamoeba* genotypes and the actual prevalence of the keratitis. The aim of this study was to determine the prevalence and genotype of AK in the patients who had referred to Bina Afarin Eye clinic in Tehran because of the incidence of potentially pathogenic *Acanthamoeba*. T4 genotype in AK patients can be a serious threat for high-risk individuals such as contact lens users. Thus, to prevent the contamination, higher hygiene considerations are recommended.

Conflict of Interest

There is no conflict to declare.

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