# Are Thermotolerant and Osmotolerant Characteristics of Acanthamoeba Species an Indicator of Pathogenicity?

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# Abstract

*Acanthamoeba* species are amoebae commonly found in nature. *Acanthamoeba* species were classified using the 18S ribosomal RNA gene sequence and have been divided into 23 genotypes called T1-T23 to date. The aim of this study is to compare the osmotolerance and thermotolerance characteristics of *Acanthamoeba* strains isolated from environmental and keratitis cases among genotype groups, within species with the same genotype, and to evaluate the relationship between pathogenicity in *Acanthamoeba* strains with T4, T5, T11 and T12 genotypes. In this study, after axenic cultures of 22 Acanthamoeba strains with T4 (Neff, A, B, D, E), T5, T11 and T12 genotypes isolated from clinical and environmental samples, thermotolerance (37°C, 39°C and 41°C) was determined and osmotolerance (0.5, 1 M) tests were performed. As a result; All strains showed growth ability at both 37°C and 0.5 M osmolarity. While all 5 strains isolated from patients with *Acanthamoeba* keratitis showed growth ability at 37 $^{o}$C and 0.5 M osmolarity, no growth was detected at 41 $^{o}$C and 1 M osmolarity. When the tolerance characteristics of the strains with the same genotype were evaluated, the strains with the T5 and T4E genotypes exhibited the same characteristic. When *Acanthamoeba* strains with T4 genotype were evaluated in general, 31.25% of the strains were found to grow at 39 $^{o}$C and 6.25% at 41 $^{o}$C. While the strain with the T11 genotype grew at all temperatures, the strain with the T12 genotype did not grow at 41 $^{o}$C. According to our research results, we believe that tolerance to 39 $^{o}$C and 1M mannitol is not an indicator of pathogenicity. In order to clarify this issue, more studies with Acanthamoeba strains are needed.


# Introduction

Species in the genus *Acanthamoeba*, an amoeba, can live in natural ecosystems such as soil, freshwater resources and seas, as well as in man-made areas such as pools, ventilation systems, operating rooms. At the same time, it has also been isolated from contact lenses, lens cases and solutions (Fuerst & Booton, 2020; Marciano-Cabral & Cabral, 2003). These opportunistic pathogens can cause *Acanthamoeba* keratitis, Granulomatous Amoebic Encephalitis, *Acanthamoeba* pneumonia, and cutaneous acanthamoebiasis (Kot, Lanocha-Arendarczyk, & Kosik-Bogacka, 2021). The global incidence rate of *Acanthamoeba* keratitis cases continues to increase and is associated with contact lens wear as a major factor (Kim, et al., 2022). Different strains of *Acanthamoeba* spp. cause amoebic encephalitis, which causes fatal brain damage in immunocompromised individuals (Kot, Lanocha-Arendarczyk, & Kosik-Bogacka, 2021). At the same time, *Acanthamoeba* species act as endosymbionts for viruses, yeasts, protists and bacteria that are highly virulence and antibiotic resistant (Siddiqui & Khan, 2012).

In the life cycle of *Acanthamoeba* spp., there is a trophozoite form that actively feeds, grows, multiplies and moves, and a cyst form, which is more resistant to external environmental conditions (Marciano-Cabral & Cabral, 2003; Khan, 2006; Tawfeek, Bishara, Sarhan, Taher, & Khayyal, 2016). In the trophozoite form, they can survive if the temperature and pH are suitable and the food is sufficient (Siddiqui & Khan, 2012). However, in the absence of these factors, it turns into a double-walled cyst consisting of endocyst and ectocyst. Cysts that are resistant to harsh conditions can survive for a long time and are resistant to

antibiotics, chlorine and disinfectants (Lacerda & Lira, 2020; Sriram, Shoff, Booton, & Fuerst, 2008). When conditions are favorable again, it can return to the trophozoite stage with excistation (Bouheraoua, et al., 2014).

The genus *Acanthamoeba* has been named more than 20 species, consisting of three different groups based on features of cyst morphology (Pussard & Pons, 1977). However, it is very difficult to differentiate *Acanthamoeba* species at the species level using morphological criteria (Marciano-Cabral & Cabral, 2003). In order to overcome this problem, in parallel with the developments in molecular biology and bioinformatics, a new solution proposal has been developed using the 18S ribosomal RNA gene (18S rRNA) sequence (Gast, Ledee, Fuerst, & Byers, 1996; Fuerst P. A., 2014). In this approach, called the genotype system, strains with a total of less than 5% differences in the partial sequence of the 18S rRNA gene were collected under a single genotype (Stothard, et al., 1998; Fuerst & Booton, 2020). In this method, known as the genetic approach, only the amount of total difference or similarity is evaluated (Schroeder, et al., 2001). As a result; Although it is divided into three groups morphologically, it has been divided into 23 genotypes called T1-T23, until today, by using molecular methods (Fuerst, Booton, & Crary, 2015; Putaporntip, et al., 2021). Acanthamoeba keratitis is predominantly caused by genotype T4 (Satitpitakul, Putaporntip, & Jongwutiwes, 2021). In this genotype, it consists of T4A, T4B, T4C, T4D, T4E, T4F and T4Neff subgroups according to the defined sequence characteristics (Fuerst & Booton, 2020).

In the genus *Acanthamoeba*, both the ability to reproduce at high salt concentrations where amoebae in tear fluid can survive and body physiological temperatures may also be important factors in pathogenicity (Booton, et al., 2004). In studies, positive *Acanthamoeba* strains that reproduced in thermotolerance and osmotolerance experiments isolated from environmental and clinical specimens were accepted as potentially pathogenic species (Kiss, et al., 2014; Vijayakumar, 2018). There are studies suggesting that *Acanthamoeba* strains, which are in the same genotype groups and isolated from various sources, show different osmotolarity and thermotolarity, thus showing different pathogenic potential (Booton, et al., 2004; Lorenzo-Morales, et al., 2005; Pumidonming, et al., 2010; Retana-Moreira, et al., 2015; Rocha-Cabrera, et al., 2015; Behniafar, Niyyati, & Lasjerdi, 2015; Todd, et al., 2015; Tawfeek, et al., 2016; Possamai, et al., 2018; Milanez, et al., 2020; Paknejad, et al., 2020). There is no study comparing both *Acanthamoeba* genotypes and pathogen and environmental isolates in terms of osmotolerance and thermotolerance in studies on strains generally isolated from environmental sources. The aim of this study is to compare the osmotolerance and thermotolerance characteristics of *Acanthamoeba* strains with T4 (Neff, A, B, D, E), T5, T11 and T12 genotypes both between genotype groups and within species with the same genotype, and also to compare environmental and keratitis cases and to compare the osmotolerance and thermotolerance characteristics of isolated *Acanthamoeba* strains and evaluate their relationship with pathogenicity.

# Materials And Methods

1. Acanthamoeba **Strains and Culture**: In the study, 22 strains of *Acanthamoeba* with T4A, T4B, T4D, T4E, T4Neff, T5, T11 and T12 genotypes isolated from various sources such as soil, water sources, mouse

brain, cornea, contact lens case containers were used (Table 1).While 5SU, 9GU, 3ST, 1BU, 2HH, 11DS, 72/2, Pat06, Z009, BUD9 of these strains were obtained from Julia Walochnick, SVS12, SVS6, SVS11, SVS16, SVS3, SVS5, SVS7, SVS8, SVS10, X2, 4A, Ac strains were isolated from various water sources and soil from Sivas/Turkey and genotyped (in publication).

*Acanthamoeba* species used in the study were grown monoxenically on 1.5-2% non-nutritive agar (BDOA) plates smeared with heat-killed *Escherichia coli*. After ensuring monoxenic growth of all strains, axenic culture was performed. PPYG medium (0.75% [wt/vol] protease peptone, 0.75% [wt/vol] yeast extract and 1.5% [wt/vol] glucose) was used for axenic culture. After the axenically produced *Acanthamoeba* trophozoites were washed twice in sterile phosphate buffered saline solution, counted with a thoma slide, counting as $1\times10^5$ amoebae/mL (99% trophozoites) and used for tolerance tests.

Table 1
Amoeba strains, genotypes, isolation sources used in the study.

| | Genotype | Strain Name | 37 °C | 39 °C | 41 °C | 0,5 M | 1 M |
|---|---|---|---|---|---|---|---|
| **1.** | T4A | 5SU | + | - | - | + | - |
| **2.** | T4A | SVS12 | + | + | - | + | + |
| **3.** | T4A | 9GU | + | - | - | + | + |
| **4.** | T4B | 3ST | + | - | - | + | - |
| **5.** | T4B | 1BU | + | + | - | + | - |
| **6.** | T4D | SVS6 | + | + | - | + | - |
| **7.** | T4D | SVS11 | + | + | - | + | + |
| **8.** | T4D | SVS16 | + | + | + | + | - |
| **9.** | T4E | 2HH | + | - | - | + | - |
| **10.** | T4E | 11DS | + | - | - | + | - |
| **11.** | T4Neff | SVS3 | + | - | - | + | + |
| **12.** | T4Neff | SVS5 | + | + | - | + | + |
| **13.** | T4Neff | SVS7 | + | + | - | + | + |
| **14.** | T4Neff | SVS8 | + | + | - | + | + |
| **15.** | T4Neff | SVS10 | + | + | - | + | + |
| **16.** | T4Neff | X2 | + | + | - | + | + |
| **17.** | T5 | 72/2 | + | + | - | + | - |
| **18.** | T5 | Pat06 | + | + | - | + | - |
| **19.** | T5 | 4A | + | + | - | + | - |
| **20.** | T5 | Ac. | + | + | - | + | - |
| **21.** | T11 | Z009 | + | + | + | + | - |
| **22.** | T12 | BUD9 | + | + | - | + | - |

# 2. Tolerance Assays:

### Thermotolerance assay

10 µl ($10^3$ amoeba) of amoeba suspensions prepared from axenic cultures were inoculated into the BDOA center on which *E. coli* was smeared, and the plates were incubated in 37 °C, 39 °C and 41 °C

ovens. Amoeba growth was followed under the inverted microscope and at 10th day, strains with and without growth were evaluated as positive (+) and negative (-), respectively. Positive results were considered thermotolerance. Experiments were performed in four repetitions

### Osmotolerance assay

Inoculating 10 µl ($10^3$ amoeba) of amoeba suspensions prepared in the center of 1.5-2% BDOA plates containing 0.5 M and 1 M mannitol, on which *E. coli* was applied, were incubated at 25$^{o}$C. Amoeba growth was monitored 10 days later under an inverted microscope, and strains with and without growth ability were evaluated as positive (+) and negative (-), respectively. Positive results were considered osmotolerance. Experiments were performed in four repetitions.

## Results

Tolerance tests were performed on 22 strains of T4 (T4A, T4B, T4D, T4Neff), T5, T11 and T12 genotypes isolated from clinical and environmental samples. Of these isolates, 22.72% (5/22) were from keratitis cases, 45.45% (10/22) from water samples, 9.09% (2/22) from contact lens case, 4.5% (1/22) from anaconda tissue, 4.5% (1/22) from mouse brain, 4.5% (1/22) from hot tub, 9.09% (2/22) from soil isolated. T4 genotype was dominant in 72.72% (16/22) of the samples, and 4 of the samples in the subgroups of this genotype were clinical and 12 were environmental samples.

All strains in T4 (T4A, T4B, T4D, T4Neff), T5, T11 and T12 genotypes showed growth ability both at 37 $^{o}$C and 0.5 M osmolarity. While growth was observed in 72.72% (16/22) of the isolates at 39°C, only 9% (2/22) of the isolates grew at 41°C and showed high thermotolerance. In the osmotolerance test, 40.9% (9/22) showed strong osmotolerance properties by growing in 1 M mannitol (Table 2).

Table 2
Thermotolerance and osmotolerance test results of *Acanthamoeba* strains

| | Genotype | Strain Name | Isolation |
|---|---|---|---|
| **1.** | T4A | 5SU | Contact lens case (Walochnik, et al. 2000b) |
| **2.** | T4A | SVS12 | Water |
| **3.** | T4A | 9GU | Contact lens case (Walochnik, et al., 2000a) |
| **4.** | T4B | 3ST | Keratitis patient, cornea (Walochnik, et al., 2000b) |
| **5.** | T4B | 1BU | Keratitis patient, cornea (Walochnik, et al., 2000b) |
| **6.** | T4D | SVS6 | Water |
| **7.** | T4D | SVS11 | Water |
| **8.** | T4D | SVS16 | Water |
| **9.** | T4E | 2HH | Keratitis patient, cornea (Walochnik, et al., 2000b) |
| **10.** | T4E | 11DS | Keratitis patient, cornea (Walochnik, et al., 2000a) |
| **11.** | T4Neff | SVS3 | Water |
| **12.** | T4Neff | SVS5 | Water |
| **13.** | T4Neff | SVS7 | Water |
| **14.** | T4Neff | SVS8 | Water |
| **15.** | T4Neff | SVS10 | Water |
| **16.** | T4Neff | X2 | Water |
| **17.** | T5 | 72/2 | Mouse brain (Pumidonming, Koehsler, & Walochnik, 2010) |
| **18.** | T5 | Pat06 | Keratitis patient, cornea (Pumidonming, Koehsler, & Walochnik, 2010) |
| **19.** | T5 | 4A | Soil |
| **20.** | T5 | Ac | Soil |
| **21.** | T11 | Z009 | Anaconda tissue (Pumidonming, Koehsler, & Walochnik, 2010) |
| **22.** | T12 | BUD9 | Hot tub (Pumidonming, Koehsler, Leitsch, & Walochnik, 2014) |

Tolerance tests were applied to 5 strains (3ST, 1BU, 2HH, 11DS, Pat06) isolated from patients with *Acanthamoeba* keratitis. As all of these strains showed growth ability at 37 $^{o}$C and 0.5 M osmolarity, but no growth was detected at 41 $^{o}$C and 1 M osmolarity. Two of these strains (1BU, Pat06) showed growth ability at 39 $^{o}$C (Table 2).

When the osmotolerance and thermotolerance characteristics of the strains with the same genotype were evaluated, the strains with the T5 and T4E genotypes exhibited the same characteristic. When *Acanthamoeba* strains with T4 genotype were evaluated in general, 31.25% (5/16) of the strains grew at 39 $^{O}$C, while growth was found in 6.25% (1/16) of the strains at 41 $^{O}$C. Of the T4Neff strains isolated from environmental samples, because of only one strain (SVS3) did not show the ability to grow at 39 $^{O}$C, showed a different characteristic from the group (Table 2).

While the Z009 strain with the T11 genotype grew at all temperatures, the BUD9 strain with the T12 genotype did not grow at 41 $^{O}$C. These two strains were able to grow in 0.5 M mannitol but not in 1 M mannitol (Table 2).

# Discussion

Two different tolerance experiments were applied to 22 different strains as those that tolerate different temperatures (thermotolerants) and different concentrations of mannitol (osmotolerants). Subgroups of the T4 genotype, most isolated from environmental sources, all strains in the T5, T11 and T12 genotypes grew at 37°C and 0.5 M mannitol. A few strains grew at 39°C and 1 M mannitol, only two strains were observed to grow at 41°C. In previous studies, for tolerance tests applied to *Acanthamoeba* strains, those that tolerate 37–41° C temperature and 0.5 M-1 M mannitol were accepted as potential pathogenic species (Kiss, Barna, Vargha, & Török, 2014; Tawfeek, Bishara, Sarhan, Taher, & Khayyal, 2016; Vijayakumar, 2018). In a different study, it was reported that all of the strains isolated from the soil in South Florida grew at 37°C (Booton, et al., 2004). Booton et al. (2004) showed that 22.2% of the strains isolated from tap water in Cairo, Egypt were osmotolerant and 50% thermotolerant, but these rates reached 15.2% and 58% in the Delta region (Al-Herrawy et al., 2013). In different studies, it has been shown that 66% of the strains isolated from the soil in Ankara, Turkey are both osmotolerant and thermotolerant (Kilic, Tanyuksel, Sissons, Jayasekera, & Khan, 2004). This inconsistency in the results of all tolerance studies is thought to be related to the fact that different *Acanthamoeba* species isolated from various sources from each study may have different physiological characteristics. In addition, although there are many studies on tolerance tests, there is no study comparing the osmotolerance and thermotolerance characteristics of *Acanthamoeba* strains with T4 (Neff, A, B, D, E), T5, T11 and T12 genotypes, both between genotype groups and within species with the same genotype.

For an amoeba to be considered potentially pathogenic, it must exhibit thermotolerant and osmotolerant properties, among several factors. Because these features show the behavior of the amoeba under stressful conditions (Todd, et al., 2015). While many studies have shown that *Acanthamoeba* can grow at 37°C and above to exhibit pathogenic potential in thermotolerance experiments, Schuster (2002) reported that some clinical specimens grow better at 30°C (Todd, et al., 2015). It is known that the strains that can cause *Acanthamoeba* keratitis can grow at 37°C, because the temperature of the human eye is 34°C on average (Walochnik et al., 2000). Since all strains in the thermotolerance experiments in this study grew at 37°C, they may have the potential to cause AK. Two of the five *Acanthamoeba* isolates isolated from keratitis cases were higher temperature thermotolerant, while the other three isolates were less

thermotolerant and osmotolerant. Ledee et al. suggested that this may be due to the exposure of keratitis cornea samples to drugs that may alter physiological properties. Since the human body temperature is 37°C in granulomatous amoebic encephalitis infection, it has been widely accepted that thermotolerance is a prerequisite for pathogenicity (Walochnik, et al., 2000). In the study by Pumidonming et al., it was emphasized that strains that did not grow at this temperature most likely did not cause disease, and even did not cause infection in strains that reproduced (Pumidonming, Koehsler, & Walochnik, 2010).

The T4 genotype is the most common genotype in both keratitis and central nervous system infections. Potential pathogenicity may be in question in this genotype since both in this study and in the cases where Acanthamoeba infections, which are the most common in the world and more than 90% of isolated cases, gave positive results in tolerance tests (Rocha-Cabrera, 2015; Putaporntip, et al., 2021; Kao, et al., 2012). In the literature, it has been supported by these studies that T4 strains, which have a very high growth rate at 37–42°C and 1 M mannitol, reproduce both in clinical and environmental sources (Rocha-Cabrera, et al., 2015; Retana-Moreira, et al., 2015; Landell, Salton, Caumo, Broetto, & Rott, 2013; Behniafar, Niyyati, & Lasjerdi, 2015; Pumidonming, Koehsler, & Walochnik, 2010). Although not directly related to pathogenicity, the thermotolerant property of the genus *Acanthamoeba* has the ability to reproduce at 37°C or higher for some clinical specimens (Putaporntip, et al., 2021). However, as a result of this research, T4D (SVS16) and T11 (Z009) genotypes obtained from environmental sources showed reproduction at high temperature. While all subgroups of T4 genotype were grown in 0.5 M mannitol, only 56.25% (9/16) of them were grown in 1 M mannitol. Its tolerant state at 0.5 M mannitol is the result of its growth in both environmental and clinical strains. However, the most important finding in this study was that there was no growth in 1 M mannitol in T4B and T4E genotypes. The strains in these two sub-genotypes could not tolerate 1 M mannitol as they were isolated from keratitis cases. At the same time, since there are no tolerance experiments for subgroups of T4 genotype in the literature, this study will shed light on the studies to be done on this subject.

T5 genotype is the second most common genotype after T4 genotype. This genotype has been isolated from both environmental and clinical cases (Booton, Visvesvara, Byers, Kelly, & Fuerst, 2005; Siddiqui ve Khan, 2012). Of the four isolates of the T5 genotype in this study, only one was isolated from the clinical case. It has been shown in the literature that T5 isolates can grow at high temperatures (Pumidonming, Koehsler, & Walochnik, 2010; Possamai, Loss, Costa, Falqueto, & Furst, 2018; Booton, et al., 2004). In the studies, none of the strains that were thermotolerant at 40°C could tolerate 1 M mannitol (Pumidonming, Koehsler, & Walochnik, 2010). In this study, these strains of both clinical and environmental origin were able to grow up to a maximum of 39°C and 0.5 M mannitol in tolerance experiments.

The T11 genotype is among the genotypes found both in environmental sources and considered as causative agents of keratitis (Booton, et al., 2004; Pumidonming, et al., 2010). It has been reported that strains with this genotype can grow at 37–40°C and tolerate 0.5 M mannitol (Milanez, et al., 2020; Booton, et al., 2004; Possamai, et al., 2018; Hajialilo, et al., 2016). In addition, Hajialilo et al. showed that strains with the T11 genotype did not grow at 37°C and 0.5 M mannitol, while Possamai et al. showed that they tolerated 1 M mannitol (Hajialilo, et al., 2016; Possamai, et al., 2018). In our study, only one

isolate of T11 genotype was used and this strain was isolated from an environmental source. This isolate was able to tolerate all temperatures, but only 0.5 M mannitol in osmotolerance experiments.

Since the T12 genotype is the genotype that causes encephalitis in humans, it is more common in clinical cases, while its environmental niche is unknown (Booton, Visvesvara, Byers, Kelly, & Fuerst, 2005; Satitpitakul, Putaporntip, & Jongwutiwes, 2021; Blaschitz, et al., 2006; Gavarāne, et al., 2018; Kao, et al., 2012). At the same time, this genotype, which has the most different genotype, is known to be quite lethal (Yu, et al., 1999; Gavarāne, et al., 2018). In the literature, a thermotolerance test was applied to this genotype on a strain isolated from a keratitis case and it was determined that it could reproduce at 42°C. In our study, a single strain showed growth ability at 37°C and 39°C and 0.5 M mannitol in tolerance tests.

It is thought that in vitro growth of *Acanthamoeba* samples may be associated with virulence, partially under high temperature and osmotic stress. Because the virulence of an isolate is relatively related to its ability to adapt to and survive the tissues of the mammalian host (Khan, 2006; Khan ve Tareen, 2003). It is thought that in vivo experiments are needed to determine the pathogenic potential of samples isolated from environmental sources (Landell, Salton, Caumo, Broetto, & Rott, 2013). At the same time, higher ambient temperature is thought to increase the growth of thermotolerant *Acanthamoeba* (Landell, Salton, Caumo, Broetto, & Rott, 2013). It has been suggested that these tolerant strains may have evolved through natural selection to adapt to heat stress in their niche (Landell, Salton, Caumo, Broetto, & Rott, 2013)

The temperatures used in the thermotolerance tests in this study were 37°C, 39°C, and 41°C, respectively, and the concentrations used in the osmotolerance tests were 0.5 M and 1 M mannitol. According to the results of tolerance studies in the literature, for Acanthamoeba to be considered as a potential pathogenic species, it must be able to tolerate both these high temperatures and high mannitol concentrations (Tawfeek, Bishara, Sarhan, Taher, & Khayyal, 2016; Vijayakumar, 2018). However, in our study three of the four strains (3/4) in T4B and T4E genotypes isolated from keratitis cases had no growth at both 39°C – 41°C and 1 M mannitol, we think classification as insignificant according to high temperature and high mannitol concentrations for researchers to associate these studies with pathogenicity.

# Declarations

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**Authors' contributions:** Polat ZA designed and directed the protect; Polat ZA and Kahraman M performed the experiments Polat ZA and Kahraman M discussed the results and commented on the manuscript. Kahraman M wrote the manuscript in consultation with Polat ZA.

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