Turkiye Parazitol Derg 2024;48(1):15-20
DOI: 10.4274/tpd.galenos.2024.92408

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

Acanthamoeba Türlerinin Termotolerant ve Osmotolerant Özellikleri Patojenite Göstergesi midir?

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Cite this article as: Kahraman M, Akın Polat Z. Are Thermotolerant and Osmotolerant Characteristics of *Acanthamoeba* Species an Indicator of Pathogenicity?. Turkiye Parazitol Derg 2024;48(1):15-20.

ABSTRACT

Objective: The aim of this study was to evaluate the pathogenicity of *Acanthamoeba* strains with T4, T5, T11, and T12 genotypes by comparing the osmotolerance and thermotolerance characteristics of *Acanthamoeba* strains isolated from genotype groups, within species with the same genotype, and from environmental and keratitis cases.

Methods: 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) and osmotolerance (0.5 M, 1 M) tests were performed.

Results: All strains showed growth ability at 37 °C and 0.5 M osmolarity. While all five strains isolated from patients with *Acanthamoeba* keratitis showed growth ability at 37 °C and 0.5 M osmolarity, no growth was detected at 41 °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 showed the same characteristics. When *Acanthamoeba* strains with the T4 genotype were evaluated in general, 31.25% of the strains were found to grow at 39 °C and 6.25% at 41 °C. Of the T4Neff strains, only one strain did not show the ability to reproduce at 39 °C and showed a different feature from the other strains. While the strain with the T11 genotype grew at all temperatures, the strain with the T12 genotype did not grow at 41 °C.

Conclusion: According to our research results, we believe that tolerance to 39 °C and 1 M mannitol is not an indicator of pathogenicity. More studies with *Acanthamoeba* strains are required to clarify this issue.

Keywords: *Acanthamoeba*, osmotolerance, thermotolerance, genotype, pathogen

ÖZ

Amaç: T4, T5, T11 ve T12 genotipine sahip *Acanthamoeba* suşlarında genotip grupları arasında, aynı genotipe sahip türler içerisinde ve çevresel ve keratit olgularından izole edilen *Acanthamoeba* suşlarının osmotolerans ve termotolerans özelliklerini karşılaştırarak patojenlikle ilişkisini değerlendirmektir.

Yöntemler: Bu çalışmada, klinik ve çevresel örneklerden izole edilmiş T4 (Neff, A, B, D, E), T5, T11 ve T12 genotipine sahip sahip 22 tane *Acanthamoeba* suşunun aksenik kültürleri yapıldıktan sonra termotolerans (37 °C, 39 °C ve 41 °C) ve osmotolerans (0,5 M, 1 M) testleri yapılmıştır.

Bulgular: Bütün suşlar 37 °C ve 0,5 M osmolaritede üreme yeteneği göstermiştir. *Acanthamoeba* keratitli hastalardan izole edilen beş suşun tamamı 37 °C'de ve 0,5 M osmolaritede üreme yeteneği gösterirken, 41 °C ve 1 M osmolaritede üreme saptanmamıştır. Aynı genotipe sahip suşların tolerans özellikleri değerlendirildiğinde T5 ve T4E genotipine sahip suşlar aynı özellikte bir durum sergilemiştir. T4 genotipine sahip *Acanthamoeba* suşları genel olarak değerlendirildiğinde 39 °C'de suşların %31,25, 41 °C'de %6,25 suşta üreme saptandı. T4Neff suşlarından sadece bir suş 39 °C'de üreme yeteneği göstermeyerek gruptan ayrı bir özellik göstermiştir. T11 genotipine suş bütün ısı derecelerinde ürerken, T12 genotipine sahip 41 °C'de üretememiştir.

Sonuç: Araştırma sonuçlarımıza göre özellikle 39 °C ve 1 M mannitol toleransın patojenlik göstergesi olmadığı kanısındayız. Bu konunun açıklığa kavuşabilmesi için daha çok *Acanthamoeba* suşu ile çalışmaya ihtiyaç vardır.

Anahtar Kelimeler: *Acanthamoeba*, osmotolerans, termotolerans, genotip, patojen

Received/Geliş Tarihi: 06.04.2023 **Accepted/Kabul Tarihi:** 08.01.2024

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Türkiye PARAZİTOLOJİ Dergisi

INTRODUCTION

Species in the genus *Acanthamoeba*, an amoeba, can live in natural ecosystems such as soil, freshwater resources and seas, as well as in artificial areas such as pools, ventilation systems, operating rooms. Simultaneously, it has also been isolated from contact lenses, lens cases, and solutions (1,2). These opportunistic pathogens can cause *Acanthamoeba* keratitis (AK), granulomatous amoebic encephalitis (GAE), Acanthamoeba pneumonia, and cutaneous acanthamoebiasis (3). The global incidence rate of AK cases continues to increase and is associated with contact lens wear as a major factor (4,5). Different strains of *Acanthamoeba* spp. cause amebic encephalitis, which causes fatal brain damage in immunocompromised individuals (3,6). Simultaneously, *Acanthamoeba* species act as endosymbionts for viruses, yeasts, protists, and bacteria that are highly virulence and antibiotic resistant (7).

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 (1,8,9). In the trophozoite form, they can survive if the temperature and pH are suitable and the food is sufficient (7). However, in the absence of these factors, it turns into a double-walled cyst consisting of an endocyst and ectocyst. Cysts resistant to harsh conditions can survive for a long-time and are antibiotics-resistant, chlorine, and disinfectants (10). When the conditions are favorable again, it can return to the trophozoite stage with excistation (11).

The genus *Acanthamoeba* has been named as more than 20 species, consisting of three different groups based on features of cyst morphology (12). However, it is very difficult to differentiate *Acanthamoeba* species at the species level using morphological criteria (1). 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 (13). 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 (2). In this method, known as the genetic approach, only the amount of total difference or similarity is evaluated (14). 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 (15,16). AK is predominantly caused by genotype T4 (17,18). In this genotype, it consists of T4A, T4B, T4C, T4D, T4E, T4F, and T4Neff subgroups according to the defined sequence characteristics (2).

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 (19). In studies, positive *Acanthamoeba* strains that reproduced in thermotolerance and osmotolerance experiments isolated from environmental and clinical specimens were accepted as potentially pathogenic species (20). Studies have suggested that *Acanthamoeba* strains, which are in the same genotype groups and isolated from various sources, show different osmotolarity and thermotolarity, thus showing different pathogenic potentials (9,19,21-27). 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 was 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 to compare environmental and keratitis cases and to compare the osmotolerance and thermotolerance characteristics of isolated *Acanthamoeba* strains and evaluate their relationship with pathogenicity.

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

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

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

	Genotype	Strain name	Isolation
1.	T4A	5SU	Contact lens case (38)
2.	T4A	SVS12	Water
3.	T4A	9GU	Contact lens case (31)
4.	T4B	3ST	Keratitis patient, cornea (38)
5.	T4B	1BU	Keratitis patient, cornea (38)
6.	T4D	SVS6	Water
7.	T4D	SVS11	Water
8.	T4D	SVS16	Water
9.	T4E	2HH	Keratitis patient, cornea (38)
10.	T4E	11DS	Keratitis patient, cornea (31)
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 (21)
18.	T5	Pat06	Keratitis patient, cornea (21)
19.	T5	4A	Soil
20.	T5	Ac	Soil
21.	T11	Z009	Anaconda tissue (21)
22.	T12	BUD9	Hot tub (21)

thoma slide, counting as 1×105 amoebae/mL (99% trophozoites) and used for tolerance tests.

2. Tolerance Assays

Thermotolerance assay

10 µL (103 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 to be thermotolerance. Experiments were performed in four repetitions.

Osmotolerance assay

Inoculating 10 µL (103 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 °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 as osmotolerance. Experiments were performed in four repetitions.

Statistical Analysis

No statistical analysis was required in the study.

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 a 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 in both at 37 °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).

Tolerance tests were applied to 5 strains (3ST, 1BU, 2HH, 11DS, Pat06) isolated from patients with AK. As all of these strains showed growth ability at 37 °C and 0.5 M osmolarity, but no growth was detected at 41 °C and 1 M osmolarity. Two of these strains (1BU, Pat06) showed growth ability at 39 °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 characteristics.

When *Acanthamoeba* strains with the T4 genotype were evaluated in general, 31.25% (5/16) of the strains grew at 39 °C, while growth was found in 6.25% (1/16) of the strains at 41 °C. Of the T4Neff strains isolated from environmental samples, because of only one strain (SVS3) did not show the ability to grow at 39 °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 °C. These two strains could grow in 0.5 M mannitol but not in 1 M mannitol (Table 2).

DISCUSSION

Acanthamoeba species are isolated from many sources and are free-living amoebae, commonly in soil and water. Disease-causing potentials of these amoebae, which are widely found in nature and have many species, are being investigated *in vitro* and *in vivo*, but there is no definitive method on this subject. The thermotolerance and osmotolerance properties of amoebae are used to determine the potential pathogenicity of these amoeba (25,27). According to these studies, it is thought that for an amoeba to be considered as potentially pathogenic, it must exhibit thermotolerant and osmotolerant properties, as well as various factors. Demonstrating thermotolerant properties, resisting high temperatures, and exhibiting osmotolerance in the same way means resisting high concentration osmolarity. Since these features show the behavior of the amoeba under stressful conditions, they are considered as findings pointing to the pathogen effect (25).

In this study, 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, and all strains in the T5, T11, and T12 genotypes grew at 37 °C and 0.5 M mannitol. A few strains

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

	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	+	+	-	+	-

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 (9,20). In a different study, it was reported that all the strains isolated from the soil in South Florida grew at 37 °C (19). Booton et al. (19) in 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 (28). In different studies, it has been shown that 66% of the strains isolated from the soil in Ankara, Türkiye are both osmotolerant and thermotolerant (29). 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. Additionally, 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.

It is thought that the *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 (8). It is thought that *in vivo* experiments are needed to determine the pathogenic potential of samples isolated from environmental sources (30). Simultaneously, a higher ambient temperature is thought to increase the growth of the thermotolerant *Acanthamoeba* (30). It has been suggested that these tolerant strains have evolved through natural selection to adapt to heat stress in their niche (30).

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 (25). It has been reported that the growth of *Acanthamoeba* at 37 °C and above is evidence of its pathogenic potential (25). It is known that the strains that can cause AK can grow at 37 °C because the temperature of the human eye is 34 °C on average (31). Since all strains in the thermotolerance experiments in this study grew at 37 °C, they may can cause AK. Two of the five *Acanthamoeba* isolates isolated from keratitis cases were higher temperature thermotolerant, whereas the other three isolates were less thermotolerant and osmotolerant. Ledee et al. (32) 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 GAE infection, it has been widely accepted that thermotolerance is a prerequisite for pathogenicity (31). In the study by Pumidonming et al. (21), 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.

The T4 genotype is the most common genotype in both keratitis and central nervous system infections (23). 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 (16,23). 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 (21-24,30). Although not directly related to pathogenicity, the thermotolerant properties of the genus Acanthamoeba can reproduce at 37 °C or higher for some clinical specimens (16). 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 the 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 the T4B and T4E genotypes. The strains in these two subgenotypes could not tolerate 1 M mannitol as they were isolated from keratitis cases. Simultaneously, since there are no tolerance experiments for subgroups of the T4 genotype in the literature, this study will shed light on the studies to be done on this subject.

The T5 genotype is the second most common genotype after the T4 genotype (33). This genotype has been isolated from both environmental and clinical cases (7,34). 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 (19,21,26). In the studies, none of the strains that were thermotolerant at 40 °C could tolerate 1 M mannitol (21). In this study, these strains of both clinical and environmental origin could grow up to a maximum of 39 °C and 0.5 M mannitol in tolerance experiments. Additionally, the 72/2 strain isolated from the T5 genotype mouse brain is the same as the strain used in the study by Walochnik et al. (21,31). The 72/2 strain exhibited thermotolerant properties up to 42 °C in thermotolerance experiments (21,31). However, in the thermotolerance tests in this study conducted in 2022, the 72/2 strain tolerated a maximum temperature of 39 °C. In the study by Pumidonming et al. (21), in 2010, it was reported that a decrease in the pathogenicity of *Acanthamoeba* strains maintained in long-term axenic cultures was observed. One of the important findings in this study is to support the view that pathogenicity is weakened by the decrease in thermotolerance tests of the same strain after 22 years.

The T11 genotype is among the genotypes found both in environmental sources and considered as causative agents of keratitis (19,21). It has been reported that strains with this genotype can grow at 37-40 °C and tolerate 0.5 M mannitol (19,26,27,35). Additionally, Hajialilo et al. (35) showed that strains with the T11 genotype did not grow at 37 °C and 0.5 M mannitol, while Possamai et al. (26) showed that they tolerated 1 M mannitol. In our study, only one isolate of the T11 genotype was used, and this strain was isolated from an environmental source. This isolate could tolerate all temperatures, but only 0.5 M mannitol in osmotolerance experiments.

Because the T12 genotype is the genotype that causes encephalitis in humans, it is more common in clinical cases, while its environmental niche is unknown (16,34,36). Simultaneously, this genotype, which has the most different genotype, is known to be quite lethal (36,37). 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 (18). In our study, it showed growth ability at 37 °C and 39 °C and 0.5 M mannitol in tolerance tests performed on a single and environmental strain. Compared to the study by Satitpitakul et al. (18), the clinical strain showed very high growth

in thermotolerance tests, indicating that different results were obtained with the growth of the environmental isolate in this study at a lower temperature. Additionally, thermotolerance tests for the T12 genotype are quite limited in the literature. To the best of our knowledge, this is the first study to apply osmotolerance tests.

CONCLUSION

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 a potential pathogenic species, it must be able to tolerate both these high temperatures and high mannitol concentrations. 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 the classification as insignificant according to high temperature and high mannitol concentrations for researchers to associate these studies with pathogenicity.

***Information:** It is an article produced from the Master's Thesis of Merve Kahraman at Sivas Cumhuriyet University, Institute of Health Sciences, Department of Medical Parasitology.

***Acknowledgments:** The authors thank Julia Walochnik, Department of Medical Parasitology, Clinical Institute of Hygiene, University of Vienna, Vienna, Austria, for providing *Acanthamoeba* strains.

* Ethics

Ethics Committee Approval: Not applicable. The five strains of AK isolated are those sold in the ATCC culture collection. Ethics committee and patient approval were not required as it was not isolated from AK patients.

Informed Consent: Informed consent were not required as it was not isolated from AK patients.

* Authorship Contributions

Concept: M.K., Z.A.P., Design: M.K., Z.A.P., Data Collection or Processing: M.K., Z.A.P., Analysis or Interpretation: M.K., Z.A.P., Literature Search: M.K., Z.A.P., Writing: M.K., Z.A.P.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: This study was supported by the Scientific Research Project Fund of Sivas Cumhuriyet University under the project number T-938.

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