The Prevalence, Isolation and Morphotyping of Potentially Pathogenic Free-Living Amoebae from Tap Water and Environmental Water Sources in Sivas

Sivas İlinde Potansiyel Patojen Serbest Yaşayan Amip Türlerinin Musluk Sularında ve Çevresel Su Kaynaklarında Yaygınlığı, İzolasyonu ve Morfotiplendirmesi

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ABSTRACT

Objective: To our knowledge, there is no study dealing with the prevalence of free-living amoebas (FLA) in water sources in Turkey, previous studies were mostly case presentations. The aim of the present study was to investigate the prevalence of FLA from tap water and natural water sources in different parts of the city.
Methods: In the study, 250 samples were collected from the city centre, districts and villages. Two litres of water was collected from each source and filtered through a vacuum filtration system. The filter papers were washed in "Page's Amoeba Saline (PAS)" solution and incubated overnight. Filter papers were removed from the tubes and centrifuged; the final pellet was inoculated on non-nutrient agar (NNA) plates. The growth rate of FLA was checked after three days of inoculation and the flagellation test was performed to determine the presence of *Naegleria* spp. Heat tolerance of isolated strains was checked at 37, 42 and 52°C for the presence of pathogenic *Acanthamoeba* species. The cyst and trophozoite morphology of amoebas were examined under a light microscope and the genera was identified according to morphotyping keys.
Results: FLA were found in 75 (30.0%) of examined water samples. Eleven (4.4%) were identified as *Acanthamoeba* spp., 25 (10.0%) as *Naegleria* spp. and 39 (15.6%) as *Hartmannella* spp. after microscopic examination.
Conclusion: Our study revealed that FLA are common inhabitants of household water as they are in the environment, so their own potential risks as well as transferring bacteria as other pathogens is important for human health. *(Turkiye Parazitol Derg 2012; 36: 198-203)*

Key Words: Free living amoeba, *Acanthamoeba, Naegleria*, isolation

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ÖZET

Amaç: Ülkemizde günümüze kadar su kaynaklarında serbest yaşayan amip (SYA) yaygınlığına yönelik kapsamlı bir çalışma yapılmamış, genellikle olgu sunumlarında SYA varlığı bildirilmiştir. Bu çalışmanın amacı Sivas ilinde musluk sularında ve çevresel su kaynaklarında SYA yaygınlığının belirlenmesidir.
Yöntemler: Çalışma kapsamında şehir merkezinden, ilçelerden ve köylerden toplam 250 örnek toplanmıştır. Her bir kaynaktan iki litre su alınarak vakumlu filtrelerden süzülmüştür. Filtre kağıtları steril "Page's Amoeba Saline (PAS)" solüsyonunda bir gece bekletilmiştir. İnkübasyon sonrası filtreler çıkarılıp tüp santrifüj edildikten sonra dip kısımdan alınan bir iki damla örnek, Besleyici-Değeri Olmayan Agar (BDOA) plaklarına inoküle edilmiştir. İnkübasyonun üçüncü gününden itibaren besiyerlerinde üreme kontrolleri yapılmıştır. *Naegleria* spp. belirlenmesi

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için kamçı deneyi yapılmış, patojenik *Acanthamoeba* türleri için de 37°C, 42°C ve 52°C de ısı tolerans testi uygulanmıştır. Amiplerin kist ve trofozoit morfolojileri ışık mikroskobu altında incelenmiş ve morfolojik anahtarlar kullanılarak cins ayrımları yapılmıştır.

Bulgular: İncelenen toplam 250 örneğin 75'inde (%30.0) SYA tespit edilmiştir. Bu türlerin mikroskobik olarak temel morfotiplendirme anahtarlarına göre incelemelerinde 11'inin (%4.4) *Acanthamoeba* spp., 25'inin (%10.0) *Naegleria* spp. ve 39'unun (% 15.6) *Hartmannella* spp., olduğu belirlenmiştir.

Sonuç: Su örneklerinde bu kadar yaygın SYA bulunması, hem amiplerin kendileri hem de taşıdıkları çeşitli bakteriler nedeniyle insan sağlığı açısından önemli risk oluşturmaktadır. *(Turkiye Parazitol Derg 2012; 36: 198-203)*

Anahtar Sözcükler: Serbest yaşayan amipler, *Acanthamoeba, Naegleria*, izolasyon

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INTRODUCTION

Acanthamoeba and *Naegleria* are the most common Free-living amoebas (FLA) that are associated with human and animal diseases (1-4). *Balamuthia mandrillaris, Hartmannella* and *Sappinia* species are also free-living amoebae but are less common causes of clinically significant infections (5, 6). The disease of the central nervous system by infection of *Naegleria* spp. was first documented in 1965 and the disease was called primary amoebic meningoencephalitis (PAM) (2). *N. fowleri* is the only species of *Naegleria* that causes human disease (7); it can be isolated from soil and fresh water (8, 9). *Acanthamoeba* is another important group of FLA that is commonly found in the environment. Previously, it was isolated from many different environment and clinical samples: soil, water, sewage water, tap water, thermal water mud, air, sea water, ear, lung secretions and nasopharyngeal mucosa samples (2, 3, 9-23). They are the causative agents of granulomatous amoebic encephalitis (GAE) and Acanthamoeba Keratitis (AK) (1, 24-27). In the subsequent years, *Acanthamoeba* were found to be responsible for some other lesions in eyes, ears, skin and innards (1). *B. mandrillaris* may also cause GAE, and was first isolated from mandrill monkeys in 1986; to date, more than one hundred cases have been reported (6). In the early 2000s, *Sappinia diploidea* was isolated from a patient with amoebic encephalitis (5); other species of *Sappinia* have since been isolated from faecal-contaminated soils. Another FLA genera is *Hartmannella* and some records are available regarding the potential pathogenicity of *H. vermiformis* in humans (13). However, to date, both experimental and clinical studies about FLA have been rather limited regarding the isolation of parasite from environment. In addition to their pathogenic potential, these amoebas may act as a "Trojan horse" of many different types of bacteria and virus. These pathogens can lead to severe human disease as complications of amoebic keratitis. For these reasons, the health importance and pathogenic potential of FLA has been better explained in recent years (28-30).

Free-living amoeba-associated diseases are relatively rare among people when compared with their environmental abundance. However, the illnesses caused by pathogenic FLA are severe, and often challenging to treat, so a better understanding of their ecologic distribution is necessary in the places where humans interact with FLA (31, 32). In Turkey, there is no study about the current prevalence of FLA in water sources and other environments. The aim of the present study was to investigate the prevalence of FLA in natural sources and domestic water systems in Centrum, districts and villages.

METHODS

Study Area

Sivas is located at the eastern part of the Central Anatolian region of Turkey; it is the second largest province in Turkey. According to the 2007 Turkish census, its population was 300,795. The city, which lies at an elevation of 1,278 m in the broad valley of the Kızılırmak river, is a moderately-sized trade centre and industrial city, although the economy has traditionally been based on agriculture.

The study sample size was determined at α: 0.05, d: ±0.06 according to the prevalence of previous studies. The sample size was approximately 250 samples.

Collection and Filtration of Water Samples

Specimens were collected from faucets in Centrum and fountains of the villages between June and December 2010. Twenty-five of the samples were surface water (streams) in rural areas (Divriği, Şarkışla, Kangal, Suşehri, Gemerek, Altınyayla, Gürün, Ulaş, Koyulhisar, and Akıncılar districts), 8 were from hot springs (Kangal, Yıldızeli, Hafik), 2 were from creeks (branches of Kızılırmak) and 4 were from wells. The distribution of 250 samples according to regions were as follows: 24 from Centrum (fountain and faucets), 43 (tap water) from districts and 144 (tap water-fountains) from villages. Water samples were collected with 2-litre sterile glass bottles and filtered. A vacuum filtration system with 0.45 µm pore size was used in the study (Sartorius AG, Goettingen, Germany). The specimens were transported and stored at ambient temperature and cultured for amoebae within 3 days. Filter papers were stored in sterile glass tubes until examination.

The Incubation of Samples and Growth

Filter papers were incubated overnight in 15 mL sterile buffer solution. In the following day, the tubes were centrifuged at 1500 rpm for 10 minutes. A few drops of pellet were inoculated on non-nutrient agar (NNA) with a lawn of inactive *Escherichia coli*. NNA was prepared with Page Amoeba Saline (PAS) (2.5 mM NaCl, 1 mM KH_2PO_4, 0.5 mM, Na_2HPO_4, 40 µm $CaCl_2$-$6H_2O$ and 20 µm $MgSO_2.7H_2O$). Agar was dissolved in PAS (1.5%), autoclaved and dispensed onto sterile plates. After inoculation, the plates were incubated at 30°C (8, 10).

Growth Control and Passages

After 3 days of incubation, the plates were monitored for the detection of trophozoites or cysts of amoeba daily until 15 days using light microscopy. In order to obtain fresh cultures, approximately 1 cm^2 of agar was taken from the grown culture and placed at the centre of new NNA plates. The strains were maintained by serial passages in this medium (8, 10).

Table 1. The source of water samples and the prevalence of FLA

Water Source	(+)		(-)		Total examined	
	No	%	No	%	No	%
Tap water (Drinking)	62	29.4	149	70.6	211	84.4
Environmental water samples (stream, hot spring, creek, well water)	13	33.3	26	66.7	39	15.6
Total	75	30.0	175	70.0	250	100.0
(χ^2: 0.24, p>0.05)						

Identification of FLA at Genera Level

In order to determine the genera of FLA, the movement and structural properties of amoebas were examined. Additionally, the flagellation test (FT) was used to identify *Naegleria*. After examination under a light microscope permanent smears were prepared and stained with Trichrome (33). A piece of agar was placed over a slide incubated in a humid environment for an hour. The transferred amoebas were fixed with Schaudinn at 37°C for a few minutes on the slides. Then, the slides were fixed in the same solution for an hour. For the morphological identification of isolates, we utilised from the study of Smirnov and Goodkov (34).

The Flagellation test

The organism is exposed to a hypotonic environment in the test. The amoebas were collected from plates and put into 1 mL distilled water. After 2 hours of incubation at 37°C, 100 µL of sample was transferred to slides and examined under light microscope for the presence of any free-swimming flagellates (12, 18).

Heat Tolerance Test

Previously cultured FLA cysts were inoculated in three fresh NNA, as described before. One of the plates was incubated at 37°C and the others at 42°C and 52°C. After two days of inoculation, the growth rate and cell motility of FLA at different temperatures were recorded daily under light microscope (12).

Axenic Culture of FLA

The isolates were axenically cultured with protease peptone, yeast extract, and glucose (PPYG) medium in 25 cm^2 Corning® flasks and incubated at 35°C. PPYG medium was prepared as described previously: 0.4% protease peptone, 0.2% yeast extract, 1.0% glucose. Before axenisation, amoebas were removed from NNA with a spatula and washed three times in PAS by centrifugation at 500 x g. The pellet was inoculated in PPYG, and gentamicine (50 µg/mL) was added to medium to inhibit bacterial growth (33).

Statistical Analysis

Data was analysed statistically with SPSS 14.0 for Windows software. The Chi-square test was used to compare results and the p value was set at 0.05.

RESULTS

Free-living amoebas were recovered from 75 out of 250 (30%) water samples. The prevalence of FLA in tap water (29.4%) and an almost identical proportion was recovered from samples from environmental sources (33.3%; Table 1). *Acanthamoeba* spp. were identified in 11 (4.4%), *Naegleria* spp. in 25 (10.0%) and *Hartmannella* spp. in 39 (15.6%) with morphotyping (Figure 1). The statistical comparison of different regions (Centrum, districts and villages) and more detailed representation of environmental sources are given in Tables 2, 3 and 4. The prevalence of FLA was higher in villages than in Centrum (χ^2=6.424, p<0.05). Interestingly, eight of the 11 *Acanthamoeba* isolates were from a district, Kangal.

In a heat tolerance test, 50 strains were grown at 37°C, 12 strains at 42°C and 3 strains at 52°C. Additionally, we observed that as the temperature increased the growth rate of FLA decreased.

Despite being repeated twice, the flagellated form of *Naegleria* spp. could not be observed in the flagellation test. The axenisation of strains was achieved only for five (10%) of the 50 samples. All of the strains that successfully axenised were *Acanthamoeba*. Genomic DNA of strains was isolated and stored for genotyping.

We examined the cyst and trophozoites morphology of isolated strains. The vegetative forms of amoebas resembled each other. However, the difference in pseudopods is important. *Acanthamoeba* were identified according to its hyaline lobopode and *Hartmanella* were identified according to its rod shaped trophozoites. Cyst forms of *Acanthamoeba* were very typical with a star-like shape, and were easy to differentiate as Group I and II. The differentiation of *Naegleria* and *Hartmannella* could be performed according to trophozoite forms.

DISCUSSION

Free-living amoebas are distributed worldwide and have been isolated from domestic tap water, drinking water, natural and treated water, sea water and bottle water (2). In the present study, we investigated the prevalence of FLA in stream, hot spring, creek and well water, as well as in domestic tap water systems in Sivas Centrum, districts and villages.

Free-living amoebas, such as the genera *Naegleria*, *Acanthamoeba,* and *Vahlkampfia,* have been commonly found in various environments all around the world and recognised as important pathogens of humans or animals (28). *N. fowleri* is the causative agent of PAME and the transfer of infection to healthy humans occurs via contaminated waters. *Acanthamoeba* spp. and *B. mandrillaris* are opportunistic pathogens of immunosuppressed people and mostly cause GAE. The pathology of disease can be observed in the lungs, sinuses and skin in immunodeficient patients (31, 35). Additionally, *Acanthamoeba* spp. invade the cornea of the eye and cause AK due to contact lens usage (3, 11, 32, 36, 37). Besides their pathogenicity, FLA may transfer some other pathogens to the human body (30).

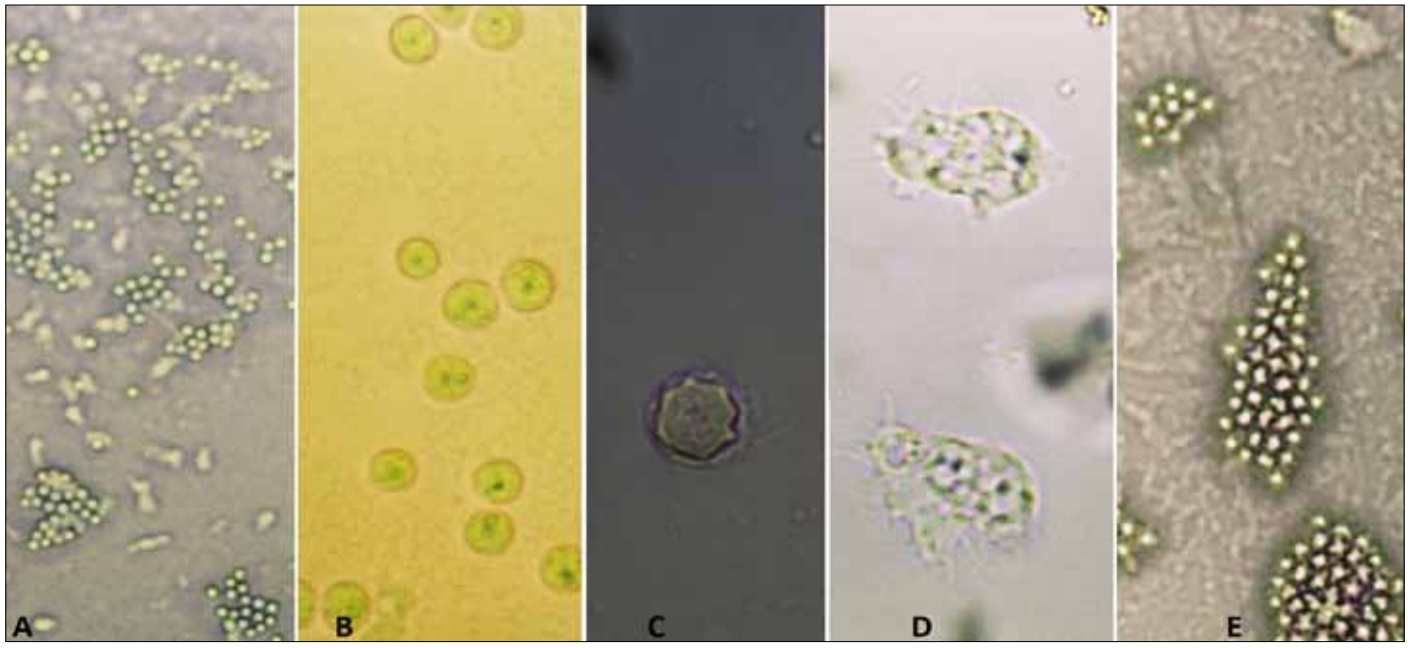

Figure 1. The isolated amoebas from water samples: A. The cysts and trophozoites of *Naegleria* spp. on NNA(x10), B. *Naegleria* spp. cysts on NNA(x40), C. *Acanthamoeba* spp. cysts in saline (x40), D. *Acanthamoeba* spp. trophozoites in saline (x40), and E. *Acanthamoeba* spp. cysts on NNA (x10)

Table 2. The regional distribution of tap water samples and the prevalence of FLA

Region	(+)		(-)		Total examined	
	No	%	No	%	No	%
Centrum	2	8.3	22	91.7	24	9.6
Districts	11	25.6	32	74.4	43	17.2
Villages	49	34.0	95	66.0	144	57.6
Total	63	30.0	175	70.0	211	100.0

Table 3. The statistical comparison of FLA prevalence according to regions

Districts-Villages	χ^2: 1.084	p>0.05
Districts-Centrum	χ^2: 2.93	p>0.05
Villages-Centrum	χ^2: 6.424	p<0.05*
*important		

Free-living amoebas species tolerate temperature ranges of 10-30°C. In the study, in the thermotolerance test, 50 strains were grown at 37°C, 12 out of the 50 clones were able to grow at 42°C after two days. Morphologically, 11 out of the twelve isolates displayed acanthapodia, and the presence of double-walled cysts was identified. While these eleven isolates were determined as belonging to the genus *Acanthamoeba* spp. one isolate was determined as *Hartmannella* spp. Three out of 50 clones were also able to grow at 52°C after two days. These isolates were also morphologically determined as *Acanthamoeba* spp. Three samples were taken from Kangal, Divriği and Suşehri town. The axenisation of strains were achieved only for five (10%) of the 50. All of the strains that successfully axenised in PPYG medium were *Acanthamoeba*.

The prevalence of FLA was reported to be between 23% and 89% from swimming pools, springs, lakes and tap water (2, 17, 20). In Germany, the following genera were identified from hot springs: *Acanthamoeba* (22%), *Naegleria* (22%), *Vahlkampfia* (20%), *Hartmannella* (15%), and *Vannella* (7%) (23). The most common was *Hartmannella* spp. in our study, which accounted for almost 50% of the isolated amoebas. FLA were detected in 80% of environmental water sources in Bulgaria and in 9.3% of tap water in USA, and 79% of river water in Germany (13, 18, 19). In our country, there has been no study dealing with the prevalence of FLA on a large scale, especially from water sources. In Kayseri, FLA were found in 5 (19.2%) samples of well water (38). In the present study, FLA were investigated in 250 samples, mostly comprising tap water. FLA were recovered from 75 out of 250 (30%) water samples. The prevalence of FLA in tap water (29.4%) and an almost identical proportion was recovered from samples from environmental sources (33.3%). *Acanthamoeba* spp. were identified in 11 (4.4%), *Naegleria* spp. in 25 (10.0%) and *Hartmannella* spp. in 39 (15.6%) via morphotyping. The prevalence of FLA obtained was higher in villages than in Centrum (χ^2=6.424, p<0.05). Interestingly, the highest *Acanthamoeba* isolates were established from one district, Kangal.

CONCLUSION

The results in this study show that potentially pathogenic FLA are widely distributed, even in drinking water. In particular in the

Table 4. The source of environmental water samples and the prevalence of FLA

	(+)		(-)		Total examined	
	No	**%**	**No**	**%**	**No**	**%**
Stream	6	24.0	19	76.0	25	64.1
Hot spring	4	50.0	4	50.0	8	20.6
Creek	1	50.0	1	50.0	2	5.1
Well water	2	50.0	2	50.0	4	10.2
Total	13	33.3	26	66.7	39	100.0

areas where tap water was possibly contaminated with soil, the prevalence of FLA was higher; the prevalence was low in Centrum, because municipal water purification systems use chlorine to remove harmful microorganisms from the water supply. However, environmental strains are more resistant to several chemicals than collection strains (14). This highlights the importance of effective disinfection in water supply systems for protection against FLA.

In the present study, FLA were recovered from a variety of ecological habitats using culture methods. It was clear that FLA were common anywhere that people can be found. The classification of FLA as potential pathogens or non-pathogens is not acceptable and knowledge of the prevalence of FLA in household water can provide a focus for the prevention of amoeba-associated illnesses.

Conflict of Interest

No conflict of interest was declared by the authors.

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