



Investigation of the Presence of *Francisella tularensis* and *Acanthamoeba* spp. in the Drinking Water of Sivas Province, Turkey

Sivas İli İçme Sularında *Francisella tularensis* ve *Acanthamoeba* spp. Varlığının Araştırılması

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Cite this article as: Ataş M, Poyraz Ö. Investigation of the presence of *Francisella tularensis* and *Acanthamoeba* spp. in the drinking water of Sivas province, Turkey. FLORA 2021;26(1):163-71.

ABSTRACT

Introduction: Tularemia is a zoonotic disease caused by *Francisella tularensis*. *F. tularensis* subsp. *holarctica* is primarily isolated in Northern Europe, the Balkans, Turkey, Siberia, and the Far East, and it is mostly responsible for waterborne outbreaks. *F. tularensis*, which is a facultative intracellular bacterium, may survive inside *Acanthamoeba castellanii*, which is a free-living amoeba in water resources; this symbiotic life allows the bacteria to survive in water and mud for months. In this study, drinking water samples taken during the tularemia outbreak and non-outbreak regions were compared in terms of the presence of *Francisella tularensis* and *Acanthamoeba* spp., and the role of *Acanthamoeba* was investigated in tularemia outbreaks observed in Turkey.

Materials and Methods: This study was conducted in the province of Sivas located in the central Anatolia region in Turkey, and a total of 300 water samples were used. Glucose-cysteine-blood agar (GCBA) and non-nutrient agar (NNA) were used for the isolation of *F. tularensis* and *Acanthamoeba*, respectively. Each isolate was identified by PCR technique.

Results: A total of 30 (10%) *F. tularensis* and 28 (9.33%) *Acanthamoeba* spp. isolates were produced by culture method. All bacterial strains were confirmed with PCR as to be *F. tularensis* subsp. *holarctica*. In our study, concurrent presence of *F. tularensis* and *Acanthamoeba* were detected in two water samples. There were no statistically significant differences between the water samples taken from outbreak and non-outbreak regions in terms of the presence of *Acanthamoeba* ($p > 0.05$).

Conclusion: Even though experimental studies have shown that *F. tularensis* may survive inside *Acanthamoeba* spp. and its viability continues, our study could not identify a relationship between tularemia outbreak and *Acanthamoeba* spp. New research on this issue may have a contribution on tularemia epidemiology.

Key Words: *Francisella tularensis*; *Acanthamoeba*; Tularemia; Tap water; PCR

ÖZ

Sivas İli İçme Sularında *Francisella tularensis* ve *Acanthamoeba* spp. Varlığının Araştırılması

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Giriş: Tularemi *Francisella tularensis* tarafından oluşturulan zoonotik bir hastalıktır. Çoğunlukla su kaynaklı salgınlardan sorumlu olan *F. tularensis* subsp. *holarctica*, başlıca Kuzey Avrupa, Balkanlar, Türkiye, Sibiry, Uzak Doğu'dan izole edilmektedir. Fakültatif bir hücre içi bakteri olan *F. tularensis*, su kaynaklarında serbest yaşayan bir amip olan *Acanthamoeba castellanii* içinde yaşayabilir; bu simbiyotik yaşam, bakterilerin aylarca suda ve çamurda hayatta kalmalarını sağlamaktadır. Bu çalışmada tularemi salgını bölgelerinden ve salgın dışı bölgelerden alınan içme suyu, *Francisella tularensis* ve *Acanthamoeba* spp. varlığı açısından karşılaştırılmış ve *Acanthamoeba*'nın Türkiye'de gözlenen tularemi salgınlarındaki rolü araştırılmıştır.

Materyal ve Metod: Bu çalışma, Türkiye'de İç Anadolu bölgesindeki Sivas ilinde gerçekleştirildi ve toplam 300 su örneği kullanıldı. *F. tularensis* ve *Acanthamoeba* izolasyonu için sırasıyla glukozlu sisteinli kanlı agar (GSKA) ve besleyici değeri olmayan agar (BDA) kullanıldı. Her izolat PCR yöntemi ile tiplendirildi.

Bulgular: Toplamda kültür yöntemi ile 30 (%10) *F. tularensis* ve 28 (%9.33) *Acanthamoeba* spp. izolatu üretildi. Tüm bakteriyel kökenlerin *F. tularensis* subsp. *holarctica* olduğu PCR ile doğrulandı. Çalışmamızda iki su örneğinde *F. tularensis* ve *Acanthamoeba*'nın aynı anda bulunduğu tespit edildi. Salgın görülen ve salgın görülmeyen bölgelerden alınan su örneklerinde *Acanthamoeba* varlığı açısından istatistiksel olarak anlamlı fark saptanmadı ($p > 0.05$).

Sonuç: Deneysel çalışmalar *F. tularensis*'in *Acanthamoeba* spp. içerisinde hayatta kalabileceğini ve yaşamını sürdürebileceğini göstermesine rağmen çalışmamızda tularemi salgınları ve *Acanthamoeba* spp. arasında bir ilişki saptanmadı. Bu alanda yapılacak yeni araştırmalar tularemiya epidemiyolojisine katkı yapabilir.

Anahtar Kelimeler: *Francisella tularensis*; *Acanthamoeba*; Tularemi; Çeşme suyu; PCR

INTRODUCTION

Francisella tularensis is a gram-negative, coccobacillus bacteria found in the northern hemisphere, causing tularemia disease. *F. tularensis* is transmitted by the inhalation of infective aerosols, the consumption of contaminated water and foods, being in contact with infectious fluid and animal tissues, and the bite of ticks and some flies. *F. tularensis* subsp. *tularensis* is transmitted to the humans by tick and mosquito, and it is found in Northern America. *F. tularensis* subsp. *holarctica* is found in Europe and Asia, and it is responsible for waterborne diseases^[1,2].

Tularemia disease has two different cycles, namely terrestrial cycle and water cycle. The main vectors of the terrestrial cycle are wild rabbits, ticks and certain fly species; whereas, beaver, muskrat, and other rat species play a role in the water cycle. The agent of the terrestrial cycle is *F. tularensis* subsp. *tularensis*, whereas, the agent

of the water cycle is *F. tularensis* subsp. *holarctica*^[3,4]. *F. tularensis* subsp. *holarctica* is primarily isolated in Northern Europe, the Balkans, Turkey, Siberia, and the Far East, and it is mostly responsible for waterborne outbreaks^[5].

Even though the most common infection route in the world are contact with infected animal and tick, the consumption of non-chlorinated drinking water or spring water constitutes the main mode of transmission in Turkey, except for some rare cases transmitted by bovine saliva or tick borne^[6,7]. The agent of tularemia outbreaks in Turkey is *F. tularensis* subsp. *holarctica*. It is known that the outbreaks are waterborne and it is acknowledged that water resources have been contaminated by rodents^[8].

More than 100 wild and domesticated mammal species, 25 bird species and some types of reptiles, ticks, and flies are accepted as reservoir for tularemia. Despite all of this

information, there is not enough knowledge about the principal reservoir of *F. tularensis*. It is stated that amoebae of the genus *Acanthamoeba* can host the bacteria that are tularemia agents^[1,5].

In nature, there are various amoeba genus and species living freely in the environment such as soil, air and water. Among these amoebas, which are defined as voluntary or opportunistic pathogens, the ones that cause infection in humans belong to the *Acanthamoeba*, *Naegleria*, *Balamuthia*, *Vahlkampfia* and *Sappinia* genus^[9,10]. In nature, free-living amoebas of the genus *Acanthamoeba* are isolated from various environments, they are commonly present in natural waters. Two phases can be distinguished in the life cycle of amoebae of the genus *Acanthamoeba*. The first phase is trophozoite form, where they are actively feeding, growing, multiplying and moving, whereas the second phase is the cyst form, where they are more resistant to external environmental conditions^[9,10].

Free-living amoebas feed by taking bacteria, algae and fungus found in the environment into the cell through phagocytosis. Numerous microorganisms having the habit of intracellular life may survive and multiply inside the amoebas through different mechanisms^[11]. *F. tularensis*, which is a facultative intracellular bacterium, may survive inside *Acanthamoeba castellani*, which is a free-living amoeba in water resources; this symbiotic life allows the bacteria to survive in water and mud for months. It has been shown that *F. tularensis* can survive and multiply in the vacuole inside of trophozoite, in vesicles released out and in cysts^[11-13].

In case of producing the culture of *F. tularensis* and *A. castellani* together, intracellular bacteria can keep their vitality for more than three weeks. In case of cultivating *F. tularensis* alone, there is no living bacteria left in the environment after two weeks^[13]. It is known that different genus of *F. tularensis* are taken inside *A. castellani* at different rates and they are multiplied inside the amoeba. In addition, it has been observed that there were bacteria in intracellular vesicles of amoeba 30 minutes after the infection of *A. castellani* by the pathogen *F. tularensis*. It is reported that live bacteria can be obtained from *A. castellani* cysts after 21 days. It is suggested

that amoebae of the genus *Acanthamoeba* may be a significant environmental reservoir for *F. tularensis*^[13,14].

This study aimed to compare drinking water samples taken from tularemia outbreak and non-outbreak regions in the province of Sivas, in terms of the presence of *F. tularensis* and *Acanthamoeba* spp. and investigate the role of amoebae of the genus *Acanthamoeba* in tularemia outbreaks observed in Turkey.

MATERIALS and METHODS

This study was conducted in Sivas, which is a city located in the Central Anatolia region of Turkey. Three hundred samples of drinking water, collected between June 2011 and March 2012, were used in the study (Figure 1). Two hundred water samples were collected from 29 settlements, where tularemia outbreak had been observed (according to the information received from Sivas Provincial Directorate of Health). Water samples of 100 villages, where tularemia disease had not been observed, were selected from the water samples sent to Sivas Public Health Laboratory for periodic analysis (Table 1,2). Water samples were put in 2-liter plastic bottles and delivered to the laboratory in cold chain.

Water samples were filtered through 0.22 µm pore diameter cellulose acetate membrane filters and after the filtration the filters were cut into two, by using a sterile scalpel. Half of the filter was placed on the antibiotic (Oxoid SR0147 and 150 U/mL Penicillin G) added Glucose Cysteine Blood Agar (GCBA) medium. The mediums were incubated at 37°C, 5% CO₂ for 4-10 days. Agglutination test was carried out on the colonies formed on the filter with *F. tularensis* antiserum (BD Bioscience).

The other half of the filter was used on the isolation of DNA and *Acanthamoeba* spp. Filters were placed in 2 mL Eppendorf tubes containing water sample, kept one night at room temperature and vortexed for 5 minutes. 1 mL of water sample was taken and DNA isolation was carried out. For the isolation of *Acanthamoeba* spp., a water sample of 100 µL was inoculated on 1.5% non-nutrient agar (NNA) plates containing *Escherichia coli*. Plates were incubated at 24°C and they

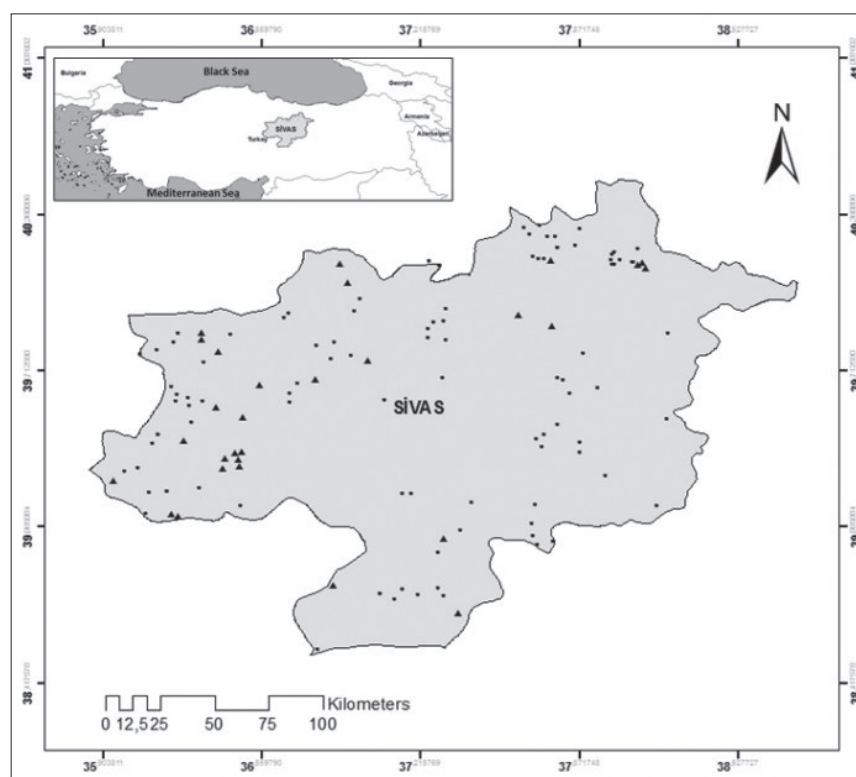


Figure 1. Geographical location of Sivas province and the distribution of the water samples used in the study (Triangles: outbreak regions, dots: non-outbreak regions).

Table 1. Distribution of examined water samples according to districts

	Central district	Gemerek	Gürün	Şarkışla	Suğehir	Yıldızeli	Zara	Kangal	Divriği	Hafik	Koyulhisar	Total
Outbreak regions	25	25	19	47	10	21	53	---	---	---	---	200
Non-outbreak regions	10	9	9	9	9	9	9	9	9	9	9	100
Total	35	34	28	56	19	30	62	9	9	9	9	300

were observed through light microscope for 15 days. Amoebas of the genus *Acanthamoeba* were distinguished by their contractile vacuole, nucleus and typical cyst appearance.

DNA isolation was carried out for *F. tularensis*, *Acanthamoeba* spp. and the membrane filters using Commercial DNA isolation kit (QIA-amp DNA extraction mini kit, Qiagen, Germany). Isolated DNAs were kept at -20°C , until PCR analysis.

TUL4 (Tul4-435 5'-GCT GTA TCA TCA TTT AAT AAA CTG CTG-3', Tul4-863 5'-TTG GGA AGC TTG TAT CAT GGC ACT-3') and RD1 (5'-TTT ATA TAG GTA AAT GTT TTA CCT GTA CCA-3', 5'-GCC GAG TTT GAT GCT GAA GA-3') primer base pairs were used for the detection of *F. tularensis* through PCR. As a result of the PCR performed with the primers belonging to Tul4 gene area, around 400 base farm areas were reproduced^[15,16]. All PCR studies were performed using GeneAmp[®] PCR

Table 2. Distribution of examined water samples according to the number of settlements

	Central district	Gemerek	Gürün	Şarkışla	Suşehri	Yıldızeli	Zara	Kangal	Divriği	Hafik	Koyulhisar	Total
Outbreak regions	3	3	3	9	3	5	3	---	---	---	---	29
Non-outbreak regions	10	9	9	9	9	9	9	9	9	9	9	100
Total	13	12	12	18	12	14	12	9	9	9	9	129

System 9700 (Applied Biosystems, Foster City, CA) thermal cycle device.

The reaction volume of 50 µL consisted of; 1x PCR buffer, 0.2 mM dNTP, 2.5 mM MgCl₂, 0.4 µM primer, 1.25 U Taq polymerase, and 5 µL DNA. The target DNA was denatured by keeping it for 4 min, at 94°C, and then a total of 40 cycles (40 sec at 94°C, 30 sec at 64°C and 45 sec at 72°C) were carried out. At the last stage, final elongation was realized by holding it 5 min at 64°C and the reaction was completed.

DNA isolation of the amoeba reproduced at NNA plates was also carried out. JDP1 (5'- GGC CCA GAT CGT TTA CCG TGA A-3') and JDP2 (5'- TCT CAC AAG CTG CTA GGG AGT CA-3') primer base pair was used to identify amoebae of the genus *Acanthamoeba* through PCR (towards 18S rDNA gene area). Approximately an area of 500 bp is reproduced in 18S rDNA gene region, among 423 to 551 base pairs^[17]. The reaction volume of 50 µL consisted of; 1x PCR buffer, 0.2 mM dNTP, 2.5 mM MgCl₂, 0.4

µM primer, 1.25 U Taq polymerase, and 5 µL DNA. The target DNA was denatured by keeping it for 7 min at 94°C, and then a total of 40 cycles (1 min at 94°C, 1 min at 65°C and 1 min at 72°C) were realized. At the last stage, final elongation was carried out by holding it 10 min at 72°C and the reaction was completed. The amplification products were evaluated after executing in agarose gel.

Data obtained from the study were analyzed using SPSS 20.0 (Statistical Package for the Social Sciences) software. Margin of error was set as α : 0.05 and Chi-Square test was used to compare the results.

RESULTS

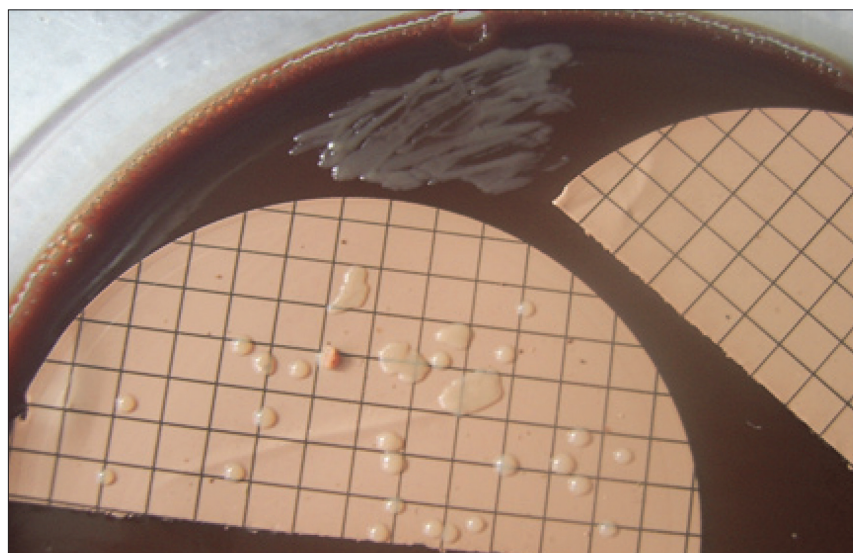
In this study, *F. tularensis* was reproduced in 30 water samples collected from 8 villages of 3 different districts where tularemia outbreak was observed (Table 3). Overall, *F. tularensis* was produced in 30 of the 200 water samples collected from outbreak areas (15.00%); however, it could not be isolated from 100 water samples

Table 3. Settlements where *F. tularensis* was isolated

County	Area	Sample Number
Gemerek	Çiçekoğlu village	T131
Gürün	Bahçeçi village	T135
Gürün	Karaören village	T173, T174, T175, T181, T182, T183
Şarkışla	Hüyük village	T142, T144, T145, T146, T148, T149, T151, T152, T153, T163, T164, T165, T166, T167, T168, T169, T170, T171
Şarkışla	Maksutlu village	T184
Şarkışla	Döllük village	T186
Şarkışla	Belkent fountain	T187
Şarkışla	Gazi village	T198

Table 4. Isolation results of *F. tularensis* and *Acanthamoeba* spp.

	n	<i>F. tularensis</i>	%	<i>Acanthamoeba</i> spp.	%	<i>F. tularensis</i> + <i>Acanthamoeba</i> spp.	%
Outbreak regions	200	30	15.00	16	8.00	2	1.00
Non-outbreak regions	100	0	0.00	12	12.00	0	0.00
Total	300	30	10.00	28	9.33	2	0.66

**Figure 2.** *F. tularensis* colonies on the GCBA.

collected from non-outbreak areas (Table 4). It was observed that 3-1000 *F. tularensis* colonies were formed on membrane filters (Figure 2). PCR analysis identified that the bacteria multiplied in the culture were *F. tularensis* subsp. *holarctica*.

Acanthamoeba spp. isolation occurred in 16 of the 200 water samples (8.00%) collected from tularemia outbreak areas and 12 of the 100 water samples (12.00%) collected from non-outbreak areas. Overall, *Acanthamoeba* spp. isolation occurred in 28 of the 300 water samples. Twenty-eight *Acanthamoeba* spp. isolates reproduced in culture environment were verified through PCR.

In our study, concurrent presence of amoebas of the genus *F. tularensis* and *Acanthamoeba* was only detected in two water samples (Table 4). There were no statistically significant differences between the water samples taken from outbreak and non-outbreak regions in terms of the presence of amoebae of the genus *Acanthamoeba* ($p > 0.05$) (Table 5). DNA isolation was carried out on the membrane filters and on 28 *Acanthamoeba* isolates, and they were examined in terms of the presence of *F. tularensis* DNA through PCR. At the end of the PCR carried out using genus specific Tul4 primers, the existence of *F. tularensis* DNA was not detected on membrane filters and on *Acanthamoeba* isolates.

Table 5. Isolation results of *Acanthamoeba* spp.

	n	<i>Acanthamoeba</i> spp. (+)	%	<i>Acanthamoeba</i> spp. (-)	%
Outbreak regions	200	16	8.00	184	92.00
Non-outbreak regions	100	12	12.00	88	88.00
Total	300	28	9.33	272	90.66

DISCUSSION

The first tularemia outbreak in Turkey was observed in 1936. Outbreaks increased after 2005, and waterborne outbreaks of tularemia have been reported from different regions of Turkey ever since. The agent of tularemia outbreaks emerged in Turkey is *F. tularensis* subsp. *holarctica*^[18,19]. In diagnosing tularemia, culture is accepted as the golden standard, and *F. tularensis* was isolated from water samples via cultivation^[5,20,21]. In Turkey, direct isolation of *F. tularensis* from water samples via cultivation was first realized in 2009. The tularemia outbreak occurred in Sivas province during 2009-2010 was waterborne, and *F. tularensis* subsp. *holarctica* was detected in water samples through cultivation and PCR methods^[22]. In our study, *F. tularensis* subsp. *holarctica* was isolated from water samples, which confirms that the outbreaks are waterborne.

The presence of *F. tularensis* subsp. *holarctica* in water is not sufficient for the emergence of the disease, the involvement of susceptible hosts is required for the disease^[23]. In a study conducted in Sweden, *F. tularensis* subsp. *holarctica* has been detected in the water samples of the regions where tularemia disease was not present^[23]. In our study, we could not detect the presence of *F. tularensis* subsp. *holarctica* through culture and PCR methods in the water samples of the regions where tularemia cases were not observed.

In our study, half of the membrane filters was used in cultivation work, whereas, the other half was used in DNA isolation. Although the formation of *F. tularensis* colonies (in the range of 3-1000 CFU) were observed on membrane filters during cultivation studies, the presence of bacteria DNA could not be detected in the same water samples through conventional PCR method. Real time detection limit of PCR was found to be 10^3 CFU/ml for the DNA obtained from *F. tularensis* using commercial DNA isolation kits^[24]. In addition, 10^2 bacteria present in 1 ml phosphate buffered water and 10^3 to 10^4 bacteria included in 1 ml serum sample containing *F. tularensis* can be detected using conventional PCR method towards Tul4 gene area^[25]. We believe that the small number of bacteria on membrane filters and the removal of bacteria from cellulose nit-

rate filters has decreased DNA efficiency, which caused the negative results obtained from PCR analysis.

It is known that amoebae of the genus *Acanthamoeba* spp. are present in water resources such as drinking water, well water, spa water, rivers, and sea water at different rates^[26-31]. *Acanthamoeba* spp. ratio found in the drinking water of Sivas province was 4.4% and 6.0% in two different studies; however, in our study this ratio was found to be 9.33%^[32,33]. This ratio was 8% for the samples taken from outbreak regions, whereas, it was 12% for the samples of non-outbreak regions.

F. tularensis is quite resistant to external environmental conditions, and it is accepted that its ability to sustain its life inside the amoebas living in water (*Acanthamoeba castellanii*) is significant in terms of waterborne epidemics and regional continuity of the disease^[11]. Experimental studies have reported that in case of cultivating *F. tularensis* and *A. castellanii* together, the bacteria can keep its vitality for longer than three weeks^[13,14].

In our study, concurrent presence of *F. tularensis* and *Acanthamoeba* were detected in two water samples taken from Şarkışla Hüyük village, numbered as T163 and T171. Although experimental studies show the complex relationship between *F. tularensis* and *Acanthamoeba*, the current study is first in terms of investigating *F. tularensis* and *Acanthamoeba* in water samples; however, no connection could be established between tularemia outbreaks and *Acanthamoeba* spp.

In conclusion, tularemia outbreaks emerged in Sivas province are waterborne and the agent is *F. tularensis* subsp. *holarctica*. Even though the bacteria were isolated from water samples through cultivation, the presence could not be detected through conventional PCR method. In case of low number of bacteria, it is more appropriate to use real time PCR method, which is more sensitive, instead of the conventional PCR method. Although experimental studies show the complex relationship between *F. tularensis* and *Acanthamoeba*, further studies investigating the role of *Acanthamoeba* in tularemia outbreaks are needed.

ACKNOWLEDGEMENT

This work was supported by the Scientific Research Project Fund of Cumhuriyet University under the project number T-428. We would like to thank Dr. Can Bulent KARAKUS, for giving us technical support to present the samples used in our study on the map. The authors would like to thank the Sivas Cumhuriyet University Faculty of Medicine Research Center (CÜTFAM) for its technical support.

ETHICS COMMITTEE APPROVAL

Since the study was produced from a PhD thesis and pre-2020 data, the ethical approval for this study is not required.

CONFLICT of INTEREST

The authors declare that they have no conflict of interest.

AUTHORSHIP CONTRIBUTIONS

Concept and Design: MA, ÖP

Analysis/Interpretation: MA, ÖP

Data Acquisition: MA

Writing: MA

Revision and Correction: MA, ÖP

Final Approval: MA, ÖP

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