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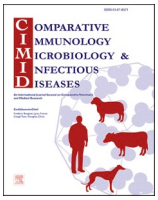

Tracking the footsteps of *Francisella tularensis*: Bacteriological and serological monitoring in epidemic areas in Ankara

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ABSTRACT

The study aimed to detect *Francisella tularensis (F. tularensis)* in water samples and to investigate the seroreactivity of sheep to tularemia in endemic areas where human tularemia cases have been reported in Ankara, Turkey. For the isolation of *F. tularensis*, 50 water samples were collected from rural areas of 5 regions of Ankara (Turkey) and selectively cultured on Francis medium supplemented with 8–9 % sheep blood and antibiotics (100 IU/ml penicillin G, 100 mg/L cycloheximide, 80,000 U/L polymixin B). No *F. tularensis* isolate was cultivated from the water samples. To determine the seroreactivity of sheep to tularemia, 1006 sheep blood samples were collected from the regions, where human tularemia is endemic. A microagglutination test (MAT) identified significant antibody titers, ranging from 1/20–1/640 in 181 (17.99 %) of the investigated sheep sera. Further investigation is required in order to evaluate and confirm a possible epidemiologic relationship between human outbreaks and probable role of sheep or other sources.

1. Introduction

Tularemia is an important zoonotic disease caused by a Gram-negative coccobacillus, *Francisella tularensis*, a bacterial pathogen of animals that can easily be transmitted to humans. The bacterium causes recurrent outbreaks in Turkey and it is a potential biological warfare agent [1,2]. The subspecies, *F. tularensis* subsp. *tularensis*, is especially found in land-dwelling mammalians such as rabbits, squirrels, rodents, and raccoons in North America [2]. However, *F. tularensis* subsp. *holartica* was often reported also from aquatic rodents such as water rats and beavers [3,4]. The bacteria continue to circulate in nature by transmission through rodents or blood-sucking arthropods such as ticks, flies, and mosquitoes [5,6]. However, flies are rarely involved in *F. tularensis* transmission to humans, and mosquitoes are only involved in specific countries such as Sweden and Finland [7]. The bacteria can survive for several weeks in water and sludge and remain infective [8]. Sheep are the most susceptible species to tularemia among domestic animals [9]. However, the disease has also been reported in cats, rabbits, dogs, pigs, and horses. Ringtail possum is a potential reservoir of *F. tularensis*, since a few human tularemia cases have been reported from southern Australia (mainly Tasmania) after possum bites [10,11]. Cattle are generally regarded to be resistant to the disease [4,12].

Tularemia is endemic in a number of countries of the Northern Hemisphere [2]. Water, arthropod and aerosol-borne epidemics have occurred in Europe and Asia, each affecting hundreds of individuals [13]. Although tularemia can be observed every year, it shows a peak in most countries in the late summer and autumn and in some countries also during winter, due to the increased activities in nature and consequently increased exposure to the vectors [3]. Human tularemia cases have been commonly reported in Turkey. Although with wide temporal and geographical variation and variable clinical presentations, oropharyngeal form is more common in Turkey [14]. Ankara has reported 550–750 human cases during a fourteen years period [15]. However, information on the prevalence and presentation of clinical diseases in domestic animals in the region is very limited [9,16].

Tularemia can be transmitted in many different ways to humans. The most frequent routes of transmission greatly vary between endemic areas. For example, vector-borne transmission is the primary mode of

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human infections only in some countries, e.g., in Sweden where most cases are mosquito-borne. Transmission by bite is also seen in origin from other animal species such as cats and dogs [17,18]. Contact with a contaminated environment (soil or water) is the potential route of transmission. Bacteria are known to survive in water supplies [19] and, in fact, almost all human outbreaks in Turkey have originated from spring water [20]. However, direct transmission of the agent to humans has not been associated with contact with animals, including sheep, in Turkey. This may be because sheep are not the best sentinels for tularemia and are rarely reported as a source of human infections [21,22]. However, in outbreaks of human tularemia, sampling from the environment and sheep which are known as probable sources of the infection may be beneficial in elucidating the epidemiology of the disease. Such studies have confirmed the epidemiological link between drinking spring water and human infections [13,23,24].

The present study aimed to investigate the presence of *F. tularensis* in water by cultural methods and antibodies in sheep sera by microagglutination test (MAT), with the purpose to establish an epidemiological link between the previous human cases and the presence of the agent in Ankara, a hot spot for recurrent human outbreaks.

2. Material and methods

2.1. Study materials

There have been 550–750 reported human cases during a 14 year period (Fig. 1). This information was supported by the cases reported recurrently in the same regions [25]. Water samples were taken from the surrounding sites of reservoirs such as houses and stables where tularemia outbreaks have been reported in the Ankara region (Table 1, Fig. 1 and Fig. 2).

Fifty water samples with a volume of 500 ml were collected from the village fountain, river, water tank and tap water for the bacterial isolation from the related regions (Table 1). Five different districts of Ankara were sampled during the study (Table 1, Fig. 2). The districts are distinguished from each other by harboring some geographic and climate characteristics. Altındağ is a central district harboring many neighborhoods where intensive stock farming is common. Bala is in the southeastern part of Ankara between the Beynam Forest and the branches of the Kızılırmak River. The district harbors many forest villages and rural settlements. Beypazarı is located in the Northwest of Ankara and is an important livestock center with large pastures, most of which are located in mountainous regions. Çankaya is the biggest district in the center of Ankara. However, the samples were taken from a rural area harboring a natural park forest and several ponds. Güdül district is a highly mountainous area with a land structure located in the Northwest of Ankara. The district harbors several branches of the Sakarya River. A rainy climate in the North-Black Sea part and a continental climate in the South-West part were described for the Ankara region [26]. However, it has been reported that there are no climatic differences between the regions sampled in this study.

Blood samples were simultaneously collected from sheep flocks those bred in the same areas where human cases were reported.

Table 1
The sample layout investigated in the present study.

Location	Location code on Fig. 2	Water	Sheep blood	Human cases
Ankara-Altındağ	1	10	312	29
Ankara-Bala	2	10	204	84
Ankara-Beypazarı	3	10	160	25
Ankara-Çankaya	4	10	116	13
Ankara-Güdül	5	10	214	8
Total		50	1006	159

2.2. Investigation of F. tularensis by culture method

The cellulose nitrate membrane filter paper (0.20 μm diameter) (Sartorius Stedim Biotech, France) was flushed with alcohol. Then 500 ml of water samples were passed through the filter paper. The filter paper was placed onto agar plates of Francis medium which was prepared with Brain Heart Infusion Agar (Oxoid, UK), 8–9 % defibrinated sheep blood, 1 % Dextrose (Difco, USA), 0.1 % L-Cystein (Sigma-Aldrich, USA), *Helicobacter pylori* Selective Supplement (Dent) (Oxoid, UK) and antibotics (Penicillin G 100 IU/ml, Cycloheximide 100 mg/L, Polymixin B 80,000 U/L) [8]. After the filtration, the plated Francis medium agar plates were incubated at 10 % CO_2 atmosphere at 37 °C for 10 days. Cultures were checked daily for the presumptive growth of *F. tularensis* after incubation. Colonies about 2–4 mm in diameter, opaque, mucoid-shiny, exhibiting greenish-white color and an opalescent sheening were considered as suspicuous for *F. tularensis* [27,28]. *F. tularensis* subsp. *holarctica* (NCTC 10857) strain was used as a positive control in the identification of cultures and for testing the media.

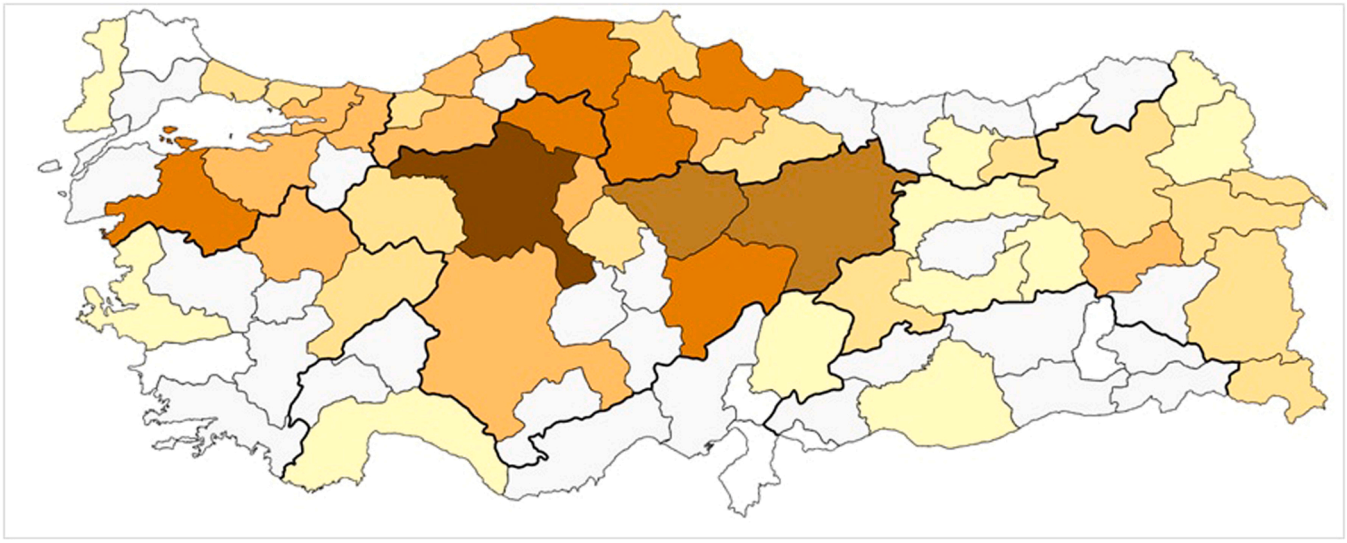

Fig. 1. Incidences of human tularemia by province between 2005 and 2018 in Turkey. The dark brown slice shows Ankara which holds the biggest incidence (550–750 cases/a fourteen-year period) of human tularemia [15].

Fig. 2. The sampling areas of the study.

2.3. Serological investigation of F. tularensis antibodies by microagglutination test

For serological analysis, 1006 sheep blood serum samples were examined by microagglutination test (MAT). Ten ml blood was collected from the *Vena jugularis* of sheep into vacuum gel tubes. Sera samples were then separated by centrifugation of the blood at 1.500 rpm for 5 min. Safranin-O stained test antigen (*F. tularensis* subsp. *holarctica* NCTC 10857 vaccine strain) was used for MAT to determine the presence of *F. tularensis* antibody [9,27]. Dilutions of serum samples were prepared in U-based microplates and the test was completed by adding an equal volume of the stained test antigen. The test was evaluated after incubation overnight at 37 °C in a humid environment. The lace-like collapse of the antigen-antibody complex and the completely clear supernatant were considered as positive reactions for MAT. The negative reaction was evaluated as a buttoned collapse in the center surrounded by light red diluent. The evaluation was performed according to the positive and negative controls and the antigen control [8]. As the diagnostic cut-off titer, a dilution of 1/20 and higher was considered positive which was previously described [29,30]. The samples were tested with Rose Bengal Plate Test and Serum Agglutination Test with regard to cross-reactivity with *Brucella* species [31].

2.4. Statistical analysis

The data were analysed with IBM SPSS Statistic 20.0. program (IBM Corp., Armonk, USA). In this context, the Pearson chi-square test was used. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Isolation findings

As a result of cultural analysis, *F. tularensis* could not be isolated from any of the water samples which were collected and cultured for 10 days.

3.2. Serological findings

The MAT assay demonstrated that 181 (17.99 %) out of 1006 sheep blood sera had a significant antibody titer against *F. tularensis*, i.e., equal to or greater than 1/20 (Table 2). The samples with lower antibody titer were double-checked with regard to cross-reactivity with *Brucella* spp., but no cross-reactivity was observed.

The distribution of positive sera according to the region was reported in Table 2. The overall seropositivity of *F. tularensis* antibody was found 17.99 % in Ankara Region. The antibody titer was identified of 1/20 in 113, 1/40 in 50, 1/80 in 11, 1/160 in 6, and 1/320 in 1 sample. Bala is the region where the highest (34.8 %) seropositivity was observed, while Altındağ is the lowest (8 %) place. The difference in *F. tularensis* seropositivity among the districts examined in this study was found to be statistically significant (The chi-square statistic is 44.5432, $P < 0.05$). The districts (Bala, Beypazarı and Güdül) harboring mountainous regions and river branches had a higher prevalence than the others have (Table 2).

4. Discussion and conclusion

The present study aimed to establish an epidemiological link between the previous human tularemia cases and the presence of *F. tularensis* by investigating water and sheep sera samples two of the probable contributing risk factors.

Tracing of the common source outbreaks with sampling environment such as water which is known as the probable source of infections may be beneficial in elucidating the epidemiology of the disease. Leblebicioglu et al. [32] reported a case-control study to evaluate the risk factors for tularemia during an epidemic in Turkey and investigated both human and water samples for the presence of *F. tularensis*. The researchers demonstrated the presence of *F. tularensis* in humans and water and concluded that case-control studies are useful for analyzing epidemics and for identifying the source of infection.

Water may serve as a source of tularemia outbreaks [33,34]. Karpoff And Antoroff [35] reported the first water-borne tularemia outbreak in Russia. Subsequently, Hüseyin [34] described the first water-borne outbreak of tularemia in Turkey. Although the routes of

Table 2
The MAT results of the samples in terms of the *F. tularensis* antibody.

Location	The number of sample	MAT positive sample	Distribution of MAT titers				
			1/20	1/40	1/80	1/160	1/320
Ankara-Altındağ	312	25 (8%)	17 (5.45%)	8 (2.56%)	–	–	–
Ankara-Bala	204	71 (34.8%)	50 (24.51%)	13 (6.37%)	6 (2.94%)	1 (0.05%)	1 (0.05%)
Ankara-Beypazarı	160	22 (13.75%)	17 (10.63%)	5 (3.13%)	–	–	–
Ankara-Çankaya	116	15 (12.9%)	5 (4.31%)	10 (8.62%)	–	–	–
Ankara-Güdül	214	48 (22.42%)	24 (11.21%)	14 (6.54%)	5 (2.34%)	5 (2.34%)	–
Total	1006	181 (17.99%)	113 (11.23%)	50 (4.97%)	11 (1.09%)	6 (0.6%)	1 (0.1%)

contamination of water have not been fully revealed in the studies, it is thought that *F. tularensis* can be transmitted by dead small rodents contaminating the water [36]. In Turkey, tularemia outbreaks have been predominantly linked with the consumption of contaminated and unchlorinated natural spring water [1,33]. The most prominent of these is that the isolation and molecular identification of *F. tularensis* subsp. *holartica* by PCR in a sample out of four which were taken from water during a human outbreak in Beypazarı district of Ankara which demonstrates that the infective agent can be spread by water [37]. In the present study, there was no isolation from any of the water samples obtained from different regions in Ankara. This result was in agreement with the previous experiences that the chance of isolation of *F. tularensis* from water could be low due to the instantaneous contamination of the water through reservoirs, the high dilution of the agent in the water, and the delayed sampling after contamination [4]. Culture failure may also be due to a condition called viable but non-culturable (VBNC), which bacterial cells cannot be cultured but retain metabolic activity and cellular integrity [38]. Even in outbreaks with a high prevalence of tularemia infection, it was found that the probability of the isolation from water samples was quite low. Chlorination of water also reduces the chance of obtaining viable bacteria during the isolation [33].

Tularemia is seasonal in many countries where it is endemic. The prevalence of the infection has been reported to be highest in late spring, summer and early autumn. This situation is probably closely related to climatic factors such as temperature and precipitation. However, as the number of cases varies greatly from one year to the next in the same region, there is almost no data to link specific climatic conditions with Tularemia outbreaks [39]. All of the tularemia cases in Turkey were related to water-sources and occurred in most of the winter months [23,24,40]. In parallel with active human infections, culture positivity from water sources were reported within these periods [23,24,40]. However, cultural failures were not absent in studies of the identical sample season [5,41]. In this study, water samples were taken in the autumn season, and both the low seasonal precipitation in the region and the absence of concurrent active human infections are considered to be the reasons for culture negativity. In addition, isolation was performed by direct cultural analysis using different media as in previous studies [23,24]. However, due to the agent's fragile structure, inoculation to experimental animals in order to increase isolation chance was not performed in the present study [40].

There are some reports on tularemia that causes simultaneous epidemics in human and animals [32,37,42,43]. Gürcan et al. [42] detected the presence of antibodies in 10 people of the 226 blood serum samples with MAT during a tularemia epidemic in Demirköy, Edirne, Turkey. The researchers have also examined tonsil swab and lymph node aspirate taken from patients and water samples from a spring and identified *F. tularensis* with PCR. In order to determine the source of the infection, the researchers evaluated the blood samples taken from 25 rabbits, 27 cows and 19 sheep belonging to the sick people with the same method. As a result, low titer antibodies against *F. tularensis* were detected in 1 rabbit and 19 cows, while all sheep were found negative. In rabbits, only three of them gave 1/40 titer.

In a study conducted by Karataş Yeni and Izgür [37], the existence of *F. tularensis* in sheep and other potential reservoirs was investigated in Anatolia in where tularemia has been observed in humans. In this previous study, one water sample yielded a culture-positive result, however, 111 (27.68 %) of the sheep had antibody titers between 1/20 and 1/640. All the more amazing, three locations of this study were the same as in the present study and the antibody titer were 36.95 % (17/46) in Altındağ, 84 % (63/75) in Bala and 4.95 % (5/101) in Beypazarı district. After the comparative evaluation of the results of the present study with the previous study, significant differences were found in the same regions which may be due to the study periods and different herds tested. However, the highest positivity in both studies was observed in Bala district and this may be a reflection of that Bala is a place being between forest and river branches and harbors many forest villages and rural settlements. Moreover, the geographical backers may have promoted the prevalence of tularemia in sheep rather than the alleged relation with human beings. Reintjes et al. [43] reported a large outbreak of tularemia in Kosovo in which an epidemiologic and environmental investigation was conducted to identify sources of infection, modes of transmission, and household risk factors. Seropositivity was found both in human and rodents and it was suggested that infection was transmitted through contaminated food or water and that the source of infection was rodents. In the present study, sera collected from sheep rearing in the areas in which human tularemia outbreaks had occurred were serologically examined with MAT to determine *F. tularensis* antibody titers. Out of 1006 serum samples, 181 (17.99 %) had antibody titers between 1/20 and 1/320, which were considered significant as supported by previous studies [21,29,30,37] since the cutoff value of MAT for sheep sera is determined as equal and greater than 1/20. This value was used as a cornerstone in the serological diagnosis of tularemia in subsequent studies [21,30,37]. However, cross-reaction with Brucellosis should also be eliminated at low titers. In this study, the samples with lower antibody titer were double-checked with regard to cross-reactivity with *Brucella* spp., but cross-reactivity was not observed. Thus, the possibility of *Brucella* cross-reaction due to low titer (1/20) is eliminated. When considering the epidemiological studies [44–46] on individual seroprevalence of tularemia in domestic animals, especially in sheep a high number of sera were positive. In conclusion, a direct link could not be established between the human cases and water contamination since the isolation of the agent from water failed. The sheep showed a high rate of positivity and it can be assumed that they indicate the presence of the pathogen in the respective environment. Nevertheless, this assumption should be confirmed by complementary tests since the seropositivity does not mean that infected sheep actively spread the agent with excretes. However, heavily tick infested sheep might spread contaminated aerosols due to contaminated feces of the ticks as was shown previously (North America; *F. tularensis* subsp. *tularensis*).

Ethical statement

In this study, animal owners gave their consent to blood sampling at the stage of collecting samples and epidemiological data. Furthermore, blood sampling was carried out under the supervision of a veterinarian in accordance with international ethical standards.

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None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Contribution statement

Derya Karataş Yeni: Data collection, laboratory analyses, statistical analyses, writing-original draft. **Fatih Büyük and other authors:** Writing, review and editing, interpretation and discussion of results, supervision.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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