Tularaemia outbreaks in Sakarya, Turkey: case-control and environmental studies

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ABSTRACT

Introduction: Tularaemia is an important zoonotic disease that leads to outbreaks. This study aimed to compare the epidemiological characteristics of two tularaemia outbreaks that occurred in the Sakarya region of Turkey, analyse the risk factors for the development of outbreaks and identify *Francisella* (*F.*) *tularensis* in the water samples.

Methods: Two tularaemia outbreaks occurred in the Kocadongel village in 2005 and 2006. A field investigation and a case-control study with 47 cases and 47 healthy households were performed during the second outbreak. Clinical samples from the patients and filtrated water samples were analysed for *F. tularensis* via real-time polymerase chain reaction.

Results: From the two outbreaks, a total of 58 patients were diagnosed with oropharyngeal tularaemia based on their clinical and serological results. Both outbreaks occurred between the months of January and April, and the number of patients peaked in February. Logistic regression analysis revealed that the consumption of natural spring water was the only significant risk factor for tularaemia infection (odds ratio 3.5, confidence interval 1.23–10.07). *F. tularensis* was detected in eight clinical samples and in the filtrated natural spring water.

Conclusion: This study is the first report of tularaemia from this region. The results show that both tularaemia outbreaks were related to the consumption of untreated natural spring water. To prevent waterborne tularaemia, community water supplies should be treated and checked periodically.

Keywords: *Francisella tularensis*, outbreaks, polymerase chain reaction, risk factors, tularaemia

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INTRODUCTION

Tularaemia is a zoonotic disease that is characterised by a variety of clinical forms caused by the bacteria *Francisella* (*F.*) *tularensis*, and it is usually found in the northern hemisphere. The disease has recently become more well known due to its bio-terroristic capacity. The microorganism has caused outbreaks in the western parts of Turkey, and drinking water springs were the sources of these recent outbreaks.[1-4] This study is the first tularaemic report from the Sakarya region and describes two recent endemic oropharyngeal tularaemia outbreaks in Kocadongel village. The first outbreak occurred between January 10, 2005 and April 8, 2005, while the second was between January 1, 2006 and April 2, 2006. During the second outbreak, a case-control study and an environmental investigation were conducted to identify the source and risk factors for infection.

METHODS

Between January and April 2005, 11 patients from Kocadongel village in Sakarya were admitted to the Faculty of Medicine, Kocaeli University with complaints of fever, sore throat and the presence of a cervical mass that did not respond to beta-lactam antibiotics. The patients' clinical data was recorded on a standard survey form; however, an environmental investigation was not performed. One year after the first outbreak, in the first week of March 2006, two patients who lived in Kocadongel village were diagnosed with oropharyngeal tularaemia using the microagglutination (MA) test. In the same week, an outbreak investigation team, which was formed in collaboration with the local office of the Ministry of Health, visited Kocadongel village. All patient records in the region were reviewed, and those who were suspected of having contracted tularaemia based on their recent medical history and physical examination were interviewed using a structured survey form. Throat swabs, lymph node aspiration

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and serum samples were obtained. Demographic data and the presence of risk factors, such as drinking of natural spring water, consumption of hunted animals, rodent bites or contact, animal breeding, consumption of food without appropriate cleaning and travel to an endemic region, were noted using a structured form. All healthcare workers who were involved during the outbreak were educated about the disease, and all newly suspected patients were referred to our hospital. A suspected tularaemia case was defined as a patient with the presence of fever, membranous pharyngitis or tonsillitis and/or cervical lymphadenopathy who came from the epidemic-affected region and did not respond to β-lactam/macrolide antibiotics. A suspected case with a positive laboratory result (a single MA titer > 1/160, or positive polymerase chain reaction [PCR] for *F. tularensis*) was considered to be diagnosed with the disease.

During the second outbreak in 2006, a case-control study was conducted. Controls were selected from among healthy household members who did not have a history of fever, sore throat or cervical lymphadenopathy within the past three months. The cases and controls were interviewed. Demographic data and the presence of the risk factors mentioned earlier were obtained using a structured form. The data was analysed using the Statistical Package for the Social Sciences version 10.0 (SPSS Inc, Chicago, IL, USA). Categorical comparisons were performed using the chi-square test, and the continuous variables were tested using the Student's *t*-test. Multivariate analysis was performed by logistic regression. The independent variables were age, gender, drinking of tap water, rodent bite and contact with a rodent, consumption of unhygienic food, breeding of animals and engaging in a high level of outdoor activities. A p-value < 0.05 was considered to be statistically significant.

In addition to the case-control study, environmental investigations were conducted. In the region in which the outbreaks occurred, natural spring water is consumed as tap water. The spring water is transported 1 km downstream from the source to a reservoir through a pipeline and then pumped into taps. It is not routinely chlorinated. One-litre water samples were taken from the reservoir and transported to a laboratory, where they were stored at 4ºC–8ºC until microbiological investigations were conducted on the same day. As the main source of income in the village is stockbreeding and farming, serum samples were obtained for the serological investigation of *F. tularensis* from ten sheep and ten cattle that belonged to some of the patients. The sera of the patients, controls and animals were screened using the MA method with a standard *F. tularensis* antigen (Becton, Dickinson and Company, Sparks, MD, USA). Antibody titers > 1:160 were considered to be positive.[3]

Water samples obtained from the reservoir of the natural spring, 12 throat swabs and four lymph node aspiration materials were analysed for the presence *F. tularensis* using real-time PCR (iCycler® IQ v 3.0, BioRad Laboratories, Hercules, CA, USA). The water samples (one litre from each source) were concentrated using 0.45 μm diameter cellulose acetate filters (GN-6 Metricel Grid 47 mm, Pall Life Sciences, Ann Arbor, MI, USA). The filter was washed with sterile distilled water in a shaker for 20 minutes. A QIAamp® DNA mini kit (QIAGEN®, Hilden, Germany) was used for the isolation of DNA from the filtered water samples, lymph node aspirates and throat swabs. Primers for *F. tularensis* (Iontek® Biotechnology, Istanbul, Turkey), which have previously been described by Versage et al,[5] were used. All reactions were performed in a final volume of 25 μl, which also contained iTaq DNA polymerase in SYBR Green I supermix (Bio Rad® Laboratories, Hercules, CA, USA). The final concentration of each primer was 5 μM. The optimum annealing temperature for all reactions was 60ºC. Thermal cycling conditions were as follows: 50ºC for 2 min, 95ºC for 8 min, 45 cycles at 95ºC for 15 s and 60ºC for 1 min, and finally 45ºC for 5 min. In each iCycler run, both negative (PCR grade sterile distilled water) and positive controls (formalin-fixed *F. tularensis* subspecies holarctica bacteria from the reference laboratory, Bursa, Turkey) were used. Cultures for *F. tularensis* (glucose cysteine heart agar included 2.5% blood) were prepared from the filtered water samples (as described above), oropharyngeal swabs and lymph node aspirates.

RESULTS

During the first tularaemia outbreak, 16 patients were identified by the local office of the Ministry of Health in the Sakarya region. The clinical data of 11 patients who were admitted to our hospital was recorded. All the patients were diagnosed with oropharyngeal tularaemia after administration of the MA test (> 1/160 titer). 47 patients were identified during the second tularaemia outbreak. The diagnosis of tularaemia was confirmed in 39 suspected cases using the MA test and in three cases which were serologically negative using PCR (Table I). All the control households were serologically negative. In both outbreaks, the distribution of the patients across the months showed the same graphical pattern, in that the number of patients

Table I. Results of the MA and PCR tests conducted on the patients during the 2006 tularaemia outbreak in the Sakarya region.

MA (n = 47)	PCR* (n = 16)	
	Positive (n = 8)	Negative (n = 8)
Positive (n = 39)	5	4
Negative (n = 8)	3**	4

* Only 16 patients underwent both the PCR and MA tests.
**All 3 samples were throat swabs.
MA: microagglutination; PCR: polymerase chain reaction

Table II. Clinical manifestations of the patients with oropharyngeal tularaemia (n = 58).

Clinical manifestation	No. (%)
Sore throat	52 (90)
Swelling on the neck	52 (90)
Fever	41 (71)
Cough	3 (5)
Lymphadenopathy	52 (90)
Cervical (unilateral)	41 (79)*
Cervical (bilateral)	5 (10)*
Submandibular (unilateral)	6 (11)*
Tonsillopharyngitis	34 (59)
Conjunctivitis	1 (2)
Erythema multiforme-like skin lesion	3 (5)

* Data calculated based on 52 patients with lymphadenopathy.

peaked in February (Fig. 1).

During the first outbreak, the mean age of the tularaemia patients was 21 ± 9.41 (range 2–31) years (Fig. 2). 64% of the patients were female, all of whom were housewives. During the second outbreak, the mean age of the tularaemia patients was 28 ± 16.7 (range 5–73) years. 32 (68.1%) patients were female, 24 (75.0%) of whom were housewives. During both the outbreaks, all the patients had the oropharyngeal form of tularaemia. Among the 58 patients, sore throat (90%), swelling on the neck (90%) and fever (71%) were the most common symptoms. Cervical lymphadenopathy (90%) was the most frequently encountered clinical finding, and 79% of the cases were unilateral (Table II). Interestingly, unilateral tonsillitis was detected in patients who had lymphadenopathy on the same side. Among the patients with both tonsillopharyngitis and lymphadenopathy, three had erythema multiforme and one had conjunctivitis (Table II). All three patients who had erythema multiforme were female.

For the case-control study, the demographic characteristics of 47 cases and 47 control households, as well as the results of the univariate analysis of risk factors, are shown in Table III. Logistic regression analysis revealed that the consumption of tap water was the only significant risk factor for tularaemia infection

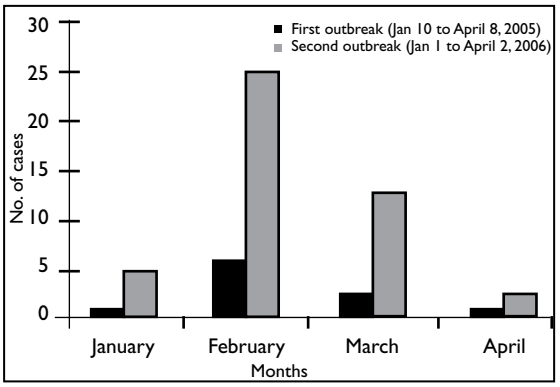

Fig. 1 Bar graph shows the distribution of patients based on the onset of their symptoms during the two tularaemia outbreaks in the Sakarya region.

(p = 0.019, odds ratio [OR] 3.5, 95% confidence interval [CI] 1.23–10.07). In the environmental investigations, all the water samples were found to be highly contaminated with coliform bacilli. *F. tularensis* was detected in the water sample obtained from the reservoir by PCR, but it could not be isolated through culturing. The MA tests of the animal sera were found to be negative. The reservoir was cleaned and chlorinated. Periodic cleaning and treatment of the reservoir was recommended. The outbreak was then kept under control after these measures had been implemented.

F. tularensis PCR was found to be positive in five out of 12 (42%) throat swabs and three out of four lymph node aspirations (75%). Three of the eight patients who had positive PCR results were serologically negative (Table I). *F. tularensis* could not be isolated from the throat swabs and lymph node aspirations by culturing.

DISCUSSION

Tularaemia is an endemic disease in Turkey. The first tularaemia outbreak was reported in 1936 in the Trakya region.[6] In the years following this outbreak, many epidemic or sporadic cases were reported from different sites in Turkey.[7-12] This is the first report of tularaemia from the Sakarya region. Tularaemia outbreaks that are associated with the consumption of hunted animals usually occur in the summer and early autumn,[3,13,14] whereas waterborne tularaemia outbreaks usually occur in the autumn and winter.[3,4,8-12] In this study, we reported two different tularaemia outbreaks that demonstrated the same epidemiological characteristics. Both outbreaks were detected between the months of January and April, and the number of patients peaked in February during both outbreaks (Fig. 1). This may be attributed to the rainy season. The stream line of the natural spring water overflows during these months due to the high level of rainfall, and inhabitants had found some dead

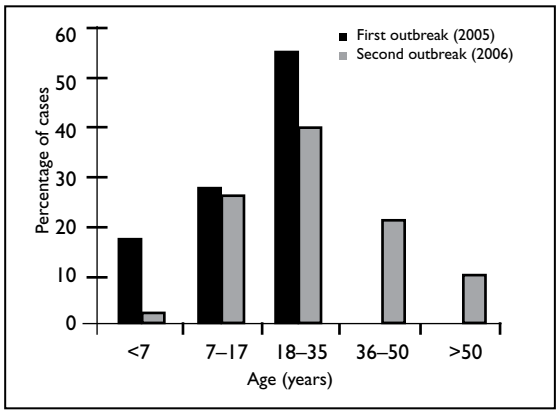

Fig. 2 Bar graph shows the distribution of tularaemia cases according to the age of the patients.

mice along the stream line which might have facilitated the contamination of the natural spring water with *F. tularensis*.

The concentration methods used for the water samples may play a role in the detection of *F. tularensis* from the water samples. In our recent study, we used the centrifugation method for the concentration of water samples, but were unable to detect *F. tularensis* in the centrifuged water samples using the same method of real-time PCR.[11] Interestingly, the detection of *F. tularensis* in a filtrated water sample by real-time PCR was observed in another study, which is similar to that observed in the present study. Based on our experience, the filtration method is more successful than the centrifugation method at detecting *F. tularensis* in water samples.[10]

There are different clinical forms of *F. tularensis*, depending on the route of transmission. The ulcero-glandular form is commonly found in Europe and the United States, while the oropharyngeal form is the most common form of tularaemia found in Turkey.[1-4,6-12] As expected, all of our tularaemia cases were of the oropharyngeal form. In both the outbreaks, most of the patients were female and 79% (31/39) of these women were housewives. Since housewives generally spend most of their time at home, they were more likely to consume the contaminated spring water than the other patients. Most of our patients (90%) had sore throat and lymphadenopathy at the onset of the disease. However, lymphadenopathy was the most common finding in our cases (90%), while tonsillopharyngitis was detected in only 59% of the patients. A lower frequency of tonsillopharyngitis may be due to delayed diagnosis. The findings of the other cases reported from Turkey are similar to those of our study.[7,8] For this reason, oropharyngeal tularaemia should be kept in mind among patients with lymphadenopathy who live in an endemic area.

We observed that the lymphadenopathies in our cases were usually unilateral. Interestingly, during the physical examination, unilateral tonsillitis that was located on the same side as the lymphadenopathy was detected in the patients who had both tonsillitis and lymphadenopathy. Further studies are required to explain why only one tonsil was infected by *F. tularensis*. Skin eruptions such as erythema nodosum and erythema multiforme may occur during the course of tularaemia.[1] Helvaci et al reported the presence of erythema nodosum in 14% of their tularaemia patients,[7] while 3% of the patients had widespread erythema multiforme-like skin eruptions in the upper and lower extremities in another study.[15] In this study, erythema multiforme-like skin eruptions were observed in 5% of our patients. It was interesting to note that all these patients were female, as was observed in another study.[15]

The results of the case-control study performed during the second outbreak support the hypothesis that the tularaemia outbreak was waterborne. In the villages, tap water generally comes from unchlorinated spring water that is stored in a reservoir and pumped through the network. *F. tularensis* was detected in the reservoir water by PCR. Rodents are the most common animals that transmit tularaemia to humans.[1,3] In rare cases, domestic animals can also transmit the infection to humans. Studies have reported cases of tularaemia transmitted from a cat[16] and a sheep.[17] We investigated tularaemia antibodies in the serum samples obtained from the domestic animals belonging to the patients; however, all of the animals were found to be serologically negative when tested using the MA test. The results of the case-control and laboratory studies confirmed that the outbreak was not associated with these animals.

Among the diagnostic tests for tularaemia, culturing is accepted as the gold standard. However, *F. tularensis* is hardly ever grown in culture; at least a Class II biosafety cabinet is required for culturing. Therefore, the MA test is routinely used for the diagnosis of tularaemia.[1,2] In general, the acceptable titer for the diagnosis of tularaemia is > 1/160 in a single serum sample.[3,14] However, antibody levels do not usually increase until the third week of the illness.[18] In addition, antibody-negative but culture-positive patients have been reported in the literature.[7] In another study, some of the tularaemia cases that tested negative with the MA test were diagnosed only by enzyme-linked immunosorbent assay or PCR.[11] In this study, *F. tularensis* could not be grown by culturing in the clinical samples. The MA titers were > 1/160 in only 39 patients, and three patients who were serologically negative had a positive PCR. Based on our experience,

Table III. Univariate analysis of the risk factors for tularaemia among the cases and controls.

Risk factors	No. (%)		p-value
	Cases (n = 47)	Controls (n = 47)	
Age (yrs)			
< 7	1 (2.1)	1 (2.1)	0.226
7–17	12 (25.5)	22 (46.8)	
18–35	19 (40.4)	11 (23.4)	
36–50	11 (23.4)	8 (17.0)	
> 50	4 (8.5)	5 (10.6)	
Gender			
Female	32 (68.1)	25 (53.2)	0.139
Male	15 (31.9)	22 (46.8)	
Drinking tap water	46 (97.9)	37 (78.7)	0.004
Rodent bite	3 (6.4)	1 (2.1)	0.307
Contact with a rodent	5 (10.6)	3 (6.4)	0.460
Eating unhygienic food in nature	12 (25.5)	6 (12.8)	0.116
Breeding animals	16 (34.0)	18 (38.3)	0.668
Engaging in a high level of outdoor activities	23 (48.9)	22 (46.8)	0.836

negative serology should not eliminate the diagnosis of tularaemia and if possible, PCR should be used for the diagnosis of tularaemia.

In conclusion, the results of this case-control study and PCR showed that the reported tularaemia outbreaks were associated with the consumption of natural spring water. In order to prevent waterborne tularaemia, community water supplies, including spring water, should be treated using the standard chlorination procedure and checked periodically. Contaminated water, should not be used or consumed. Additionally, the population at risk should be educated regarding the transmission and prevention of tularaemia. As *F. tularensis* is hardly ever detected in water samples and as the concentration methods for water samples may play a role in the detection of *F. tularensis*, the authors recommend the use of the filtration method for detecting *F. tularensis* from water samples.

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