Original Article

Tularemia, a Reemerging Disease in Northwest Turkey: Epidemiological Investigation and Evaluation of Treatment Responses

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SUMMARY: An outbreak of tularemia occurred in three provinces in Turkey in February 2004 and reemerged in the same provinces in February 2005. A total of 61 cases, 54 of which were confirmed with the micro-agglutination test, were diagnosed with oropharyngeal tularemia. No culture for *Francisella tularensis* was attempted, but PCR for *F. tularensis* was positive in aspiration material of suppurated lymphadenitis of 7 patients. *F. tularensis* detection with PCR was negative in water samples, but epidemiologic and environmental findings suggested that contaminated water or food was the cause of the outbreaks. Late initiation antibiotic therapy could not prevent suppuration and draining of the involved lymph nodes.

INTRODUCTION

Tularemia, caused by the highly infective bacterium *Francisella tularensis*, is a zoonotic disease. *F. tularensis* spp. *tularensis*, or type A, occurs mainly in North America and is usually transmitted to humans by tick bites or through contact with rabbits. *F. tularensis* spp. *holarctica*, or type B, occurs throughout the Northern Hemisphere and is associated with water and rodents living near water (1,2). In addition *F. tularensis* can be isolated from contaminated water or mud (3-5). The disease can emerge in variable clinical presentations, namely the ulceroglandular, glandular, oculoglandular, oropharyngeal, typhoidal and pneumonic (6,7). Ulceroglandular tularemia, which accounts for 21 to 87% of the cases, is the most-seen form and usually emerges in sporadic cases. Oropharyngeal tularemia represents 0 to 12% of the cases, occurs as a result of ingesting contaminated food or water, and is usually associated with outbreaks (8). In this report, we describe two oropharyngeal tularemia outbreaks that emerged in February 2004 and reemerged in February 2005 and that synchronically affected three provinces in Turkey.

METHODS

Three female patients were admitted to our university hospital in the last week of March 2004 with the same complaints: a cervical mass following fever and pharyngitis that did not respond to antibiotics such as penicillin or cephalosporin. Each patient was from a different province (province A, Zonguldak; province B, Bartın; and province C, Kastamonu), with distances of nearly 50 km between them

(Figure 1). The onset of the symptoms was in February 2004. Tularemia was the suspected disease, and it was confirmed with the micro-agglutination (MA) test. Surveillance was conducted to detect patients with tularemia and to identify possible sources and transmission modes of the bacteria. Water or fresh food contaminated with *F. tularensis* was the presumed mode of transmission, and water sanitation and training programs for inhabitants were performed. However, the tularemia outbreak reemerged in February 2005 (Figure 2).

Case definition: A suspected tularemia case was defined as a person who had the unusual syndrome (fever, pharyngitis and accompanying cervical lymphadenitis, none of which responded to betalactam antibiotics) and the onset of which was within the last 3 months. A suspected case whose MA titer showed a fourfold increase or showed a single MA titer ≥ 1/160 or for which *F. tularensis* was isolated in a clinical specimen of the case was considered to be a confirmed tularemia case (1,6). A suspected case whose MA titer was positive (in any titer < 1/160) was considered to be a probable case.

In addition, asymptomatic control cases, most of which were household members of suspected cases, were defined as people who were healthy and had none of the symptoms of tularemia. A symptomatic control case was defined as a person who had symptoms that were not compatible with tularemia or who had tularemia-like symptoms but had a definitive diagnosis of a disease other than tularemia (such as acute streptococcal pharyngitis).

Treatment response: Suppuration and draining (occurring spontaneously or by surgical means) of the involved lymph node during or after antibiotic therapy was defined as treatment failure. The treatment was considered to be successful if the sings and symptoms of the disease disappeared and if the involved lymph node recovered without suppuration. The tularemia cases were followed up at 3-month interval for a total of 12 months.

Diagnostic tests: The MA test was studied at the labora-

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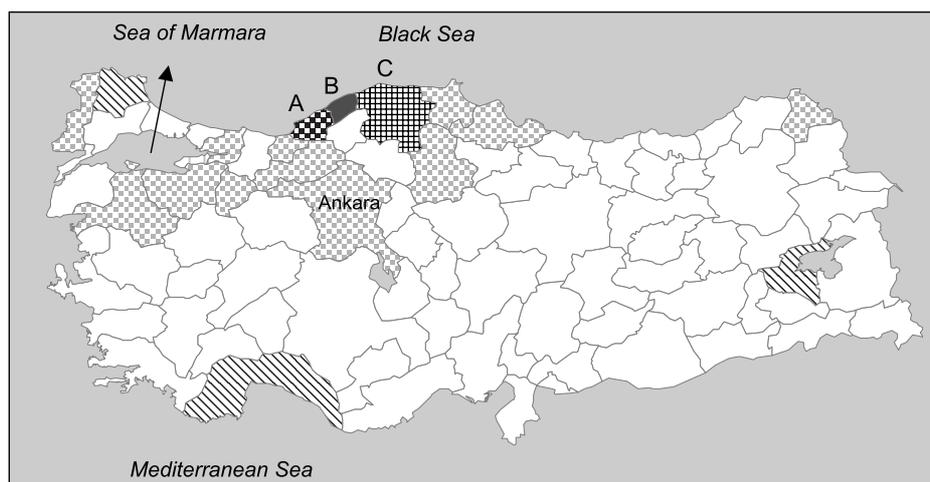
Fig. 1. Distribution of regions from which tularemia outbreaks were reported in Turkey. ⊗ area, province A (Zonguldak); ● area, province B (Bartın); ⊞ area, province C (Kastamonu). ⊡ areas show provinces tularemia outbreak emerged in the last 25 years and ⊘ areas show tularemia epidemics before 1954.
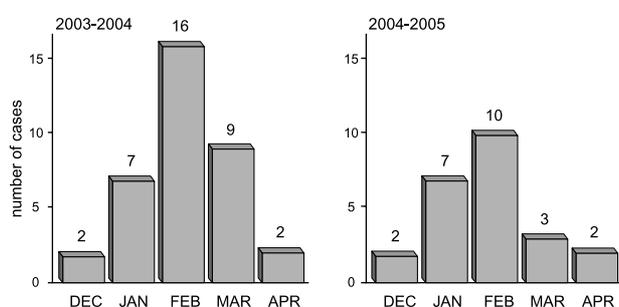
Fig. 2. Distribution of tularemia patients among months in years 2003 - 2004 and 2004 - 2005. Case distribution was based on initiation time of symptoms.

tory of the Medical Faculty of Uludağ University in Bursa (the reference laboratory of tularemia in Turkey) as described in the references (9-11).

PCR was studied at the laboratory of the Medical Faculty of Kocaeli University in Kocaeli, Turkey. Aspiration material of suppurative cervical lymphadenitis from tularemia patients and water samples obtained from 6 different sources (pipes, wells and reservoirs) in the outbreak area of province A were analyzed for *F. tularensis* by PCR. Water samples were also analyzed with routine microbiological tests but due to the high infectivity of *F. tularensis*, special conditions are necessary, the culturing of *F. tularensis* was not attempted.

Aspirates from lymph nodes were directly inserted into tubes containing 500 μl lysis buffer for DNA isolation (guanidine isothiocyanate [5M], Na acetate [1/10 (v/v)], 0.5% sarcosil). Water samples (2 liters from each source) were concentrated through 0.22-μm diameter cellulose acetate filters. The surfaces of the filters were washed with sterile distilled water for 15 min in a shaker, and lysis buffer was added to this pellet as described above. DNA isolation was performed depending on the binding of the DNA to glass beads (Glassmilk; Bio 101, La Jolla, Calif., USA) in the presence of the chaotropic nuclease inhibitor guanidine isothiocyanate. All samples were stored at −20°C until use. Regions targeted for TaqMan assay were specific for *F. tularensis* and included the *tul4* (91 bp) and *fopA* (87 bp) genes. *Tul4* and *fopA* primers and 5′-FAM and 3′-TAMRA labeled probes for *F. tularensis* TaqMan 5′ nuclease tests were

used. Primers and probes for TaqMan assay were as follows;
tul4,
Tul4-F-5′ ATTACAATGGCAGGCTCCAGA 3′,
Tul4-R-5′ TGCCCAAGTTTTATCGTTCTTCT 3′, Tul4-P-TTCTAAGTGCCATGATACAAGCTTCCCAATTACTAAG
fopA,
FopA-F-5′ ATCTAGCAGGTCAAGCAACAGGT 3′,
FopA-R-5′ GTCAACACTTGCTTGAACATTTCTAGATA 3′,
FopA-P-CAAACTTAAGACCACCACCCACATCCCAA
(F, forward primer; R, reverse primer; P, probe).

TaqMan 5′ nuclease assay was performed using a Quantica real-time PCR device (Techne Inc., Cambridge, UK). As a passive reference dye, ROX was used. Reactions were run in 50 μl volumes containing 5 μl of sample. For the *tul4*, and *fopA* TaqMan assays, the final MgCl$_2$ concentration was 5 mM, whereas the primers and probes were 500 nM and 100 nM, respectively. The annealing temperature for TaqMan assays was 60°C. The TaqMan PCR conditions were as follows: activation involved 1 cycle of 94°C for 10 min, and amplification and detection involved 40 cycles of 94°C for 30 sec and 60°C for 2 min. In this study, both negative (not containing any template) and positive controls (10-fold dilutions of *F. tularensis* DNA) were included. *F. tularensis* subsp. *palearctica* (LVS species) was used as positive control DNA (12,13).

RESULTS

Tularemia patients: In total, 61 tularemia-suspected cases were detected, and 54 of these were confirmed (≥1/160) with the MA test. The MA titers were ≤ 1/80 in 4 cases (probable) and were negative for the remaining 3 cases (suspected). With the exception of one asymptomatic control case (whose MA titer was 1/40), the MA tests were negative for all control cases (24 asymptomatic and 18 symptomatic). The mean time period for the MA test, from the onset of the symptoms to blood sample collection, was 9 weeks (1 - 21 weeks), and the median MA titer was 1/640 (Figure 3). The patients whose MA titers were negative or lower than 1/160 were in the 3rd month of their illness when blood samples were obtained for the MA test. Control MA tests were not studied for these patients.

The mean age of the tularemia cases was 37 years (min 12, max 76), and most of them (62.3%) were female (Figure 4). Among the 61 tularemia patients, 35% were in the same households, and the remaining patients were from different families.

All tularemia cases had symptoms or clinical findings compatible with the oropharengeal tularemia form. In addition, 4 patients considered to exhibit both oculoglandular and oropharengeal tularemia reported chemosis and conjunctivitis in the early period of the illness in their interviews. Glandular, ulceroglandular, typhoidal and pulmonary forms of tularemia were not observed.

Swollen cervical lymph nodes were the most common symptom in the tularemia patients (97%). Other symptoms were fever (84%), sore throat (82%), cough (26%), ulcer in oral mucosa (23%), sputum (15%), diarrhea (15%), skin rash (13%) and chemosis (16%). In the physical examination, all patients were observed to have swollen cervical lymphadenitis or scars of lymph node excision on the cervical region (Figure 5).

PCR: The detection of *F. tularensis* in cervical lymph node aspirates by PCR was positive for 7 of 7 tularemia patients, who were also confirmed with the MA test. *F. tularensis* detection by PCR was negative in all water samples obtained from 6 different sources.

Outbreaks and environmental findings: In both of the outbreaks, the case distributions among months showed the

Fig. 5. Suppurated cervical lymph adenitis of a tularemia patient.
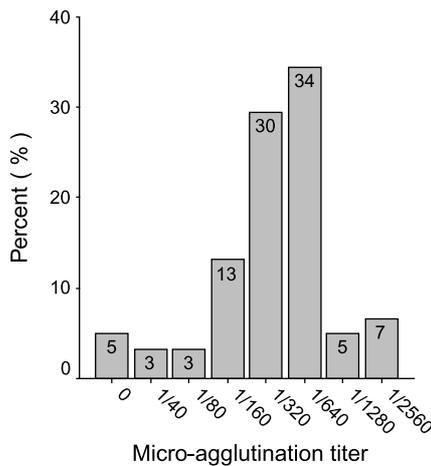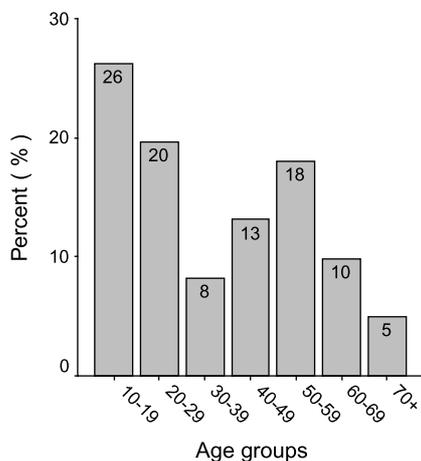
same graphical pattern, as follows. The first case (according to the initiation time of the symptoms) was detected in December. The number of cases increased in January, reached the maximal level in February, and decreased in March, and the last cases were detected in April (Figure 2). No tularemia patients were detected in the other seasons in this outbreak area. The areas where the tularemia outbreaks occurred are located in the West Black Sea Region. These regions are mostly surrounded by mountains covered with forests containing many kinds of trees, herbs and rodents. In agreement with what the villagers described, we could not find any evidence suggesting water contamination by rodents (such as carcasses or feces of rodents). However, the chlorination of water was not regular, and routine microbiological analysis showed that the bacteria count (colony-forming unit per milliliter) in the water that the villagers drink was above the acceptable limit. No increased rodent population was observed by the inhabitants in the 6 months before the outbreak.

Treatment: A combination of streptomycin (1 g/day i.m.) plus doxycycline (200 mg/day p.o.) was recommended for most of the patients, and ciprofloxacin (1 g/day p.o.) was recommended for a few patients, both for 14 days (Table 1). Some patients, with a diagnosis of suspected tularemia or other diseases, had used antibiotics that are also effective for *F. tularensis* (such as tetracycline, gentamicin, ciprofloxacin, levofloxacin, telitromycine and chloramphenicol) before the surveillance. The mean delay time for the initiation of an appropriate antibiotic was 8 weeks (1 - 17 weeks). Overall, 53 of 61 (87%) patients used appropriate antibiotics for tularemia in the course of their illness. The remaining 8 (13%) patients (4 of whom recovered spontaneously) did not use any antibiotic that is used for the treatment of tularemia (Table 2).

Within a 4-month period, 21 of 61 (34.4%) patients completely recovered from the illness. However, there was a therapeutic failure in the remaining 40 (65.6%) patients who went to lymph node excision or draining. No mortality or major complications (such as pneumonia, meningitis, deep neck infection) due to tularemia was observed. However, wound scars requiring reconstructive surgery occurred on the excision or draining region of a few patients. Most of the patients were reevaluated for prognosis on the 6th and 12th month after the first visit, and no relapse of the disease was observed.

We investigated the correlation between the treatment responses and the following parameters: age, age group, sex,

Fig. 3. Micro agglutination titers of tularemia patients.

Fig. 4. Age distribution among tularemia patients.

Table 1. Distribution of antibiotic usage and antibiotic choice among tularemia patients

Antibiotic usage status of tularemia patients	No. of patients n (%)
Streptomycin + doxycycline combination	36 (59.0)
Streptomycin monotherapy	2 (3.3)
Gentamicin + doxycycline combination	3 (4.9)
*Fluoroquinolone + doxycycline combination	4 (6.6)
*Fluoroquinolone monotherapy	8 (13.1)
No appropriate antibiotic usage	8 (13.1)
Total	61 (100.0)

*: Ciprofloxacin or levofloxacin

Table 2. Treatment response rates of tularemia cases according to some patient variables

Patient variables	No. of patients whose lymph nodes were suppurated and drained n (%)	No. of patients who showed complete healing n (%)
All cases	40 (65.6)	21 (34.4)
Patients under 50 years old	25 (61.0)	16 (39.0)
Patients 50 years old and over	15 (75.0)	5 (25.0)
Male	15 (65.2)	8 (35.8)
Female	25 (65.8)	13 (35.2)
Appropriate antibiotic usage in the early period of the disease (within 3 weeks)	2 (25.0)	6 (75.0)
Appropriate antibiotic usage in the late period of the disease (after 3 weeks)	30 (73.2)	11 (26.8)
No appropriate antibiotic usage	4 (50.0)	4 (50.0)
Antibiotic therapy includes amino glycoside	27 (65.9)	14 (34.1)
Antibiotic therapy includes fluoroquinolone	9 (75.0)	3 (25.0)

underlying diseases, appropriate antibiotic usage, antibiotic choice, and point at which the antibiotic was begun (Table 2). While 6 of 8 (75%) tularemia patients who used appropriate antibiotics within the first 3 weeks of their illness recovered completely; only 11 of 41 (26.8%) patients who used appropriate antibiotics within 4 or more weeks of their illness recovered completely. The difference was found to be significant with the chi-square test ($P < 0.016$). A significant correlation was not detected between the other parameters described above. When the correlation was investigated for only confirmed tularemia cases, the findings did not change.

DISCUSSION

Tularemia, not a nationally notifiable disease until 2005, was first reported in 1936 with an epidemic affecting 150 persons in Trakya in Turkey (14). In the following years, tularemia epidemics and sporadic cases were reported at different sites in Anatolia (12,15-23). The tularemia-affected areas of the last 25 years have commonly been located in the Black Sea and Marmara regions of Turkey (Figure 1). This is the first report of tularemia epidemics affecting these three provinces of Turkey.

In recent years tularemia outbreaks were reported in a number of European countries (2,5,24-27). The ulceroglandular form was the most prevalent clinical form of the disease in Spain, Sweden and Finland (27). However, oropharengeal

tularemia, not a frequent form in western or northern Europe or in America, is the most common form in Turkey, as it is in Bulgaria and Kosovo (18,24,25).

Oropharyngeal tularemia occurs as a result of the ingestion of food or water contaminated with *F. tularensis*. Children have contracted the disease more often than adults, and several family members may be affected simultaneously (8). However, a large proportion of tularemia patients in Bulgaria were elderly people, and researchers have found that age might be associated with increased susceptibility to oropharyngeal tularemia (24). In contrast, in a report including 205 tularemia cases form Turkey, most of the patients were in 16-40 years old (18). In another reported epidemic involving 21 cases in Turkey, most of the patients were elderly people (20). In our study, age distribution was not significant, but most of the patients were between 10-50 years old, and there was no patient under 10 years old. In Turkey, tularemia patients under 10 years old were extremely rare. It is interesting that children under 10 years old were not affected while the main route of transmission was considered to be food or water that was commonly ingested.

The source and transmission mode of *F. tularensis* are not clear in Turkey. It is well known that tularemia is a highly infective bacterium, and even 10 bacteria are enough to cause the disease (6). Another well known fact is that food- or water-borne diseases generally affect most of the members of a family due to the common consumption of food or water. Members of the households of individuals who contracted the disease made up only 35% of the tularemia cases in this epidemic. Food related transmission was not suggested to be the main transmission route of the disease in our region. First, there were no vegetables or fruits (that might be contaminated with the bacteria) that could be collected in nature and ingested in the spring time in this region. Second, most of the inhabitants did not describe any increased number of rodents or evidence suggesting contact between food and rodents. Thus, we considered that ingestion of contaminated water was the transmission mode of the bacteria. However, we could not explain why the other members of affected families were not affected if they ingested common food or water contaminated with a highly infectious bacteria. Individual differences of immunity might play a role in this setting or the transmission route might not be limited to drinking water as supposed. In addition, almost all of the sera obtained from control cases, most of whom were household members of individuals with the disease, were found to be negative on the MA test. In contrast, almost all of the suspected cases of tularemia were found to be positive by the MA test. It is difficult to accept that the MA-negative household members were free of contact with contaminated water that was commonly used in the house. Thus, if contact with contaminated water was the means of transmission, our data suggest that antibody response (detectable with MA test) against *F. tularensis* generally occurred in the patients who were symptomatic.

Tularemia outbreaks in these provinces emerged in the same period (between December and April) and showed similar case distributions among months in both years. Although some tularemia epidemics have emerged in the summer, most of the reported tularemia epidemics have occurred between the fall and the spring in Turkey (14-20). We did not find any definitive evidence to explain why both outbreaks occurred in the same seasons with similar graphical patterns of case distributions in both years in these provinces. The climate is rainier and colder in these months than in the summer in

Turkey. Contaminated water was the supposed mode of transmission in these epidemics, and the rainy climate might play a facilitative role in the contamination of water sources with *F. tularensis*.

The gold standard of diagnosis is a positive culture for tularemia, but a 2 or 3 biosafety level laboratory is required to such a culture (6-8). However, growing bacteria is not a preferred way to diagnose the disease (except in reference laboratories) because of the difficulty of growing the bacteria and the high risk of infection in laboratory studies involving *F. tularensis* (1). Thus, nonculture methods, mainly the agglutination test, immune assay and PCR, have generally been preferred for diagnosing this disease (13,28). We used the MA test for diagnostic confirmation (≥1/160). According to CDC criteria, a fourfold or greater change in the serum antibody titer to *F. tularensis* antigen is required for a confirmed diagnosis. However, a single titer of 1/160 or greater is considered to be a confirmed diagnosis by some authors (1,6). In addition, *F. tularensis* was detected in aspiration material of the suppurated lymph node of 7 patients by PCR. Furthermore, most of the patients had used appropriate antibiotics for 14 or more days before the aspiration material was obtained. In most cases, antibodies to *F. tularensis* appeared 6-10 days after the onset of symptoms (29). Thus, the MA test has a limited diagnostic value in the early period of the disease. However, PCR provides diagnostic advantages by showing highly sensitive results in the early stages of tularemia (28,30-32). Our limited findings suggested that PCR is a useful diagnostic test not only in the early period of tularemia but also in the late period, even if an antibiotic has been used.

Tularemia is not a well recognized disease, whereas tuberculosis is an endemic disease in this region of Turkey, and this is the first report of tularemia in these provinces. Interestingly, 5 of these 61 tularemia patients had been misdiagnosed with tuberculous lymphadenitis (based not on microbiological but rather clinical and histopatological findings) and started anti-tuberculosis drugs before the surveillances began. Tularemia should be kept in mind as a differential diagnosis of cervical mass etiology and granulomatous lymphadenitis in the regions where a tularemia risk is present.

Streptomycin or gentamicin is the drug of choice for the treatment of tularemia (1,6-8). Alternative drugs include ciprofloxacin, doxycycline and chloramphenicol, but there is a lack of supporting clinical data to evaluate the effectiveness of these alternatives (8,33). In a recent tularemia outbreak in Spain, ciprofloxacin was the antibiotic with the lowest level of therapeutic failure (26). Apart from the choice of antibiotic, Helvaci et al. found that early antibiotic therapy (within the first 3 weeks) was much more effective for resolving the infection (18). Most of our patients used a combination of streptomycin plus doxycycline, and the others used fluoroquinolone monotherapy or a combination of fluoroquinolone and doxycycline. Antibiotic choice was not a factor affecting the treatment response, however, treatment response was significantly related to any delay in the appropriate antibiotic therapy. Indeed, 75% of the tularemia patients who started the appropriate antibiotic within the first 3 weeks of the disease recovered, whereas only 26.8% of patients who used appropriate antibiotics within 4 or more weeks of the disease recovered ($P < 0.016$).

In conclusion, tularemia, commonly presenting in the oropharyngeal form, is endemic and may cause sporadic or epidemic diseases, mostly in the Black Sea and Marmara regions of Turkey. Antibacterial therapy has a limited benefit if it is initiated in the late period of oropharyngeal tularemia. Further investigations are needed to expose the reservoir of the bacteria and mode of transmission in Turkey.

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