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An outbreak of oropharyngeal tularaemia linked to natural spring water

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A tularaemia outbreak was investigated involving 188 suspected cases in the Kocaeli region of Turkey between December 2004 and April 2005. A case–control study comprising 135 laboratory-confirmed cases and 55 controls was undertaken to identify risk factors for the development of the outbreak and to evaluate laboratory diagnostic methods. Tularaemia was confirmed by a microagglutination test (MAT) titre of $\geqslant$1 : 160 in 90 of the patients. In MAT-negative sera, 23/44 (52 %) were positive by ELISA with *Francisella tularensis* LPS and 1/9 (11 %) by Western blotting with this antigen. A species-specific PCR was positive in 16/25 (64 %) throat swabs and 8/13 (62 %) lymph node aspirates. Multivariate analysis showed that drinking natural spring water was the leading risk factor for the development of tularaemia (P=0.0001, odds ratio 0.165, 95 % CI 0.790–0.346). The outbreak ceased after abandonment of the suspected natural water springs.

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INTRODUCTION

Tularaemia is a zoonotic disease characterized by a variety of clinical forms caused by virulent *Francisella tularensis* species. It is most common in the northern hemisphere, and outbreaks linked to ingestion of contaminated natural spring waters have been described in western Turkey (Helvaci *et al.*, 2000). *F. tularensis* is also classified as a Category A bioterrorist agent (Tärnvik & Berlund, 2003; Tärnvik *et al.*, 2004). The incubation period for tularaemia is approximately 3–5 days, and the clinical picture varies according to the virulence, dose and portal of entry of the bacteria, and to the immunity of the host. The disease may be asymptomatic, or may progress rapidly to sepsis and death if not treated properly. The major clinical forms are ulceroglandular, glandular, oculoglandular, oropharyngeal, typhoidal and pneumonic tularaemia (Ellis *et al.*, 2002). Oropharyngeal infection is the most common presentation in Turkey and in other Eastern European countries, and this has been attributed to the consumption of contaminated water and food (Tärnvik *et al.*, 2004).

Abbreviation: MAT, microagglutination test.

Kocaeli is an industrial city located 111 km east of Istanbul, with a population of approximately 1.5 million inhabitants. The city is surrounded by forests with many natural water springs. The outbreak of tularaemia occurred in a new settlement area constructed after the earthquake of 1999 (magnitude 7.4) between December 2004 and March 2005. The clinical, laboratory and therapeutic features of patients affected by this outbreak have been reported previously (Meric *et al.*, 2008a). This study addressed the risk factors for the infection and evaluated different diagnostic methods available for oropharyngeal tularaemia.

METHODS

Index cases. Five patients were referred to Kocaeli University Hospital, Turkey, in January 2005 with persistent fever, sore throat and massive swelling of the cervical lymph nodes, despite at least 10 days' therapy with β-lactam/macrolide antibiotics for a

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pre-diagnosed upper respiratory tract infection. On physical examination, an exudative tonsillitis and cervical lymphadenitis were detected in all cases.

Outbreak investigation. Following the diagnosis of index cases as oropharyngeal tularaemia, an outbreak investigation team was formed in collaboration with the local office of the Ministry of Health. The study was approved by the institutional review board of the Medical Faculty, Kocaeli University. A suspected case of tularaemia was defined as the presence of fever, a membranous pharyngitis or tonsillitis and/or cervical lymphadenopathy in a patient who came from the epidemic region, and who did not respond to β-lactam/macrolide antibiotics or who improved with antibiotics active against tularaemia. All hospitals and health-care centres in the region were visited, and patients who had been admitted with fever, sore throat and a cervical mass were visited at their homes. Data were collected through a structured questionnaire that addressed patient demographics and risk factors such as drinking of natural spring water, eating hunted animals, rodent bites or contacts, eating food without appropriate cleaning and heating, and travel to an endemic region. Blood, throat swabs and lymph node aspirates were obtained. Antibiotics against *F. tularensis* were given to suspected and confirmed tularaemia cases.

In the outbreak region (areas 1, 2 and 3; Fig. 1), people drank both tap and natural spring water; the latter was transferred by a pipeline from 1 km downstream of the source to six separate fountains (Fig. 1). These fountains were not under the control of the local municipality and the water was not collected in a reservoir and was not chlorinated. Tap water was routinely controlled by the local office of the Ministry of Health. One litre samples from each fountain and tap water were taken for microbiological investigations, transported to the laboratory and stored at 4–8 °C until culture on the same day. All attendant health-care workers were educated about the illness and newly suspected patients were directed to a health-care centre in the region. Control subjects who did not have a history of fever, sore throat or cervical lymphadenopathy within the last 3 months were selected randomly from among the neighbours and family members of the patients.

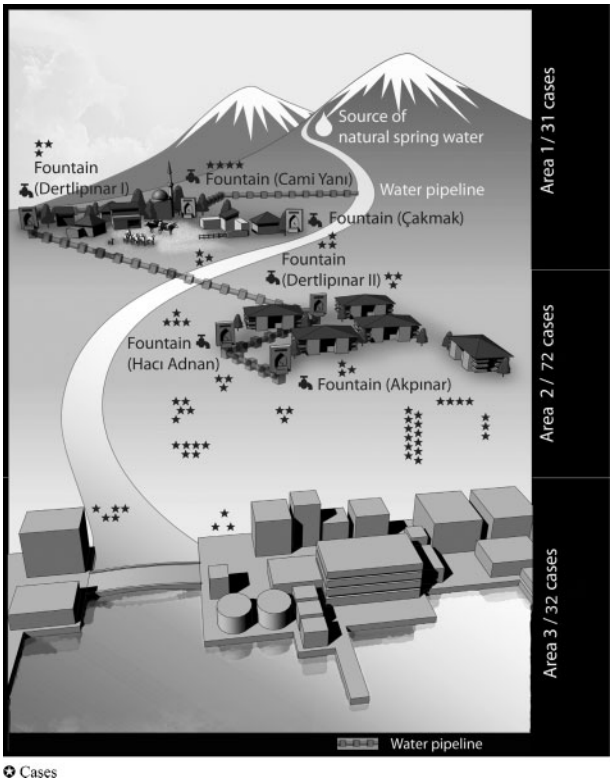

Fig. 1. Diagram showing the outbreak region and the three areas where the cases originated. Asterisks indicate cases and tap symbols indicate the positions of the six fountains.

Laboratory diagnosis. Blood samples from the first 10 patients and throat swabs from 25 patients were obtained for routine bacteriological culture. All sera from patients and control cases were tested by a microagglutination test (MAT) at Uludag University, Turkey. PCRs of serum, lymph node and throat swab samples were performed for *Mycobacterium tuberculosis* and *F. tularensis* at Kocaeli University, Turkey. Samples were also dispatched to the Bundeswehr Institute of Microbiology, Munich, Germany, for confirmation of the PCR results, identification of the *Francisella* subspecies and for ELISA and Western blot tests. The laboratory confirmation of tularaemia was based on the presence of at least one of the following test results: an MAT titre of $\geqslant$160 (Chu & Weyant, 2003; Tärnvik & Berlund, 2003), a fourfold increase in the MAT titre within 7–14 days, detection of antibodies by ELISA and/or Western blotting, and a positive result in the PCR assay.

MAT. Serum samples were tested with an antigen preparation of a *F. tularensis* subsp. *holarctica* strain according to the method of Gedikoğlu (1996).

PCR assays. An IQ real-time PCR (iCycler IQ, v3.0a; Bio-Rad Laboratories) was used. The primers (Versage *et al.*, 2003) were prepared by Iontek; primer sequences are given in Table 1. All reactions were performed in a final volume of 25 µl containing iTaq DNA polymerase in SYBR Green I supermix (Bio-Rad); the final concentration of each primer was 5 µM and the optimum annealing temperature was 60 °C. Cycling conditions were 50 °C for 2 min and 95 °C for 8 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min, with a final extension at 45 °C for 5 min. Negative (PCR-grade, sterile distilled water) and positive (formalin-fixed *F. tularensis* subsp. *holarctica* cells) controls were included in each run. All three gene targets were required to be positive to confirm the presence of *F. tularensis*.

DNA was isolated from all samples using a QIAamp DNA extraction mini kit (Qiagen). Water samples were centrifuged for 10 min at 5000 ***g*** and the pellet was suspended in 180 µl ATL buffer from the DNA mini kit. After the addition of 20 µl proteinase K (20 mg ml^{-1}), samples were incubated for 1 h at 56 °C before DNA extraction. Swabs from lymph node suppuration and throat samples were dispersed in 2 ml PBS in an Eppendorf tube and incubated for 3–4 h at room temperature before DNA extraction. Lymph node aspirates were mixed with 200 µl PBS and 20 µl proteinase K (20 mg ml^{-1}) with 200 µl AL buffer from the DNA mini kit and incubated for 10 min at 56 °C.

Water analysis. Bacteriological analysis for coliform bacilli in water samples was performed by a standard multiple-tube method.

Serological tests. ELISA and Western blot analysis were performed as described previously (Schmitt *et al.*, 2005). An absorbance value >0.250 by ELISA and a typical lipopolysaccharide ladder at a serum dilution of 1 : 500 by Western blot were considered positive.

Data analysis. Statistical analysis was performed using the SPSS 10.0 software program. Categorical comparisons were performed by the χ^2 test and continuous variables were tested by Student's *t*-test.

Table 1. *F. tularensis* primers used in this study

F, Forward primer; R, reverse primer.

Target gene	Primer	Sequence (5′→3′)	Amplicon size (bp)
IS*Ftu2*	ISFtu2 (F)	TTGGTAGATCAGTTGGTGGGATAAC	97
	ISFtu2 (R)	TGAGTTTTACCTTCTGACAACAATATTTC	
23 kDa	23 kDa (F)	TGAGATGATAACAAGACAACAGGTAACA	84
	23 kDa (R)	GGATGAGATCCTATACATGCAGTAGG	
Tul4	Tul4 (F)	ATTACAATGGCAGGCTCCAGA	91
	Tul4 (R)	TGCCCAAGTTTTATCGTTCTTCT	

Multivariate analysis was performed by logistic regression. The independent variables were age, gender, drinking spring water, contact with the excreta of rodents, consumption of unhygienic food and outdoor activities. ELISA and Western blot analysis were evaluated against the MAT as the standard.

RESULTS

The outbreak of oropharyngeal tularaemia occurred in Gölcük, Kocaeli, between December 2004 and April 2005; it was the first outbreak of tularaemia in this region. The first five patients were diagnosed and hospitalized based on the clinical picture and confirmed by the MAT titre. In total, 188 suspected cases were identified and 135 (72 %) were confirmed by laboratory tests. These confirmed cases were compared with 55 controls serologically negative by MAT. Of the 135 case patients, 76 % were living in areas 1 and 2, where the six fountains were located, whilst the remainder lived in area 3 and also had a history of drinking natural spring water (Fig. 1). Healthy individuals in the control group lived in the same region and had similar socio-economic status to the tularaemia case patients. Table 2 shows that the only statistically significant difference in demographic features and risk factors between the case and control groups was the consumption of spring water (P=0.0001, odds ratio 0.165, 95 % CI 0.79–0.346).

The majority (73 %) of the patients had received penicillin, ceftriaxone or a macrolide, and four had been given antituberculosis therapy, based on pathological examination of their lymph nodes. All patients were seronegative for Epstein–Barr virus, cytomegalovirus, *Toxoplasma gondii* and antistreptolysin O. No acid-fast bacteria were observed in lymph node aspirates and none of the samples were positive for *M. tuberculosis* by PCR or in culture. Routine blood cultures and throat swabs from patients were negative for *F. tularensis* and other pathogenic bacteria.

Among the initial 188 suspected cases, blood samples were obtained from 177 patients, throat swabs from 25 and lymph node aspirates from 13. There were 11 patients from whom no samples were available and another 42 suspected cases where the patients' first serum samples were negative by MAT. The MAT titre was diagnostic of tularaemia ($\geqslant$1 : 160) in 90/177 patients (51 %) from single serum samples and the diagnosis was confirmed in a further 22 patients by a fourfold rise in titre in paired samples; 21 were positive by ELISA and two by PCR to give 135 confirmed cases. In non-serum samples, the PCR result was positive in 16/25 throat swabs (64 %) and 8/13 lymph node aspirates (62 %). Approximately half (52 %) of the MAT-negative sera were positive in the ELISA, and 11 % were positive by Western blotting.

Water samples from the six fountains were highly contaminated with coliform bacilli, whereas the local municipal tap water met hygienic water-quality standards. All water samples were negative in the PCR assay.

DISCUSSION

Tularaemia usually presents with different clinical forms, but the oropharangeal form dominates if the infection is acquired through contaminated food or water (Ellis *et al.*, 2002). Most of the reported tularaemia cases in Turkey in the last 20 years have been oropharyngeal and related to the

Table 2. Demographic characteristics and risk factors of tularaemia case patients and controls

	Cases (n=135) (%)	Control group (n=55) (%)	P value
Median age (years)	38.14±18.09	37.25±19.02	0.707
Female gender	81 (60)	39 (71)	0.157
Housewives	72 (53)	32 (58)	0.682
Consumption of spring water	119 (88)	29 (53)	0.0001
Consumption of unhygienic food	13 (10)	2 (4)	0.165
Contact with rodent excreta	11 (8)	1 (2)	0.104
Outdoor activities	18 (13)	3 (5)	0.116

consumption of contaminated water (Helvaci *et al.*, 2000; Celebi *et al.*, 2006; Ozdemir *et al.*, 2007); other reports of cases from different European countries support this association (Luotonen *et al.*, 1986; Reintjes *et al.*, 2002). In this study, the risk of infection was found to be significantly correlated with the consumption of water from natural springs in the outbreak region, and the outbreak was controlled by the abandonment of the fountains. These fountains received water from the same source and were not hygienically controlled, unlike the tap water, which was clean and available in all houses. The spring water was heavily contaminated with coliform bacteria, but some people in rural areas prefer the taste of natural spring water. Indeed, 88 % of the 135 confirmed case patients obtained their drinking water from the natural springs and 60 % were women, who were more likely than men to gather spring water (Table 2). The Kocaeli region is highly populated by victims of the 1999 earthquake, and the ensuing disruption of the infrastructure may have facilitated the proliferation of the rodent population in this area (Reintjes *et al.*, 2002). No cases of tularaemia were reported in the region prior to 2004.

Outbreaks of tularaemia associated with contaminated water usually occur in the autumn and winter months (Tärnvik & Berlund, 2003; Tärnvik *et al.*, 2004), whereas disease associated with the consumption of hunted animals, as in the USA, is more common in the summer and early autumn (Anda *et al.*, 2001; Ellis *et al.*, 2002). The Kocaeli outbreak occurred at the end of autumn, with high rainfall, and some dead mice were seen on the stream line of the water springs by inhabitants of the region. These findings may relate the development of the outbreak to mass dying of wild rodents with a preceding tularaemia epizootic. The water and other environmental habitats could have been highly contaminated with the pathogen, leading to exposure of humans through consumption of water and food contaminated with animal excreta.

Despite the high statistically significant link between spring water and tularaemia in this outbreak, the PCR assay for *F. tularensis* was negative for the water samples. This may have been due to the insensitivity of centrifugation as a means of concentrating the organism, because, in another outbreak, PCR following filtration of the water proved positive for *F. tularensis* (Meric *et al.*, 2008b).

Four patients were initially diagnosed as having tuberculosis based on histopathological examination of their lymph nodes, and were given antituberculosis treatment, including streptomycin, which is also effective against tularaemia. The majority of patients (73 %) had been given penicillin, cephalosporin or macrolides because of a suspected non-specified bacterial upper respiratory tract infection. In endemic regions, tularaemia should be highly considered in patients with membranous pharyngitis and/or lymphadenopathy.

The MAT is the most common method used for the serodiagnosis of tularaemia (Ellis *et al.*, 2002; Tärnvik & Berlund, 2003), but antibodies to *F. tularensis* can be demonstrated by a variety of other methods including haemagglutination and ELISA with specific antigens (Schmitt *et al.*, 2005). However, it is noteworthy that, in this study, 23 cases were negative in the MAT assay and were diagnosed by ELISA and/or PCR. We suggest that, in addition to repeating the MAT after 7–14 days, ELISA and Western blot assays should also be considered for MAT-negative subjects. The species-specific PCR proved particularly useful for the detection of the organism in throat swabs (64 %) and lymph node aspirates (62 %) of the patients tested.

In conclusion, in endemic areas, surveillance and early alert systems for tularaemia and field investigation teams are necessary for prevention, detection and control of outbreaks, and physicians and other health-care workers should be cognizant with the basic clinical and epidemiological features of the disease. Diagnostic centres should be established and equipped, and the population at risk after natural disasters in potentially endemic/enzootic regions of tularaemia should be made aware through local education programmes of the significance of mass dying of rodents and the need for good hygiene along with anti-epidemic measures and pest control. Community water supplies, including spring waters, should be adequately chlorinated and controlled periodically to ensure that contaminated water is not used or consumed.

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