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Epidemia of Tularemia in Central Anatolia

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Abstract: Tularemia is a zoonotic disease caused by *Francisella tularensis* which can be transmitted to humans by tick bites contaminated water handling infected materials and inhalation. The different clinical types are ulceroglandular, glandular, oropharyngeal, oculoglandular, typhoidal and pneumonic tularemia. Streptomycin and tetracycline are commonly used to treat this infection. In this study approched other patients after the suspected tularemia patient attended to Kirikkale University, Faculty of Medicine with the servical mass complaint at January 2010. To find the reservoir for tularemia, laboratory research was carried on the mice suspected to be infected with *Francisella tularensis* in the town of infection.

Key words: Tularemia, zoonoses, mice, *Francisella tularensis*, glandular, Turkey

INTRODUCTION

Tularemia is a zoonotic disease caused by *Francisella tularensis* (Helvaci *et al*., 2000; Nordahl *et al*., 1993; Tarnvik and Berglund, 2003). The disease can emerge in variable clinical presentations namely the glanduler, ulseroglanduler, oculoglanduler, oropharyngeal, pneumonic and typhoidal.

The *F. tularensis* bacteria are found in widely diverse animal hosts and habitats and can be recovered from contaminated water, soil and vegetation (Ellis *et al*., 2002). Natural reservoirs of the infection include voles, mice, water rats, squirrels, rabbits and hares. Being bitten by flies, ticks or other bugs carrying the disease (Tarnvik and Berglund, 2003; Rinaldo *et al*., 2004). Humans can get the disease through:

- Direct contact through a break in the skin with an infected animal or its dead body
- The bite of an infected tick, horsefly or mosquito
- Eating infected meat (rare)

Tularemia can emerge in variable clinical presentations namely the glandular, ulseroglandular, oculoglandular, oropharyngeal, pneumonic and typhoidal. Ulceroglandular tularemia is the most common form of the disease (Helvaci *et al*., 2000; Collison and Adams, 2003; Wills *et al*., 1982; Luotonen *et al*., 1986; Kantardjiev *et al*., 2007).

Routine laboratory testing is generally not helpful in tularemia except to aid in excluding other diseases from the differential diagnoses. Routine blood culture results are usually negative for tularemia. Successful cultivation requires media that contains cysteine for growth. Cultivation in the laboratory poses a hazard for workers thus, laboratory personnel should always be advised if tularemia is suspected so that they may take appropriate precautions. The diagnosis of tularemia is usually based on serology results. Tularemia tube agglutination testing is the most commonly used serological test. Diagnosis is confirmed by a 4-fold increase in titer. An acute-phase titer of 1:160 is suggestive but such titers seldom develop until 11-21 days after onset of illness (Kantardjiev *et al*.,

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2007; Tarnvik and Chu, 2007). Lymph node biopsy is generally not needed for diagnosis. Polymerase chain reaction on material from wounds is being studied in some centers and appears promising as a means of earlier and easier diagnosis. This diagnostic modality is also being evaluated for potential use on other body substances (Kantardjiev *et al*., 2007). The goal of treatment is to cure the infection with antibiotic treatment. Streptomycin and tetracycline are commonly used to treat this infection. The early administration of these antibiotics (before the 3rd week of disease) was found to be much more effective to resolve the infection (Kantardjiev *et al*., 2007; Ellis *et al*., 2002).

The incidence of this infection is variable in different geographic locations. In various reports, variable seroprevelance rates and endemics in different areas are published (Helvaci *et al*., 2000; Gurcan *et al*., 2004; Kilicturgay *et al*., 1989; Tarnvik *et al*., 2004; Arikan *et al*., 2003).

Tularemia epidemics were reported in Gerede, Bursa (Gurcan *et al*., 2004; Arikan *et al*., 2003) and Thrace Region in the country. Recently cases of tularemia were reported from cities of Corum and Yozgat. When the cases determined in Kirsehir are added to the cases determined in Corum and Yozgat, an epidemic of tularemia in Central Anatolia can be defined.

In this study examined the patients with the servical mass complaint and suspected the tularemia. All the cases are diagnosed with serological and molecular technique. Clinical characteristics of this disease are discussed with a review of the literature. Mice around the location of epidemic were thought to be the vectors for this infection. Thus, they were collected and *F. tularencis* was searched with cultures and PCR in liver and spleen specimens of these rodents.

MATERIALS AND METHODS

In this study, after the clinical suspicion of *F. tularensis* in the patient attended to Kirikkale University, Faculty of Medicine with the complaint of neck mass at January 2010, it communicate with the Refik Saydam Hifz-i Sihha Presidency (RSH), Tularemia Reference Center. A team was created including doctors of the Clinic. This team went to Hamit town, Kaman County of Kirþehir Province to see other cases. Serum samples were taken from all patients. Suspicious cases were admitted to Hospital in Kirikkale University Faculty of Medicine and Kirikkale State Hospitals.

A diagnosis of tularemia was suspected due to clinical findings. In all patients, complete blood counts, Erythrocyte Sedimentation Rates (ESR) and C-Reactive Protein (CRP) levels were measured. For specific diagnosis, sera of the patients were sent to RSH Center Laboratory for microagglutination assay. The antigen used in the serological tests was prepared from an *F. tularensis* strain (Helvaci *et al*., 2000). Antibody titers of 1/160 and above were accepted to be significant for diagnosis.

Biopsies from the neck masses were taken from the patients in Otorhinolaryngology Department of Kirikkale University. Biopsy specimens were sent to Pathology Department of Kirikkale University for Pathological Examination and also sent to RSH Center for Molecular Evaluation (PCR). Treatments of patients were given. An informed consent from all study participants was received.

Reservoir source: To find the reservoir for tularemia by the cooperation with Veterinary Faculty of Kirikkale University and RSH Center in Hamit town, Kaman County of Kirþehir Province, mice were caught from different places. Fields and houses and rain water accumulation depots and their redirection basins in which mice may have been seen were investigated in day-time. Than, traps were established at midnight.

Captured alive mice were referred to laboratory in the early hours of the next morning. Studies were performed in mobile P2 level laboratory. Total of 42 mice were collected. In class II B-type safety cabinet, autopsy was performed to mice without anesthetic agent application and spleen and liver were removed in aseptic conditions. Collected samples were taken to 2 mL tubes containing small ceramic beads and approximately 500 mL of saline were added on. Tissues were homogenized with Magna Lyser (Roche) by holding for 30 sec at 6000 rpm and kept at -20°C.

The samples taken from the liver and spleen of the mice were examined by culture and PCR. Determinations of the type of mice were performed. Notification of cases was made to the Health Directorate of Kirikkale Province.

Culture: Francis medium (with L-cysteine, antibiotic supplement of *H. pylori* and 9% sheep blood) was used for this purpose. Human biopsy samples, throat swabs and liver and spleen samples of mice were planted separately to 100 μL mediums incubated at 37°C in environment with CO_2 for 10 days and evaluated. When growth occurred, colony morphologies were assessed. Suspected colonies were confirmed by slide agglutination method (with antiserum of BD Company) and PCR.

PCR: To obtain DNA from the biopsy specimens of human and liver and spleen tissues of mice. The PureLink™ Genomic DNA Purification Kit (Invitrogen) was used. House PCR method was used to increase the isolated DNA. To determine *F. tularensis*, Tul 4 (type-specific, 420 bp) and RD1 (sub type-specific) primer pair

were used (15). Used 1st primary was TUL4-435 (5'-GCT GTATCATCATTTAATAAACTG CTG and 2nd primary was TUL4-863(5'-GGAAGCTTGTATCATGGCTTGACT).

Other materials: Reaction reagents (40 μL reaction volume, 1× PCR buffer (Invitrogen), 0.2 mM dNTP (Amersham GE), 1 mg mL^{-1} BSA, 2.5 mM $MgCl_2$, 0.4 μM of each primaries, 1.25 U Taq polymerase, 5 μL DNA) and reaction conditions 4 min denaturation at 94°C, 40 cycles, 40 sec denaturation at 94°C, 30 sec anneling at 64°C, 45 sec elongation at 72°C and finally terminated by holding 5 min at 72°C. To view the products, agarose gel with 2% ethidium bromide was used.

Serology: By Microagglutination method, serum titers were evaluated. BD antigen was used for this purpose. Microagglutination procedure was performed as written below:

- U plate for micro agglutination was used
- About 90 μL to the first column of 8 wells and to other 6 wells 50 μL 0.85% saline was added
- About 10 μL of patient serum to first well was added
- It was mixed well by a pipette and transferred 50 μL from 1st well to 2nd well and serial dilute through 6th well and discarded 50 μL
- About 7th well was not added patient serum (Antigen control)
- About 50 μL of positive serum was added to 8th well
- About 50 μL of antigen with A + B stained to each well was added and mixed gently
- To prevent evaporation plate was sealed with paraffin and incubated at 37°C
- Negative samples had a button of cells at the bottom of the well

Since positive specimens agglutinated the antigen, a mat that looks like a fiffissed net of cells formed at the bottom of well. The results of 1/160 and over were considered as positive.

RESULTS AND DISCUSSION

Characteristics and physical examination results of the patients (9 cases) were shown on Table 1. Diagnostic tests and follow-up results of the patients were shown in Table 2. There were 6 male and 3 female patients. Their mean age was 34.22±16.37 (Ranged from 11.0-63.0).

In almost all cases, the patients used antibiotic (ineffective for tularemia) before admitted the clinic. Majority of cases were in glandular form. The first complaints of the patients were sore throat, fever and lymphadenopathy. Physical examination revealed fever and lymphadenopathy. Laboratory findings were in normal limits. Microagglutination test results were >1/160 in all cases. PCR was positive in 5 cases. Pathological

Table 1: Characteristics and physical examination results of the patients

	Cases with tularemia								
Characteristics	1	2	3	4	5	6	7	8	9
Age	63	16	45	11	35	38	19	39	42
Gender	K	E	K	K	E	K	K	K	E
Occupation	House wife	Student	House wife	Student	Farmer	House wife	House wife	House wife	Livestock
Coming place	Kirşehir/ Hamit	Kirşehir/ Hamit	Kirşehir/ Hamit	Kirikkale/ Çerikli	Kirikkale/ Balişeyh	Kirşehir/ Haciselimli	Kirşehir/ Çaliburnu	Kirşehir/ Tatik	Kirşehir/ Hanyeri
Antibiotics before application	β lactam	β lactam	β lactam	β lactam	-	β lactam	β lactam	β lactam	β lactam
Complaints	Sore throat Fever LAP	Sore throat LAP Weight loss	Sore throat Fever LAP	Fever LAP	LAP Conjunctivitis	Sore throat LAP	Sore throat Fever LAP	Sore throat Fever LAP	Sore throat Fever LAP
Physical examination results									
Lap	+	+	+	+	+	+	+	+	+
Tonsillitis	-	-	-	-	-	-	-	+	+
Fever	+	+	+	+	-	-	+	+	+
Organomegaly	-	-	-	-	-	-	-	-	+
Animal feeding	+	-	-	+	+	-	+	+	+
Contact of rodent animal									
Contaminated food	-	-	-	-	-	+	+	+	+
Bite	-	-	-	-	-	-	-	-	-
Found in the area	+	+	+	+	+	+	+	+	+
Suspicious contact	-	+	-	-	-	-	-	+	+
Cases from family /environment	+	+	+	-	-	+	-	-	-

*LAP = Lymphadenopathy

Table 2: Diagnostic tests and follow-up results of the patients

	Cases with tularemia								
Results	1	2	3	4	5	6	7	8	9
Laboratory findings									
Leukocyte count ($\times 10^9$/L)	7.800	8.900	8.800	11.700	8.000	8500	7100	9700	7100
Platelet count	228000	250000	252000	263000	329000	234000	315000	362000	252000
Erythrocyte sedimentation rate (mm h^{-1})	50	5	17	-	17	20	35	79	94
C-reactive protein (CRP) (mg L^{-1})	61	9	2	11	3	8	34	164	61
Elisa	>1/160	>1/160	>1/160		1/640	1/640	1/1280	1/1280	1/1280
PCR	+	+	+	+	+	+	+	-	+
Patological examination	+ Necrotizin granulomatous inflammation	+ Necrotizin granulomatous inflammation	+ Chronic infl. process inc. giant cells	-	-	-	-	-	-
Clinical follow-up									
Secondary infections	+	+	+	+	-	-	-	-	-
Treatment									
Drug	streptomycine	doxycycline	doxycycline	doxycycline	streptomy	streptomy -cine doxycy cline	streptomy -cine	streptomy -cine	streptomy -cine
Time	21 days	21 days	21 days	21 days	20 days	21 days	14 days	14 days	14 days

examination results were evaluated as necrotizing granulomatous reaction in 2 cases and chronic inflammatory process in 1 case (Table 1 and 2).

Reservoir source results: To find the reservoir for tularemia, captured in hamit town 42 mice were studied. All of the mouse was detected as Microtus socialis. Nothing was growthed in the cultures from liver and spleen of 42 mice in RSH Center. By PCR, *F. tularensis* was found as negative in examples taken from both liver and spleen of 42 mice.

Various different pathological situations may be associated with mass formation in the neck region. Upper respiratory tract infections, tooth problems, congenital malformations, tuberculosis, primary and metastatic malignancies are most common clinical entities related with mass formation in neck region. Thus, differential diagnosis is very important in clinical practice. Tularemia, as an etiological factor for mass formation in the neck is usually not remembered in differential diagnosis. It can be easily overlooked when there is not an endemia and misdiagnosed if epidemiological properties of this clinical entity are not well known (Rinaldo *et al*., 2004; Stupak *et al*., 2003).

The preferred antibiotics in upper respiratory system infections, proceeding with mass formation in the neck are penicillins and other beta lactam antibiotics. *F. tularensis* is resistant to all these antibiotics. Improper antibiotic administration in the case of *F. tularencis* infection may cause a chronic infection with poor prognosis. Thus, epidemiological data must be analyzed in details and anamnestic findings as contact with rodent and suspicious cutaneous lesions must be examined in details. All of the above mentioned patients had a history of attending to at least two other clinicians and non-specific antibiotic usage.

Bacterial cultures, pathological and molecular examinations may be performed in aspirates of neck masses or LAPs in suspected patients. Reproduction and proliferation of *F. tularensis* in laboratory practice is so difficult and necessitates selective culture mediums. Chronic granulomatous reactions seen in pathological examination may be confused with tuberculosis which leads to selection of inaccurate treatments. However, streptomycin used for tuberculosis may produce clinical improvement in tularemia. Definitive diagnosis of tularemia can be made by isolation of the microorganism with cultures or by molecular techniques as PCR. In 6 of the cases PCR was positive in excisional biopsy specimens. The reason for performing biopsies was Elisa test sometimes showed false negative results.

However, no reproduction was obtained in bacterial cultures against incubation for 2 weeks. In 4 of the cases, chronic granulomatous reactions were reported in pathological examinations. Serological tests were positive in 9 of the patients. In 9 of the 10 patients, the diagnosis of tularemia was proved with laboratory and clinical findings. However in investigations to determine the resource of the disease, all water samples were negative. It recognized that the populations of the voles and mice were increased in the nearby town where the most of the patients were living. This increase in population of rodents was thought to be related with high mean temperature and less snowing during the winter months and with inappropriate usage of agricultural combat

agents. In examination of rodents entrapped from this area, bacterial reproduction was not obtained with cultures. PCR studies were also negative. No resource was mentioned in any of the previous reports of tularemia in the country. The difference of the study was we investigated reservoir of tularemia; 42 mice were captured. For the cases, *F. tularensis* with PCR was also negative in rodents.

CONCLUSION

This result may be concluded that the captured mice were in the non-infected mice group. Tularemia must be kept in mind in differential diagnosis in patients with or without mass formation in neck region. Although, a history of contact with a tularemia patient is important in order to suspect for this infection, it is not a prerequisite. The public must be informed in details for both the clinical properties of tularemia and protection from these forms of infections.

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