Surveillance of tularaemia in Kosovo*, 2001 to 2010

R Grunow (Grunowr@rki.de)[1,2], A Kalaveshi[2,3], A Kühn[1], G Mulliqi-Osmani[3], N Ramadani[3]
1. Robert Koch Institute, Centre for Biological Security (ZBS 2), Berlin, Germany
2. The first two authors contributed equally to the paper
3. National Institute of Public Health of Kosovo*, Department of Microbiology, Pristina, Kosovo*

Tularaemia, caused by *Francisella tularensis*, had not been registered in Kosovo* before an outbreak in 1999 and 2000. A national surveillance system has been implemented in Kosovo* since 2000 to monitor a number of diseases, including tularaemia. Antibody detection in human sera was used for laboratory diagnosis of tularaemia and *F. tularensis* lipopolysaccharide antigen was used as a marker of infection. The purpose of this study is to describe the incidence of tularaemia in Kosovo* after the 1999–00 outbreak. In 2001 and 2002, a second outbreak occurred, with 327 serologically confirmed cases. From 2001 to 2010, 25–327 cases were registered per year, giving a mean annual incidence of 5.2 per 100,000 population. The most likely sources of infection were contaminated drinking water and food. The dominant clinical manifestations were the glandular (79%) and ulcero-glandular (21%) forms. By 2010, the disease had spread throughout Kosovo*. Presumably as a result of war and subsequent environmental disruption, mass population displacement and breakdown of sanitation and hygiene, the two major outbreaks of tularaemia resulted in the establishment of an active endemic area of tularaemia in Kosovo*.

Introduction

The causative agent of tularaemia, a relatively rare zoonotic disease, is *Francisella tularensis*. The bacterium is widely distributed in the northern hemisphere and is found in Europe [1]. Most countries in central and southern Europe reported single cases over the past decades. The disease is a more serious public health problem in Balkan countries and has also been reported in Turkey [2-4].

The cells of this Gram-negative, non-motile, capsule-forming, facultative intracellular bacterium are pleomorphic, typically appearing as short rods or coccoid forms [5]. Two subspecies of *F. tularensis* cause tularaemia in man. Biovar A1 of the subspecies *tularensis* (or type A) is the most virulent type of *Francisella* bacteria and can be associated with lethal pulmonary infections in humans [6]. Subspecies *holarctica* (or type B) is assumed to be less virulent. *F. tularensis* ssp. *tularensis* has been isolated almost exclusively in North America, whereas *F. tularensis* ssp. *holarctica* could be found in the entire northern hemisphere [5]. Interestingly, a second biovar of *F. tularensis* ssp. *tularensis*, A2, has a lower morbidity and mortality in humans than ssp. *holarctica* [6].

Depending on the site of entry of the pathogen, tularaemia can occur as ulcero-glandular and glandular forms, as well as oculo-glandular, oropharyngeal, respiratory or typhoidal [5]. Humans acquire the bacterium through contact with infected animals and/or vectors, by inhaling contaminated dust or aerosols, or by consumption of contaminated food or water. Human-to-human transmission is unlikely [5].

Francisella is capable of infecting a large number of animal species such as hares, rabbits, mice, lemmings and even fish [5]. Birds are known to be carriers, but probably do not develop the disease themselves [5]. Various vectors could play a role in transmission of *F. tularensis* from animals to humans: in Scandinavia, mosquitoes probably play a major role, while in North America, ticks are considered to be the most important vector [5].

F. tularensis is able to survive in the environment under cool and humid conditions, probably for weeks, and has been found in water and soil [7]. The mechanisms for survival of the bacteria are not yet well understood. Protozoa and nutrient conditions could play a role in protecting the bacteria [8-10].

History shows that tularaemia outbreaks are associated with poor hygienic conditions, especially in war and post-war situations, accompanied by a large increase in rodent populations and subsequent mass death of these animals [1,11-13]. Natural outbreaks occur in various endemic areas and can involve several hundred patients [1]. *F. tularensis* has been listed as a potential biowarfare agent [13,14].

Tularaemia had not been recorded in Kosovo* until an outbreak of the disease in 1999–2000 [13,15]. An intensive case investigation was carried out during the first outbreak [15]: specific antibodies against *F. tularensis* were detected in 247 serum samples from 912 suspected cases. Kosovo*, in south-east Europe, covers an

area of 10,908 km2, with a population of approximately 2.1 million inhabitants and population density of 192 per km2. It proclaimed its independence in 2008.

In January 2000, the national Institute of Public Health in Pristina implemented a surveillance system for 20 communicable diseases, based on syndromic approaches and clinical diagnoses. Timely reporting of disease syndromes from the different municipalities (administrative regions) allows a number of the most important infectious diseases in Kosovo* to be monitored simultaneously.

This study aims to provide a follow-up on the incidence of tularaemia in Kosovo* after the first outbreak in 1999–00 until 2010.

Methods

Case definition
A suspected case of tularaemia was defined as a person with fever and enlargement of cervical lymph nodes. The following indicators could also be present: skin ulcers and perspiration; weakness, body pain and headache; and throat pain and ingestion problems. Confirmed cases were individuals with the above clinical picture in whom laboratory confirmation of the infection was obtained.

To ensure that all individuals with the tularaemia were identified, the case definition was rather broad. The clinical manifestation of *F. tularensis* infection can be quite similar to that of tuberculosis, brucellosis or mumps – all of these diseases have a relatively high prevalence and incidence in Kosovo* (data not shown). Therefore, laboratory conformation by detecting specific antibodies or the pathogen itself is required for the final diagnosis of tularaemia.

Collection of epidemiological data
All outpatient clinics and medical centres are obliged to fill in official reporting forms every week to record aggregated and individual data on the 20 specified communicable diseases, including tularaemia. The forms are sent to the regional Institute of Public Health, which subsequently passes them on to the national institute. Double reporting is prevented by checking the personal data of reported cases. At the national institute, the data are entered into a central database, to be regularly analysed by means of Epi-Info software.

Diagnostic sera were obtained by local physicians from suspected cases and sent for analysis to the national Institute of Public Health. The sera were analysed for specific antibodies to *F. tularensis*, routinely using a microagglutination assay [16]. Positive sera and additional sera from persons suspected to be infected with *F. tularensis* were checked by a highly specific enzyme-linked immunosorbent assay (ELISA) and western blot analysis, as described below.

Microagglutination
The live vaccine strain of *F. tularensis* biovar *holarctica* (ATCC 29648) was grown for 2–3 days at 37 °C in a 5% CO_2 humid atmosphere on heart-cysteine-blood agar and harvested into sterile distilled water or isotonic sodium chloride. Bacterial concentrations were adjusted photometrically at 580 nm to an optical density of 1.0. The suspension was inactivated with paraformaldehyde and prepared as agglutination antigen as described elsewhere [16]. The assay was adjusted by the optimal antigen concentration and evaluated on the basis of a titre of 1:16 or higher being considered positive.

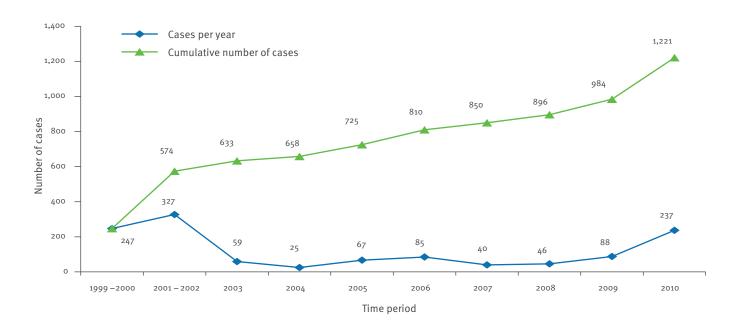### Antibody detection in human sera by ELISA and western blot
The ELISA was used for screening and western blot for confirmation. Both have been described elsewhere [16-18] and were used with some modifications. Briefly, a 96-well microtitre plate (Polysorb, Nunc, Germany) was coated with purified lipopolysaccharide (LPS) from the live vaccine strain as antigen. Bound human antibodies to *F. tularensis* were detected by polyvalent goat anti-human IgA-IgM-IgG horseradish peroxidase-conjugated secondary antibody (dianova, Germany) and subsequent substrate reaction. For the western blot, the soluble fraction of formalin-inactivated live vaccine strain was separated using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (ImmobilonP, Millipore, United States). Using polyvalent horseradish peroxidase-conjugated secondary antibodies, the typical LPS ladder revealed the presence of specific anti-*F. tularensis* antibodies. The final results were given after confirmation of the ELISA results by western blot: 'positive' denoted strong bands, 'negative' – almost no bands and 'borderline' – weak but clearly visible bands).

Samples for antigen detection and capture ELISA
Environmental samples (faeces from rabbits and mice, water samples), tissue samples (spleen, liver) from dead mice, rats and rabbits, as well as clinical samples from serologically confirmed tularaemia patients were analysed using a capture ELISA. It was essentially performed as described previously, using the *F. tularensis* LPS-specific murine monoclonal antibody 11/1/6 as capture antibody bound to the solid phase [19-21].

Faeces from pathogen-free inbred and outbred mice and rabbits were kindly provided by the National Research Centre for Environment and Health (GSF) in Munich, Germany, which were used as negative controls. Samples were homogenised and pre-treated with LPS-extraction buffer containing chenodeoxycholic acid in phosphate buffered saline/ethylenediaminetetraacetic acid (PBS/EDTA). Large particles were allowed to sediment for 5 minutes and the supernatant was analysed for the presence of *F. tularensis* LPS using the cELISA.

Reported confirmed cases of tularaemia, Kosovo*, 1999–2010 (n=1,221)

* This designation is without prejudice to positions on status, and is in line with United Nations Security Council Resolution 1244/99 and the International Court of Justice Opinion on the Kosovo declaration of independence.

Results

Tularaemia outbreak in 2001 and 2002

After the tularaemia outbreak in 1999–00, a second outbreak occurred from November 2001 to June 2002, which was investigated by a Kosovar/German team from the Bundeswehr Institute of Microbiology, Munich, Germany. This outbreak has not been previously described in the scientific literature. During this period, 1,168 serum samples from suspected cases were tested; 327 cases laboratory confirmed by ELISA and western blot (Figure 1). Although the second outbreak started in other parts of Kosovo* (east and south-east), the affected areas generally overlapped those of the previous outbreak.

The epidemiology of the second outbreak was quite similar to that of the first [15]. It can be assumed that the main reason for the spread of the disease seemed to be again the bad sanitary conditions, especially in rural areas of Kosovo*. As described for the first outbreak [15], housewives (37%) and farmers (27%) were the most affected occupational groups. Similarly, cases were mainly female (60%) and the age group 20–40 years (52%) were also most affected.

It was characteristic of both outbreaks that people in affected regions reported an enormous increase in the rodent population, especially field, forest and domestic mice before the outbreak among humans.

The investigation of animal and environmental specimens by the capture ELISA, which is highly specific for *F. tularensis* [19], showed that the antigen was detected mainly in mouse and hare faeces (Table). During the outbreaks, faeces of small rodents were regularly found by the investigation teams in products stored in food stores of affected households and showed the most striking positive results in antigen detection of *F. tularensis*. During the first and second outbreaks, 145 and 220 samples were collected respectively from similar sources, of which 10 and 22, respectively, were positive. We could not detect *F. tularensis* antigen in a very limited number of available clinical specimens: throat swabs (n=18), pus and wound secretions (n=4). At that time, a more sensitive polymerase chain reaction (PCR) was not available.

As in the first outbreak, the predominant manifestation of the disease during the second was oropharyngeal. The main route of transmission leading to this oropharyngeal form was probably ingestion of contaminated food or water [15]. More than 90% of the patients (305/327) had as the leading symptom enlarged cervical lymph nodes, whereas the other patients had enlarged lymph nodes in other locations such the axilla or inguinal region. This clinical manifestation of oropharyngeal tularaemia was dominant throughout the study period.

Francisella tularensis antigen detection in animal and environmental specimens during tularaemia outbreaks in Kosovo*, 1999–2002 (n=365)

Sample type	Source	1999–00		2001–02		Total without controls	
		Number of samples	Number positive	Number of samples	Number positive	Number of samples	Number positive
Faeces	Mice	55	7	58	9	113	16
	Control: pathogen-free mice	NT	NT	100	0	–	–
	Hares	NT	NT	104	12	104	12
	Control: pathogen-free rabbits	NT	NT	57	0	–	–
Animal tissue	Mice, rats, hares	63	3	35	1	98	4
	Control: pathogen-free mice	NT	NT	25	0	–	–
Water	Wells, ponds	27	0	23	0	50	0
Total without controls	–	**145**	**10**	**220**	**22**	**365**	**32**

NT: not tested.

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Surveillance of tularaemia from 2001 to 2010

After the first outbreak, the surveillance system revealed the presence of cases every year (Figure 1). From 2003 to 2010, the annual number of tularaemia cases was between 25 and 237, with a mean annual incidence of 3.9 ±3.2 standard deviation (SD) per 100,000 population. During this period, a total of 647 cases were reported.

In 2010, more than 200 tularaemia cases were registered. The reason for this high number of cases is unknown: no specific source of infection nor a specific outbreak scenario could be identified. The cases were distributed equally throughout Kosovo* and throughout the year. This could indicate a high epizootic and zoonotic activity in that year, for some unknown reason.

Housewives and farmers were the most affected occupational groups, representing about 33% (n=216) and 24% (n=153) of cases, respectively. This is also reflected in the sex distribution of all cases during this period: 57% (n=372) of cases were female and 43% male (n=275) (Figure 2). Most cases (n=309) were in the age group 20–40 years, of whom 61% (n=188) were female and 39% (n=121) male. In addition, quite a high proportion, about 20% (n=128) of children and teenagers (aged under 20 years) were infected.

Since 2001, the clinical manifestation in the 974 patients was oropharyngeal or glandular tularaemia: about 93% (n=906) of the cases had unilaterally enlarged cervical lymph nodes or swollen axillar or inguinal lymph nodes; 7% (n=67) had the ulcero-glandular form.

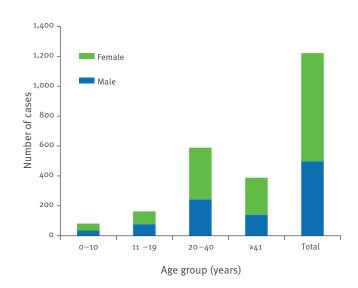Since the first outbreak, tularaemia has spread to other parts of Kosovo*. As a result, all municipalities participating in the surveillance system have reported human tularaemia cases (Figure 3). The three municipalities marked in grey in Figure 3B were not participating in the surveillance system.

Discussion

By mid-1999, more than 10 years of political crisis and warfare in Kosovo* had resulted in environmental disruption, mass population displacement and a breakdown of sanitation and hygiene [15]. Many essential public health functions, such as disease surveillance and outbreak response, had collapsed [15]. It

FIGURE 2

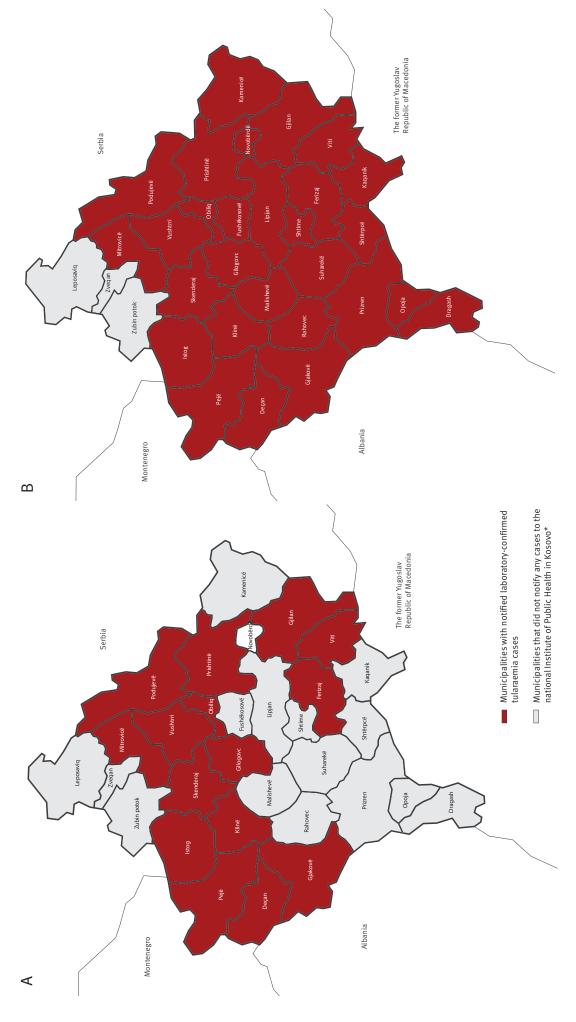Age and sex distribution of confirmed tularaemia cases, Kosovo*, 1999–2010 (n=1,221)

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FIGURE 3

Distribution of confirmed tularaemia cases, Kosovo*, Panel A: 1999–2000 (n=247), Panel B: 1999–2010 (n=1,221)

A

B

■ Municipalities with notified laboratory-confirmed tularaemia cases

▢ Municipalities that did not notify any cases to the national Institute of Public Health in Kosovo*

The three municipalities marked in grey in Panel B were not participating in the surveillance system.

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is suspected that tularaemia had been present in the region during and/or after the Second World War, but there are no official data about the disease in Kosovo* during that time. Although the disease was notifiable in the former Yugoslavia, local representatives stated that no cases of tularaemia had been detected before the war in Kosovo* in 1998 to 1999 [13].

The number of laboratory-confirmed cases of tularaemia in the first outbreak in 1999–00 was unexpectedly high, given that Kosovo* had been considered non-endemic for the disease at that time. An even larger number of cases was seen during the second outbreak in 2001–02. The circumstances of a typical post-war situation in the autumn of 1999 were probably responsible for the outbreaks: people left their homes and did not harvest the fields, which led to an oversupply of food for rodents [13]. Consequently, an unusually large increase in the rodent population was observed until January 2000. It is known that an increased density of the rodent population can facilitate the spread of zoonotic infectious pathogens including *F. tularensis* among animals and induce an epizootic spread to man [22]. We found *F. tularensis* antigen in a relatively high percentage of samples from collected rodents and hares. However, for further identification of the infectious source and characterisation of the causative agent, attempts should still be made to obtain isolates of *F. tularensis* from the samples collected between 1999 and 2002.

As almost all the tularaemia patients during 2001 to 2010, as in the first outbreak [15], had the oropharyngeal form with fever and a unilateral cervical lymph node enlargement as the main symptoms, obviously the main route of infection was alimentary ingestion of *F. tularensis*. It was rather surprising that during the first outbreak, cases were spread over a large part of Kosovo*. It can be speculated that either the pathogen had already been present in these regions in spite of not having been observed or it was spread by human and animal migration as a consequence of the war. It was assumed by the national Institute of Public Health and the outbreak investigation team at that time that an emerging or re-emerging endemic region with periodic outbreaks of tularaemia might develop in Kosovo*. In fact, the data for 2003 to 2010 indicate a continuous activity of tularaemia after the initial outbreaks.

The clinical manifestation in 2010 was similar to that during the outbreaks in 1999–2002, which suggests that the routes of transmission have remained the same. In comparison, in other parts of the world, the ulcero-glandular form of tularaemia is primarily detected, which can arise due to direct exposure of the patients' skin to infected animals, carcasses, water or other materials, or to arthropod vectors [1,5]. Climate change is believed to influence the spread of vectors and therefore of tularaemia [23]. Obviously alimentary ingestion of the pathogen has been the major route of infection in Kosovo*. Given the situation, ingestion

of contaminated food and water arising from the poor hygiene conditions seem to be the most likely risk factors for the infection.

Interestingly, the mean incidence of tularaemia in Kosovo* from 2001 to 2010 (5.2±4.6 SD per 100,000 population) is comparable to that in Sweden (3.2±2.08 SD per 100,000 population, calculated for the same time period from data from the Swedish Institute for Communicable Disease Control [24]), which is known to be endemic for tularaemia, and about 100 times higher than that in Germany (0.013±0.012 SD per 100,000 population; calculated for the same time period from SurvStat data from the Robert Koch Institute in Germany [25]). Tularaemia in Germany is less evident than in some other countries, but little is known about the epizootic activity of the disease in Germany. Thus, low numbers of reported human cases in Germany may not reflect the actual prevalence of the pathogen in nature and the potential risk of epidemics [26].

In 2010, Kosovo* represented an emergent endemic region for tularaemia. The reasons for this development are not fully understood. More recent data are in the process of being evaluated at the national Institute of Public Health in Pristina. Further surveillance of this disease is important in order to detect possible outbreaks in a timely manner and to take adequate measures to prevent the further spread of the disease. The main reason for ongoing activity of the disease seems to be the still poor sanitary conditions, especially in rural areas of Kosovo*. In addition, animal control and surveillance, including that of rodents, should be carried out to prevent further outbreaks. Further field investigation is required to obtain *Francisella* isolates for clarification of the subspecies prevalent in Kosovo* and to further identify reservoirs and routes of transmission of the pathogen. Additional resources are required to manage this serious health problem, although other infectious diseases may have an even higher impact on public health in Kosovo*.

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