Final Report

Ecology of *Francisella tularensis* and its impact on biological safety

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Content:

4) Origgi, F.C., König, B., Lindholm, A.K., Mayor, D., Pilo, P. Tularemia among Free-Ranging Mice without Infection of Exposed Humans, Switzerland, 2012. **Emerg. Infect. Dis**. In Press

5) Origgi, F.C., Pilo, P. Pathology, bacteriology and proposed pathogenesis of Tularemia in European Brown Hares (*Lepus europaeus*): a model for human tularemia. In preparation

6) Dobay, A., Pilo, P., Lindholm, A.K., Origgi, F.C., Bagheri, H.C., König, B. Epizootic of tularemia in a closely-monitored free-roaming population of wild house mice. In preparation

7) Origgi, F.C., Pilo P. Role of ticks (*Ixodes ricinus*) in transmission of *Francisella tularensis* subsp. *holarctica* subgroup B.FTNF002-00 in European Brown Hares (*Lepus europaeus*). In preparation

Bullet points

- Tularemia is a complex disease and the environment has an important impact in the biological cycle of *F. tularensis*.
- Tularemia is endemic in Switzerland.
- Two phylogenetic clusters of *F. tularensis* subsp. *holarctica* are co-circulating in Switzerland (B.FTNF002-00 and B.13).
- The cluster B.13 is resistant to macrolides.
- Humans, non-human primates, rodents and lagomorphs are mainly affected by tularemia in Switzerland.
- 2012 was a tularemic year for humans, non-human primates, rodents and lagomorphs in Switzerland.
- Tularemia was a relevant infectious disease in hares in 2012-2014 in Switzerland.
- Probable route of infection in hares: inhalation and ingestion.
- The phylogenetic cluster B.FTNF002-00 shows a different pathology than the B.13 in hares.
- Outbreaks of tularemia in small rodents are probably overlooked.
- The first estimation of the basic reproduction number R0 of an epizootic caused by *F. tularensis* in mice using quantitative modeling was calculated as being 1.16 and 1.91 (two different assumptions).
- Beavers are essentially not affected by tularemia in Switzerland and are supposed to play a marginal role if any in the ecology of the disease
- Recommendations for standard operative procedures (SOPs) for necropsy and bacteriological analyses of affected small mammals.
- Found-dead or sick free-ranging animals should not be manipulated with bare hands.
- Highest risk for tularemia outbreaks: water-borne tularemia.
- Carcasses or diseased (animal showing abnormal behavior, lethargy, inability to move) free-ranging rodents and lagomorphs should not be touched with bare hands or opened in the field. The local game warden should be alerted.
- A long-term study to trace sources of infection in the environment is necessary to evaluate the reservoir of *F. tularensis* and to understand epizootics-inter-epizootics periods.
- Serological and clinical history assessment of individuals either at risk or not to evaluate the likelihood of contracting the disease.

1. Brief description of the project.

1.a Specific aims.

Tularemia is a life-threatening infectious disease and a major concern for public health and biosafety authorities. It mainly affects free-ranging animals, including endangered and protected species. Despite the recent large ongoing research efforts focused on the causative agent of tularemia, *Francisella tularensis*, the ecology of this bacterium including its maintenance and amplification in the environment are still virtually unknown. To better understand these issues, which are a matter of biological safety, we started a broad investigative project aiming to dissect the life cycle of this microorganism on June, 1st 2012. In 2012, an increasing number of tularemia cases in humans and animals was observed and the following specific aims were identified:

1) Safety of the SOPs associated with the diagnosis of tularemia cases (pathology and bacteriology; *F. tularensis* subsp. *holarctica* in small mammals),
2) Identification of the infection route of *F. tularensis* and associated pathology in European brown hares (*Lepus Europaeus*),
3) Assessment of beaver's role, and other semi-aquatic mammals, in the maintenance of *F. tularensis* in the environment,
4) Monitoring of an outbreak of tularemia in a free-ranging mice population associated with human exposure.

This interdisciplinary project provides crucial information to better understand the maintenance and spread of *F. tularensis* in the environment, and to formulate recommendations to address biosafety issues, with expected positive outcomes such as the limitation of the spread of tularemia among free-ranging animals, with particular emphasis on relocation projects of affected species and reduction of the risk of accidental exposure and infection of people.

This project led to the publication of 4 original articles in internationally recognized peer-reviewed scientific journals and 3 additional manuscripts are in preparation.

1.b. Introduction.

F. tularensis is a Gram-negative, facultative intracellular, coccoid-shaped bacterium. It is a highly virulent microorganism capable of infecting a large number of animal species and it is the causative agent of the zoonotic disease tularemia (Hopla, 1974). Due to its high infectivity, *F. tularensis* is of major concern to public health officials and has been classified as a Category A Select Agent by the CDC (Petersen and Schriefer, 2005).

The species is divided in three subspecies: *F. tularensis* subsp. *mediasiatica*, *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* but only the two latter are clinically relevant (Brenner et al., 2005). *F. tularensis* subsp. *tularensis* is restricted to North America, while *F. tularensis* subsp. *holarctica* surfaces the whole Northern Hemisphere (Olsufjev and Meshcheryakova, 1982). It has to be noted that the taxonomic position of *F. tularensis* subsp. *novicida* or *F. novida* is currently under revision (Huber et al., 2009; Busse et al., 2010; Johansson et al., 2010). Historically, the disease was differentiated in two forms, type A (*F. tularensis* subsp. *tularensis*) and type B (*F. tularensis* subsp. *holarctica*) both sharing similar clinical features, yet showing distinctive severity (Olsufjev and Meshcheryakova, 1982). However, recent studies suggest that subgroups within subspecies may be associated to different grades of virulence (Kugeler et al., 2009).

Tularemia is primarily a disease affecting wild mammals such as lagomorphs and rodents (Mörner, 1992). These orders seem to be quite sensitive to the infection and prone to develop disease, and may play a role as bacterial amplifiers more than reservoirs *sensu stricto* (Hofstetter et al., 2006). However, the role of specific species within these orders as reservoirs is still unknown. The cycle of *F. tularensis* in the environment is not clear. Blood-sucking arthropods have been suggested to maintain this microorganism in nature but clear scientific and experimental data are missing. It is worth to note that tick endosymbionts, closely related to *Francisella* sp, have been recently described and impede the identification of *F. tularensis* (Niebylski et al., 1997; Sun et al., 2000; Scoles, 2004; Kugeler et al., 2005; Escudero et al., 2008; Machado-Ferreira et al., 2009; Baldridge et al., 2009). A study performed in collaboration between the Institute for Veterinary Bacteriology (IVB) in Bern and the Cantonal Institute for Microbiology in Bellinzona found single ticks harboring both *Francisella* sp endosymbionts and *F. tularensis* subsp. *holarctica* (Dr. Pilo, unpublished results). For this reason, PCRs targeting 4 distinct DNA loci or sequencing of the PCR products are necessary to confirm the presence of *F. tularensis* in ticks.

1.c. Background and significance.

Recently, it has been shown that a specific cluster of *F. tularensis* subsp. *holarctica*, the subgroup B.FTNF002-00, is circulating in Switzerland and more generally in Western Europe (Coolen et al., 2013; Dempsey et al., 2007; Gyuranecz et al., 2013; Müller et al., 2013; Pilo et al., 2009). Interestingly, pathological lesions in Swiss hares seemed to be different than the ones described commonly in the same species of lagomorphs including the absence of obvious grossly detectable foci of necrosis in the spleen, lung, kidney, liver and pericardium which often seems to be the only organ significantly affected (Dr. Origgi, unpublished results; Gyuranecz et al. 2010b). Moreover, while beavers have been reported as susceptible to tularemia in North America (Jellison et al., 1942), none of those tested until now at the Institute for Veterinary Bacteriology (IVB) in Bern was positive for *F. tularensis* (Dr. Pilo, unpublished results). These observations suggest a likely different biology of the Western European cluster (subgroup B.FTNF002-00) compared to other *F. tularensis* clusters and subgroups from other geographical regions. Tularemia has been confirmed in few free-ranging animal species in Switzerland (Burgisser, 1974; Haerer et al., 2001; Friedl et al., 2005; Pilo et al., 2009). At the moment, no active surveillance program for tularemia in animals is established, which leads to under-estimation of the current case number and probably also of the number of affected species.

Tularemia is emerging in Europe (Bellido-Casado et al., 2000; Anon, 2000; Gurycova et al., 2001; Eliasson et al., 2002; Byström et al., 2005; Al Dahouk et al., 2005; Christova and Gladnishka, 2005). The specific genetic background of the cluster circulating in Switzerland may influence the ecology and pathogenicity of these strains. Free-ranging animals are involved in the environmental cycle of this bacterium (Hopla, 1974). However, essential information about the ecology and the way of transmission of the disease, including the infection route, the spectrum of animal species affected and their specific epidemiological role within *F. tularensis* life cycle is still lacking. Indeed, several case reports describe different pathological lesions. These divergences may reflect species specificity features or those concerning the bacterial strains and/or the way of transmission (Park et al., 2009; Gyuranecz et al., 2010a; Gyuranecz et al., 2010b). In humans, the route of infection is associated with distinct clinical manifestations of the disease (Tarnvik and Berglund, 2003).

Another point that needs to be addressed is the role played by other potential reservoirs *sensu stricto* in the environment. Several studies and reports propose blood-sucking arthropods (mosquitoes, ticks, deer flies) (Hubalek et al., 1996; Hubalek and Halouzka, 1997; Hubalek et al., 1998; Goethert et al., 2004; Goethert and Telford, III, 2009; Gyuranecz et al., 2010a) and protists (Abd et al., 2003) as reservoirs. However, conclusive scientific and experimental evidence supporting these hypotheses has not yet been provided.

2. Work performed and final results.

2.a. Standard operating procedures for processing and analysis of carcasses and samples.

The aim of this chapter is to describe the standard operating procedures (SOPs) used during the project and that resulted safe for the personnel performing the analysis.

2.a.A. Necropsy

Animal carcasses were necropsied either in the routine necropsy room of the institute of animal pathology (ITPA) of the University of Bern afterhours or in a separate necropsy room with limited access to not authorized personnel. Necropsies were performed using disposable overall, surgical mask, double gloves, disposable head cap and cover shoes. The necropsy table was covered with absorbent disposable drapes to avoid blood and other fluids contaminations. Tissues samples for histopathology were placed in a container with 10% buffered formalin with a tight cap and the external side of the container was disinfected before exit the necropsy room. The tissue samples collected for bacteriology examination were placed in a clean plastic tray and covered with transparent plastic paper, sealed and making sure that no external surface was contaminated with potential septic material. The carcass of the hares and of the remaining tissues were wrapped into the disposable drapes, and together with all the protective material, were placed into double biohazard bags and directly autoclaved. Instruments used for the necropsy were placed in a stainless steel casserole, closed and directly autoclaved. The necropsy table was disinfected with household bleach.

2.a.B. Bacteriology

Samples for bacteriological analyses were processed under an aspirating hood. Cultures and lysates (eppendorfs with screw caps) for PCR analyses were performed. Agar plates were

placed in an aluminum box and placed in an incubator at 37°C with 5% CO_2 atmosphere. Lysates were heat-inactivated 1 hour at 60°C followed by 15 minutes at 97°C as denaturation step before PCR. When PCRs resulted positive, the aluminium box containing the agar plates was brought to the BSL3 for further analyses. All manipulations of live cultures were carried out in the BSL3. All remaining tissue samples were placed in double biohazard bags and directly autoclaved, while instruments used for inoculation and production of lysates were inactivated.

2.b. Animal received for bacteriological and pathological investigation and included in the project.

During the project, 192 animals were tested for the presence of *F. tularensis* (Table 1) whereof 55 resulted positive for *F. tularensis* (Table 1). They have been received from 12 cantons: GE, NE, FR, BE, JU, SO, BL, BS, AG, ZG, ZH and TG.

Table 1. Summary of the animals submitted for tularemia investigation and of the results of positive and negative detection of *F. tularensis*.

Species	Total	Positive	Negative
Bat (*Rhinolopus* sp)	**1**	**0**	**1**
Beaver (*Castor fiber*)	**38**	**0**	**38**
Brown Hare (*Lepus europeus*)	**53**	**28**	**25**
Mouse (*Mus musculus*)	**69**	**24**	**45**
Non-human primates (*Saguinus oedipus, Saguinus labiatus, Macaca fascicularis*)	**9**	**2**	**7**
Rabbit (*Oryctolagus cuniculus forma domestica*)	**2**	**0**	**2**
Rat (*Rattus norvegicus*)	**2**	**0**	**2**
Red fox (*Vulpes vulpes*)	**6**	**0**	**6**
Roe deer (*Capreolus capreolus*)	**5**	**0**	**5**
Squirrel (*Sciurus vulgaris*)	**1**	**0**	**1**
Stone marten (*Martes foina*)	**1**	**1**	**0**
Wild boar (*Sus scrofa*)	**1**	**0**	**1**
European polecat *(Mustela putorius)*	**1**	**0**	**1**
European otter ***(Lutra lutra)***	**1**	**0**	**1**
Marmot *(Marmota marmota)*	**1**	**0**	**1**
Nutria *(Myocastor coypus)*	**1**	**0**	**1**
Total	**192**	**55**	**137**

The project was mainly focused on hares and semi-aquatic mammals but as mentioned above animals with obvious ectoparasitism were also included in the study. Moreover, in June-August 2012, an outbreak of tularemia in free-ranging house mice from a research facility was recorded and animals from this epizoozotic were included in the project. Finally, few non-human primates with suspicion of tularemia were also tested.

Figure 1. Cantons with animals diagnosed positive animals for *F. tularensis* during the project.

2.b.A. Pathology and bacteriology results.

Of all the species investigated, and with the limitations associated with the presence of not representative sample sizes for different species, the most commonly infected species was the brown hare (*Lepus europeus*) with 28 positive individuals out of 53 (Table 1; for more details see paragraph 2.b.A.1 Hares). The second most commonly affected species is that of house mice (*Mus musculus*) with 24 positive animals out of 69, although the bulk of the positive animals was detected during a restricted time window overlapping with the peak of an outbreak (Table 1; for more details see paragraph 2.b.A.2 Mice). Non-human primates (*Saguinus oedipus, Saguinus labiatus*) represent the third most commonly affected group with

positive individuals (Table 1; for more details see paragraph 2.b.A.3 Non-human primates). A sporadical positive animal was a stone marten (*Martes foina*) (Table 1; for more details see paragraph 2.b.A.4 Stone martens). The epidemiological meaning of this finding still needs to be evaluated since only one subject of this species was tested and so it is not clear if this positivity represents a casual finding or a significant epidemiological indicator. All the other species tested were negative. Among these, the most relevant result concerns beavers (*Castor fiber*). Of the 38 individual tested, none was positive, suggesting that this species might play a marginal or debatable role in the ecology of F. *tularensis* in Switzerland and more broadly in Europe (Table 1; for more details see paragraph 2.b.A.5 Beavers).

2.b.A.1 Hares.

Brown hare (*L. europaeus*): Twenty-eight out of 53 hares resulted positive for *F. tularensis*. Tularemia cases came from a large geographical area of Switzerland suggesting a wide spread of *F. tularensis* within the national territory (Figure 1). If we add the cantons reporting human cases of tularemia since 2004, *F. tularensis* is endemic in the whole country (BAG, 2013). Moreover, typing of strains led to the discovery of a novel cluster *F. tularensis* in Switzerland. Very interestingly, this new cluster is associated with macrolide resistance (for details see paragraph 2.c Phylogeography of *F. tularensis* in Europe and associated antimicrobial resistances).

See annex 2:

Origgi, F.C., Frey, J., Pilo, P. Characterisation of a new group of *Francisella tularensis* subsp. *holarctica* in Switzerland with altered antimicrobial susceptibilities, 1996 to 2013. **Eurosurveillance**. 2014. Jul 24;19(29). pii: 20858

Our findings show that tularemia is a relevant infectious disease for European brown hares in Switzerland. The relative high number of infected hares among those collected during our investigation from February 2012 to May 2014 parallels the increased number of human infections reported during the same period (BAG, 2013). It has to be mentioned that the number of reported case of tularemia in humans in Switzerland increased more than 150% in 2012 compared to 2011 (BAG, 2013). The overrepresentation of tularemia as infectious disease affecting free-ranging brown hares in Switzerland is a relatively new finding considering that during a similar investigation carried out in Switzerland in the late 90's, only 15% of the examined hares (total n=167) were determined to have died because of infectious

diseases, which included besides tularemia, also pasteurellosis, brucellosis, pseudotuberculosis, listeriosis, and toxoplasmosis (Haerer et al., 2001). The reason for this apparent increase of the incidence of tularemia in hares is not clear but inter-epizootic periods have been described in the literature (Gyuranecz et al., 2010a).

The largest array of lesions consistent with *F. tularensis* infection was observed in this species. A core of most frequent lesions was associated to a secondary group of lesions not systematically occurring. The "primary" core of lesions included spleen, lung, liver and tracheal disease along with an always occurring lymphoid depletion. A second most-common core of lesions included heart and lymph nodal disease. Additional lesions, more sporadically occurring, included changes in endocrine tissue (ovary, accessory structure of the testicle, adrenal glands) and other findings less specific, possibly representing contextual, but unrelated diseases.

Our findings show that European brown hares infected with *F. tularensis* belonging to the B.FTNF002-00 cluster develop lesions with morphology and distribution different from those infected with the Eastern European clusters (subgroup B.13). In a previous study (Gyuranecz et al., 2010b), investigating the tularemia-associated pathology in European brown hares infected with the *F. tularensis* B.13 cluster in Hungary, the authors described as common finding the presence of granulomatous and necrotic foci observed grossly in multiple organs, but primarily in lung, pericardium and kidney. Strikingly, in our investigation it was relatively uncommon to find gross lesions consistent with detectable necrotic foci in the lung, pericardium and kidney, with only exceptions including the tracheal mucosa and the lymph nodes. Differently, the most common gross lesion consisted in a variable degree of splenomegaly. Also, the histological lesions were invariably detected in the spleen, liver and lymph nodes. In the lung, the microscopic lesions were observed relatively frequently, but most of the time were relatively mild and with patchy distribution. Differently from what observed with the B.13 cluster, lesions in the kidney were uncommon. Pericardial lesions were even more infrequent. It appears then that the B.13 and the B.FTNF002-00 clusters of *F. tularensis* are associated with distinct pathology. Curiously, the only hare showing a necrotic focus on the kidney capsule was one of the two infected with the B.13 cluster. However, this might just reflect a coincidence.

It has to be mentioned that although the gastro-intestinal tract was observed to be a relatively marginal area as concern the severity and extent of the *F. tularensis*-associated lesions, the

involvement of the lymphoid follicles of the cecal appendix was not uncommon. In one case germinal center of the cecal follicles was clearly IHC-positive, suggesting that *F. tularensis* might have gained access to the lymphoid tissue by the migration of macrophages or/and dendritic cells. This might be a way for *F. tularensis* to become systemic following infection of the host by the oral route (KuoLee et al., 2007).

Concerning ectoparasites, 193 ticks were collected from 19/28 *F. tularensis*-positive hares. All ticks were identified as *Ixodes ricinus*. Eleven were larvae, 129 were nymphs and 53 were adults (43 females and 10 males). Thirty-seven ticks collected from 4 tularemic hares were positive for all 4 DNA targets by PCR confirming the presence of *F. tularensis*. Among ticks positive for *F. tularensis* 6 were larvae, 17 were nymphs and 14 were adults (14 females and 0 males). These findings need more long-term data but raise the question of an active role of ticks, at least of *I. ricinus* in transmission of *F. tularensis* subsp. *holarctica* subgroup B.FTNF002-00. Moreover, we recently published a human case report of tularemia in a patient living and working at 1700 meters above sea level, where ticks are rare (Ernst et al. In Press).

See Annex 5 and 3:

Origgi, F.C., Pilo, P. Pathology, bacteriology and proposed pathogenesis of Tularemia in European Brown Hares (*Lepus europaeus*): a model for human tularemia. In preparation

Ernst, M., Pilo, P., Fleisch, F., Glisenti, P. Tularemia in the Southeastern Swiss Alps at 1,700 m above sea level. **Infection**. In Press

2.b.A.2 Mouse.

Mice (***M. musculus***): An outbreak of tularemia in free-ranging house mice, monitored in the frame of a distinct research project, occurred in summer 2012 in the Canton of Zürich. Data concerning natural outbreaks of tularemia are difficult to obtain, especially from house mice, whose carcasses rarely remain available for collection because of predators and scavengers (DeVault et al., 2003). In this study, a large population of mice (around 360 animals at the beginning of the outbreak) could be monitored under natural conditions, in the absence of antimicrobial drug treatment, during a tularemia outbreak. PCR confirmed that during the 3-month outbreak of tularemia, 7% of the mouse population died from the disease. This number is relatively low considering the high sensitivity of mice to *F. tularensis* (Lyons and Wu,

2007); however, the number of exposed mice is unknown, and not all dead mice were available for testing.

A unique aspect of this investigation is that we were able to evaluate sero-conversion of humans with known exposure to infected animals. Eleven researchers entered the barn inhabited by house mice and monitored/handled the animals every 2–3 days without the use of specific personal protective equipment, except for disposable gloves; some of the mice were later found to be infected with *F. tularensis*. The barn is a closed environment filled with bedding; mouse excrement is present on all surfaces and has the potential for aerosolization. Nevertheless, sero-conversion was not detected in any of the researchers, bringing to question whether shedding of *F. tularensis* in urine and feces of mice is a relevant source of *F. tularensis* transmission for humans.

Thus far, reports about *F. tularensis* shedding in rodents have had inconsistent findings (Bell and Stewart, 1975; Shlygina and Olsuf'ev, 1982; Inzana et al., 2004). However, this is a crucial point to investigate because *F. tularensis* shedding through urine and feces would not only affect outdoors but also household environments via rodent infestation and contamination of the water (also with carcasses).It is important to note that a 150% increase of human tularemia cases was reported in 2012 in comparison to 2011 in Switzerland; most cases occurring in the same area where the mouse barn mentioned in this study is located (BAG, 2013). This demonstrates the importance of monitoring sentinel animals for tularemia to better understand the ecology of *F. tularensis*.

At last, modeling infectious diseases is relevant to understand their dynamics and is critical for authorities making decisions on outbreak management. Reports and analyses of tularemia outbreaks represent important data for the understanding of the basic dynamics behind the spread and transmission of *F. tularensis* and its survival rate in the environment. In collaboration with the Institute of Evolutionary Biology and Environmental Studies at the University of Zürich, we performed the first estimation of the basic reproduction number R0 of an epizootic caused by *F. tularensis* using quantitative modeling based on a susceptible-infected-recovered framework. We applied that model to data collected during the mice outbreak. The model was based on two assumptions and the basic reproduction number R0 of the current outbreak was calculated as being between 1.16 and 1.91.

See annexes 4 and 6:

Origgi, F.C., König, B., Lindholm, A.K., Mayor, D., Pilo, P. Tularemia among Free-Ranging Mice without Infection of Exposed Humans, Switzerland, 2012. **Emerg. Infect. Dis.** In Press

Dobay, A., Pilo, P., Lindholm, A.K., Origgi, F.C., Bagheri, H.C., König, B. Epizootic of tularemia in a closely-monitored free-roaming population of wild house mice. In preparation

2.b.A.3 Non-human primates.

Non-human primates (*S. oedipus, S. labiatus)*: Of the 4 non-human primates examined, two were positive for the presence of *F. tularensis*.

A better understanding of the transmission and maintenance of *F. tularensis* in zoos with particular emphases on possible vectors and reservoirs for the pathogen is relevant because of the hazard for endangered species and the zoo personnel. We just started a collaboration with a Swiss zoo who experienced 2 tularemia cases in monkeys in October 2014 in order to evaluate this aspect.

2.b.A.4 Stone marten.

Stone marten (***M. foina***): The lesions observed in the stone marten were not specific and not consistent with those more frequently recorded in *F. tularensis* infected animals. The relevance of tularemia in *Mustelidae* is not clear and unfortunately these animal species are usually not considered for studies investigating *F. tularensis*. Investigations about the ecology of *F. tularensis* would deserve the inclusion of other species than only species belonging the orders of rodents and lagomorphs.

See annex 1:

Origgi, F.C., Wu, N. and Pilo, P. *Francisella tularensis* infection in a stone marten (*Martes foina*) without classic pathological lesions consistent with tularemia. **J Vet Diagn Invest.** 2013 Jul;25(4):519-21. doi: 10.1177/1040638713489124

2.b.A.5 Beavers.

Beaver (*C. fiber*): In the frame of this project 65 carcasses of beavers, including those collected in the present study (38) and those from a previous pilot study (27) were tested. None of them was positive for *F. tularensis*. In contrast to the situation in North America, beavers do not seem to be affected by tularemia in Europe and their epidemiological role in the disease cycle is questionable at this time. Several reasons might explain these results. Among the most important:

1) The species of beaver present in Europe (*C. fiber*) may be more resistant to *F. tularensis* than the species present in North America (*C. canadensis*).
2) The clusters of *F. tularensis* present in Switzerland may be less pathogenic than the clusters present in North America.

2.c Phylogeography of F. tularensis in Europe and associated antimicrobial resistances.

In the frame of this study all isolates from our strain collection were genetically characterized and antibiograms were performed. All strains were identified as *F. tularensis* subsp. *holarctica*. All strains belonged to the Western European cluster (B.FTNF002-00) with the exception of five of them that belong to the cluster B.13 detected for the first time in Switzerland. The newly detected strains were isolated both from humans and hares. All strains were shown to be sensitive to first-line antibiotics used to treat tularemia. However, the cluster B.13 was associated with resistance to macrolides (mutations in the *rrl* genes). This finding is relevant for the treatment of tularemia in cases when first-line antibiotics cannot be used and alternative antimicrobial agents should be proposed (for example pregnant women).

See annex 2:

Origgi, F.C., Frey, J., Pilo, P. Characterisation of a new group of *Francisella tularensis* subsp. *holarctica* in Switzerland with altered antimicrobial susceptibilities, 1996 to 2013. **Eurosurveillance**. 2014. Jul 24;19(29). pii: 20858

Direct PCRs and cultures specific for *F. tularensis*

In order to evaluate the sensitivity of the methods used for the detection of *F. tularensis*, PCR and culture were compared. Twenty-eight hares out of 53 were positive by bacteriological analysis. All samples were tested by direct PCR and by culture. In general, PCR detection of *F. tularensis* from all organs and clinical sampleswas more sensitive than culture (Table 3), although in one single case the PCR failed to give a positive result, while cultures of *F.*

tularensis were recovered from the organs. Moreover, since PCRs were directly performed from the samples withhout DNA extraction, results could be obtained in few hours and without needing any centrifugation step. The most appropriate organs to test for *F. tularensis* are major organs including lung, spleen, liver and kidney (Table 3). Interestingly, trachea and ovary gave more than 80 % recovery. *F. tularensis* is described as a fastidious bacterium to grow and culture might be hampered by overgrowth or inhibition due to other microorganisms. As expected, the in-house selective medium Ftsel gave better results in terms of bacterial isolation.

Spleen, liver, trachea and enlarged lymph nodes generally showed the highest level of detection by direct PCR, even if in positive animals most of the organs tested were positive.

Table 3. Comparison of the detection of *F. tularensis* by PCR and culture from organs of hares positive for *F. tularensis*.

	PCR [positive/n (percentage)]	**Culture** [positive/n (percentage)]		
		BA	**ChocIso**	**Ftsel**
Lung	27/28 (96.43%)	5/28 (17.86%)	14/28 (50.00%)	22/28 (78.57%)
Spleen	26/28 (92.86%)	11/28 (39.29%)	19/28 (67.86%)	23/28 (82.14%)
Lymph node	18/20 (90.00%)	5/20 (25.00%)	10/20 (50.00%)	15/20 (75.00%)
Liver	25/28 (89.29%)	9/28 (32.14%)	13/28 (46.43%)	22/28 (78.57%)
Kidney	24/28 (85.71%)	6/28 (21.43%)	13/28 (46.43%)	17/28 (60.71%)
Trachea	22/26 (84.62%)	3/26 (11.54%)	6/26 (23.08%)	15/26 (57.69%)
Ovary	8/10 (80.00%)	2/10 (20.00%)	4/10 (40.00%)	7/10 (70.00%)
Heart	20/27 (74.07%)	2/27 (7.41%)	4/27 (14.81%)	11/27 (40.74%)
Urinary bladder	17/23 (73.91%)	0/23 (0.00%)	2/23 (8.70%)	7/23 (30.43%)
Esophagus	17/24 (70.83%)	0/24 (0.00%)	1/24 (4.17%)	8/24 (33.33%)
Uterus	7/10 (70.00%)	0/10 (0.00%)	1/10 (10.00%)	4/10 (40.00%)
Brain	16/23 (69.57%)	2/23 (8.70%)	5/23 (21.74%)	10/23 (43.48%)
Tonsil	8/12 (66.67%)	1/12 (8.33%)	3/12 (25.00%)	7/12 (58.33%)
Adrenal	14/21 (66.67%)	2/21 (9.52%)	8/21 (38.10%)	13/21 (61.90%)
Small intestine	18/27 (66.67%)	0/27 (0.00%)	1/27 (3.70%)	2/27 (7.41%)
Testicle	9/16 (56.25)	1/16 (6.25%)	5/16 (31.25%)	7/16 (43.75%)
Stomach	14/27 (51.85%)	0/27 (0.00%)	1/27 (3.70%)	4/27 (14.81%)
Large intestine	14/27 (51.85%)	0/27 (0.00%)	0/27 (0.00%)	3/27 (11.11%)
Appendix	2/9 (22.22%)	0/9 (0.00%)	0/9 (0.00%)	2/9 (22.22%)

BA: blood agar plates; ChocIso: chocolate agar supplemented with isovitalex; Ftsel: selective medium for *F. tularensis.*

3. General discussion and comments

The study of tularemia is challenging because of the complexity of the disease and the many unknown aspects of the biology of *F. tularensis.* For this project a multidisciplinary approach was chosen in order to tackle this major task. The several aspects of the ecology of *F. tularensis* were considered and led to the collection of new information that has been integrated with results derived from the long-term research on tularemia, comprising human tularemia, carried out at the IVB.

Tularemia was shown to be endemic in Switzerland (Burgisser, 1974; Haerer et al., 2001; Friedl et al., 2005; Pilo et al., 2009) and affects at least humans, non-human primates and some species of the orders of rodents and lagomorphs (Pilo et al., 2009; Origgi et al., 2014). This was confirmed during the performed study. The project also showed that tularemia was the most relevant infectious disease for European brown hares during the time-period of 2012-2014. In this species, the routes of infection seem to be principally inhalation and ingestion. This aspect has a direct implication for human tularemia because the source/s of infection for people is in the environment. However, none of its constitutive elements can clearly and indubitably be pointed out as major source of infection. A recent study from a Swedish group led to the identification of ecological risk factors for exposure to *F. tularensis* (Svensson et al., 2009). Interestingly, they identified proximity to rivers and lakes as an important risk factor for human tularemia, consistently with the association between human tularemia and mosquito bites reported in Sweden. In Switzerland the ecology of *F. tularensis* seems to be different since mosquitos have not been shown to play a significant role in the dynamic of the disease. Furthermore the historical separation between the terrestrial and aquatic cycles associated with *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* (Hopla, 1974), respectively, might be worth of reconsideration at the light the diverse ecosystems where *F. tularensis* is present and the phylogenetic clusters of *F. tularensis* involved. As a matter of fact, *F. tularensis* subsp. *holarctica* was not identified in semi-aquatic mammals during the project. Several of the “axiomas” formulated in the past concerning tularemia might need to be re-thought, at least in part depending on the diversity of ecosystems. In particular, it appears that the chances to pin point the actual environmental sources of *F. tularensis* might be magnified only by a long-term study tracing the potential vectors and reservoirs in the environment.

Among the vectors traditionally reported as relevant for the transmission of *F. tularensis,* hematophagous arthropods, and in particular ticks, have always been ranked as very likely involved. However, our results suggest that the role of ticks deserves further and detailed

studies. We identified *F. tularensis* only in ticks (*I. ricinus*) collected from 4/19 infected hares and no conclusions can be drawn at the moment because of the limited number of positive ticks.

We identified 5 strains not belonging to the B.FTNF002-00 cluster; 2 isolated from hares in this project and 3 from humans. These strains were genetically characterized and belong to the cluster B.13 that is known to circulate in Eastern Europe. Our finding changes the phylogeography of *F. tularensis* in Europe. Moreover, the pathological lesions in hare affected with this cluster are different from the ones associated with the cluster B.FTNF002-00 suggesting a possible different biology between the 2 clusters. A critical finding associated with this discovery was the different antibiotic susceptibility profiles of the two clusters with the strains belonging to the cluster B.13 being resistant to macrolides, while all strains belonging to the cluster B.FTNF002-00 being sensitive. Genetic characterization of this phenotype led to the detection of mutations in the *rrl* genes common to all 5 strains. Given that strains belonging to the cluster B.13 are circulating in Switzerland, it is critical typing the strains to choose the appropriate antimicrobial agent to treat cases of human tularemia when first-line antibiotics are not an option.

Impact of tularemia on people health is particularly relevant for a group of distinct categories of professionals including veterinarians, microbiologists, biologists, zoo keepers, game wardens, hunters and hikers who are more likely to get in contact with contaminated materials. Concerning professional involved in animal inspection, necropsies and sample collection such as veterinarians and in samples handling and analysis, such as microbiologists, the SOPs applied during the project appeared to be sufficient to prevent the contagion if strictly followed in the case of small mammals affected by *F. tularensis* subsp. *holarctica*. Professionals and people outdoors are strongly suggested not to touch dead or sick free-ranging animals with bare hands should be implemented and carcasses of those animals should not be opened in any case. Hunters are encouraged to be suspicious of hares, in particular, with abnormal behavior, not escaping and/or visibly sick. In this case, it is preferable not to shoot the animal but to warn a game warden so that proper and official measures can be taken to assess if the animal was actually sick with tularemia. Hunters almost invariably hunt hares with the aid of hunting dogs and when possible, dogs should be hold by the hunter and prevented to capture a sick hare. As a matter of fact, in a number of occasions we received hares that were captured by hunting dogs. Although dogs are usually described as resistant to tularemia, a recent article from a group in Norway challenges this common

thought, describing the exposure of a hunting dog to *F. tularensis* by serology (Nordstoga et al., 2014).

A portion of the Swiss population is still unaware that tularemia is endemic in Switzerland and it is important to make the general public aware that few simple rules could strongly limit human infections. Moreover, we plan to further study the issue of shedding of *F. tularensis* via urine and feces of rodents because of their potential source of infection in households with various degrees of rodent's infestation. The other important issue is contamination of water by rodent and lagomorph carcasses since this source of infection has been described as an important cause of human outbreaks in various countries and resulting in more than 500 human cases in Spain in 2007-2008 (Ariza-Miguel et al., 2014; Larssen et al., 2011; Willke et al., 2009).

In summary, the project "Ecology of *Francisella tularensis* and its impact on biological safety" used a multidisciplinary approach to collect data to better understand the biological cycle of *F. tularensis* and to identify risks associated with tularemia in Switzerland. This approach was successful as it brought new knowledge that was reported in 4 international scientific peer-reviewed journals and three more articles are in currently preparation. Moreover, SOPs for necropsy and bacteriological analyses for small mammals affected by *F. tularensis* subsp. *holarctica* were established and validated during the study. At last, the circulation of *F. tularensis* among free-ranging animals stresses the question of contamination of water by carcasses and /or urine since this source of infection is of highest risk for tularemia outbreaks as the human epidemic in Spain in 2007-2008 (Ariza-Miguel et al., 2014).

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Annex 1:

Origgi, F.C., Wu, N. and Pilo, P. *Francisella tularensis* infection in a stone marten (*Martes foina*) without classic pathological lesions consistent with tularemia. **J Vet Diagn Invest.** 2013 Jul;25(4):519-21. doi: 10.1177/1040638713489124

Francisella tularensis infection in a stone marten (*Martes foina*) without classic pathological lesions consistent with tularemia

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Francisella tularensis infection in a stone marten (*Martes foina*) without classic pathological lesions consistent with tularemia

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Abstract. The current report describes the isolation and typing of a strain of *Francisella tularensis*, the causative agent of tularemia, from the spleen of a stone marten (*Martes foina*) showing no classic lesions consistent with the disease. The identification of this bacterium, belonging to the World Health Organization risk 3 category and considered to have a low infectious dose, could be performed only because of an ongoing project screening *F. tularensis* in the environment sensu lato. The findings described herein should alert diagnostic laboratories of the possible presence of *F. tularensis* in clinical samples in countries where tularemia is endemic even in cases with no consistent anamnesis and from unsuspected animal species.

Key words: Diagnostics; *Francisella tularensis*; inconsistent pathological lesions; martens.

Tularemia is a very complex emerging zoonotic disease caused by the Gram-negative bacterium *Francisella tularensis.* Depending on the route of infection, involved strains, and hosts, tularemia presents numerous clinical forms.[6,14,15] It is assumed that people become infected via the environment sensu lato, mainly after contact with infected animals, contaminated water, and aerosols or by bites from blood-sucking arthropods.[6] However, besides outbreak situations, the source of infection remains essentially unknown and difficult to demonstrate.[17] Although certain wild animal species are recognized as playing a role in the biological cycle of this bacterium, especially lagomorphs and rodents, the real spectrum of naturally infected species remains hypothetical.[11] This is principally due to the fact that *F. tularensis* grows only on special culture media[5,18] and is not commonly considered in routine diagnostic investigations. Tularemia may be overlooked in some animal species and more particularly when anamnesis and/or tissue changes are inconsistent. Additionally, similarly to other fastidious organisms, the presence of *F. tularensis* might be masked by the overgrowth of other microorganisms or go undetected because of poor growth on inadequate isolation media. The current report describes the isolation and genetic characterization of *F. tularensis* subsp. *holarctica* belonging to the cluster circulating in Western Europe[4] from the spleen of a stone marten (or beach marten, *Martes foina*), with no pathological observations suggestive of tularemia.

A stone marten was found unresponsive and did not attempt to escape when approached by a game warden in Graenichen, Canton Aargau, Switzerland, in July 2012. The animal was culled and sent to the Centre for Fish and Wildlife Health, University of Bern, Switzerland, for a postmortem examination. A full necropsy was performed on the submitted marten, and representative tissue sections of multiple organs were routinely processed and stained with hematoxylin and eosin according to the standard protocol.[16] Additionally, special stains were used as appropriate.

Grossly, a 4-mm perforation of the skin just caudal to the left elbow region associated with the presence of subcutaneous light tan creamy material consistent with pus was observed. The suppurative exudate was dissecting the subcutis and extended over the entire left thoracic region and the cranial two-thirds of the left abdominal region. Additionally, a 0.4 cm × 1 cm perforating wound was found on the right front paw. No additional gross findings were observed.

Histologically, the lesions observed were consistent with complication of traumatic wounds, parasite infestation, and kidney disease. None of the lesions were suggestive of tularemia. The most severe tissue change was a chronic-active suppurative cellulitis with intralesional cocci observed in correspondence to the gross skin wounds. A mild myocarditis on a background of mild multifocal fibrosis and focal moderate thickening of the epicardium were seen in the heart. A parasitic pneumonia, with large numbers of nematode parasites, and multifocal parenchymal consolidation with rare small foci of parasite-associated necrosis were seen in the lungs. Grocott and Gram stains did not reveal the

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presence of detectable organisms. A mild multifocal lymphoplasmacytic interstitial nephritis with segmental tubular necrosis and mineralization along with occasional collection of tubular casts was seen in the kidneys. Multifocal mild mineralization in the lamina propria of the stomach and moderate numbers of cestodes in the lumen of the small intestine were seen in the gastrointestinal tract. Finally, a minute focus of coagulation necrosis was seen in the liver, while a diffuse, minimal lymphoid depletion was observed in the spleen.

Samples from spleen, lung, liver, kidney, and subcutaneous suppurative exudate were submitted for bacteriology analyses. Stone martens are unconventional hosts for tularemia. However, 2 factors were considered: an ongoing project investigating *F. tularensis* in the environment was being carried out in the laboratory, and an outbreak of tularemia in farmed *Mustela* sp., another genus belonging to the family *Mustelidae*, had been previously reported.[12] Hence, the decision was made to test the stone marten for *F. tularensis*, despite the absence of typical signs or lesions. Lysates[20] from the organs and a swab of the subcutaneous suppurative exudate were tested by real-time polymerase chain reaction (PCR) for *F. tularensis* as previously described.[22] The lung, spleen, and subcutaneous pus were positive by this *F. tularensis*–specific PCR.

All samples were cultivated onto trypticase soy agar (TSA) with 5% sheep blood,[a] chocolate agar with IsoVitaleX[b] (ChocIso), and *F. tularensis* selective agar (Ftsel; chocolate agar with brain–heart infusion broth[b] as base, 1% soluble hemoglobin powder,[a] 0.1% L-cysteine hydrochloride monohydrate, 0.1% D-glucose and Skirrow *Campylobacter* selective supplement[a]) for 3 days at 37°C with 5% CO_2. Mixed flora grew from all samples on TSA, ChocIso, and Ftsel. After colony purification, a strain with colony morphology consistent with *F. tularensis* could be isolated from Ftsel agar plates inoculated with the spleen.

The strain isolated from the spleen was then genetically characterized. A lysate[20] from the culture was prepared as DNA template. The strain was identified as subspecies *holarctica* by PCR.[1] An additional PCR targeting the region of difference 23 was performed as previously described,[4] and the amplicon size corresponded to the emerging clone from Western Europe of *F. tularensis* subsp. *holarctica* that is circulating in France, Germany, Italy, Spain, and Switzerland.[4,7,10,19] Sequence analysis of the single nucleotide polymorphism markers B.18 (derived state) and B.19 (ancestral state) confirmed the subclade B4.FTNF002-00,[21] which is endemic in Switzerland. Multiple-loci variable number of tandem repeats (VNTR) analysis[2,13,19] was performed, and the isolated strain showed a VNTR profile that is circulating in Switzerland.[19]

Awareness of tularemia is emerging in Europe, and increased resources are being invested in order to better understand this disease.[3,8,9,19] The focus on the improvement and refinement of diagnostic methods for *F. tularensis* identification of the past decade may contribute to an increase in the number of cases reported in human beings and in animals. However, only long-term systematic surveillance programs may answer if the present scenario is the outcome of an actual increment of *F. tularensis* on the territory or simply an increased attention for this bacterium.

In veterinary diagnostic laboratories, *F. tularensis* is normally investigated only in cases of suspicion of tularemia (mainly in primates, rodents, and lagomorphs) and typically only when consistent anamnesis is present. In the reported case, the marten was tested in the frame of a study investigating the biological cycle of *F. tularensis* in the environment sensu lato. It should be emphasized that the bacterium could be isolated only on an appropriate selective medium used specifically for this project and was present as a mixed culture. In other circumstances, it is very likely that this case would have been missed, which raises serious concerns not only for the missed diagnosis of a serious pathogen but also for biosafety, especially for that of the personnel who handled the carcass and samples. The isolation of *F. tularensis* from an animal species generally not screened for this bacterium and with no consistent pathological lesions may be challenging and dangerous for the personnel of diagnostic laboratories. Awareness of the potential presence of *F. tularensis* in unconventional hosts should be raised in countries where tularemia is endemic.

Finally, the biological role of martens, if any, in the environmental cycle of *F. tularensis* is not clear as is the case for many other wild animal species. More investigations are needed because the environmental "reservoir(s)" for tularemia is still unknown.

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Annex 2:

Origgi, F.C., Frey, J., Pilo, P. Characterisation of a new group of *Francisella tularensis* subsp. *holarctica* in Switzerland with altered antimicrobial susceptibilities, 1996 to 2013. **Eurosurveillance**. 2014. Jul 24;19(29). pii: 20858

Characterisation of a new group of *Francisella tularensis* subsp. *holarctica* in Switzerland with altered antimicrobial susceptibilities, 1996 to 2013

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Molecular analysis of *Francisella tularensis* subsp. *holarctica* isolates from humans and animals revealed the presence of two subgroups belonging to the phylogenetic groups B.FTNF002-00 and B.13 in Switzerland. This finding suggests a broader spread of this group in Europe than previously reported. Until recently, only strains belonging to the Western European cluster (group B.FTNF002-00) had been isolated from tularaemia cases in Switzerland. The endemic strains belonging to group B.FTNF002-00 are sensitive to erythromycin, in contrast to the strains of the newly detected group B.13 that are resistant to this antibiotic. All the strains tested were susceptible to ciprofloxacin, streptomycin, gentamicin, nalidixic acid and chloramphenicol but showed reduced susceptibility to tetracycline when tested in a growth medium supplemented with divalent cations. The data show a previously undetected spread of group B.13 westwards in Europe, associated with changes in the antibiotic resistance profile relevant to treatment of tularaemia.

Introduction

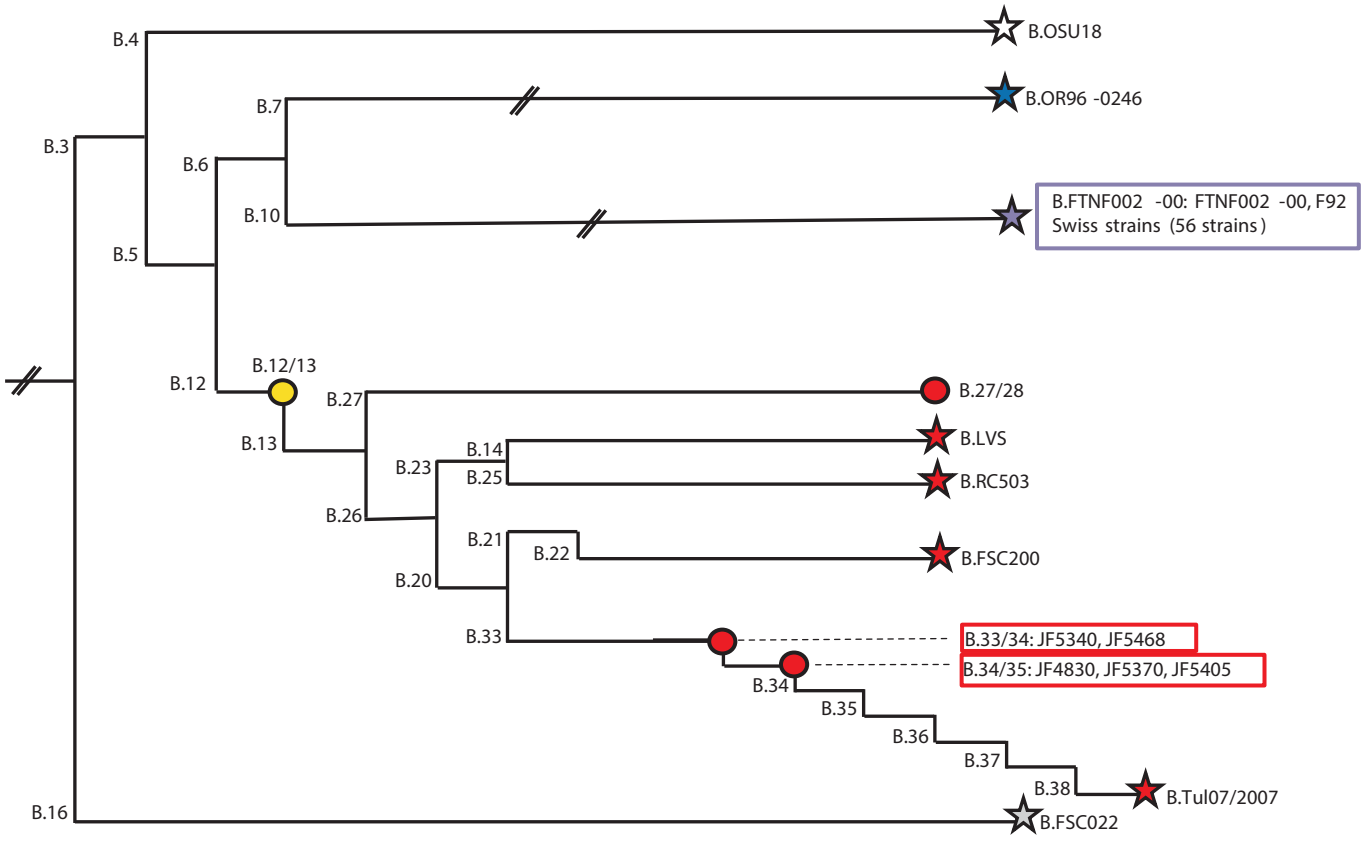
Francisella tularensis is a Gram-negative bacterium causing the zoonotic disease tularaemia. The two clinically relevant subspecies are *F. tularensis* subsp. tularensis and *F. tularensis* subsp. *holarctica*. Of the two, only the latter subspecies is present in Europe. Human infections mainly occur through inhalation, ingestion, or by direct contact with infected animal species and contaminated animal tissues, water and aerosols [1]. In general, it is difficult to trace the source of infection [2]. Analyses of molecular genetic markers of the strains circulating in the environment provide valuable information on the dynamics of infection in people and in animals, and improve the knowledge of the biology of this bacterium. Genetic and phenotypic data are also necessary to determine the most suitable antimicrobial substances to use to treat humans and animals [3-8].

With the recent growing interest in *F. tularensis* biology, several tools have been developed to investigate the molecular epidemiology of this genetically monomorphic bacterium following a hierarchical scheme [9-13]. Genomes of different strains are screened for canonical single nucleotide polymorphism (canSNP) signatures. With the advent of novel technologies, an increasing number of strains are sequenced, leading to the discovery of new canSNP markers and signatures specific to new subgroups. The growing information involves adjustments in the phylogenetic nomenclature of *F. tularensis* and allows better resolution within the subgroups [11,12,14]. Throughout this manuscript, we will follow the nomenclature based on the canSNPs (for nomenclature clarity, refer to schema in Figure 1). In Europe, strains belonging to groups B.13 and B.FTNF002-00 are those predominantly isolated [12]. The group B.13 extends geographically from Scandinavia to the eastern European rim, with co-circulation of several of its subgroups in some countries. In Western Europe, a specific group, B.FTNF002-00, is circulating in France, Germany, Italy, Spain and Switzerland [11-19]. Recently, it was observed that Germany represents a geographical diaphragm virtually separating group B.13 from group B.FTNF002-00 [18].

Interestingly, strains belonging to group B.FTNF002-00 are described as sensitive to erythromycin, whereas strains belonging to other groups show variability in this marker [3-5,7,18]. Historically, strains of *F. tularensis* subsp. *holarctica* have been separated in two biovars: biovar I, strains sensitive to erythromycin, and biovar II, strains resistant to erythromycin [20]. Although this marker is principally used for epidemiological purposes, there may be significant clinical implications in areas with co-circulation of different groups. A paradigmatic example of this is the recent recommendation of treating pregnant women infected with *F. tularensis* with azithromycin in geographical

Figure 1

Schematic of *Francisella tularensis* subsp. *holarctica* nomenclature based on canonical single nucleotide polymorphisms



Canonical single nucleotide polymorphisms (canSNP), adapted from and according to the colours and symbols previously described [11-13], showing the position of Swiss strains within the subspecies. Stars represent terminal subgroups (sequenced strains), while circles indicate collapsed branches. The length of branches is not scaled. Only canSNP relevant to this study are presented to clarify the phylogenetic position of groups and subgroups discussed. Parallel bars indicate missing intermediate canSNPs and corresponding nodes and branches. CanSNPs are indicated to the left of the nodes. Strains for which whole-genome sequencing information is available and which were used for comparisons, are highlighted in bold at the end of the branches. Groups and subgroups identified in this study are boxed. Dashed lines do not represent branches.

areas where strains sensitive to erythromycin are circulating [21].

This study describes the first isolation of erythromycin-resistant strains of *F. tularensis* subsp. *holarctica* belonging to group B.13 in Switzerland. We discuss how these findings impact on the phylogeography of *F. tularensis* subsp. *holarctica* in Europe and on antibiotic treatment of affected individuals living in areas with co-circulating groups [12].

Methods

Bacterial strains, DNA templates, identification and typing

All manipulations with live cultures were performed in a BSL3 containment laboratory. *F. tularensis* strains were cultivated on chocolate agars with IsoVitaleX (Becton Dickinson, Allschwil, Switzerland) for three days at 37 °C with 5% CO_2. Lysates from cultures were prepared, filter-sterilised [22] and tested by real-time PCR for the presence of the fopA gene to confirm the species *F. tularensis* [22,23]. The subspecies was subsequently determined by amplification of the region of difference (RD)1 [24]. Strains were further characterised by PCR for the presence of deletions in two different markers, RD23 and Ft-M24, specific to the group B.FTNF002-00 [16,17,24] and by multilocus variable-number tandem repeat (VNTR) analysis (MLVA) with six VNTRs markers (Ft-M3, Ft-M6, Ft-M20, Ft-M21, Ft-M22 and Ft-M24) [17,25]. The MLVA results were further confirmed by analysis of the following canSNP markers B.11, B.12 B.20, B.21, B.22, B.23 and B.33 to B.38 [11,12].

Minimal inhibitory concentrations of antimicrobial agents

The minimal inhibitory concentration (MIC) values of antibiotic drugs relevant to clinical use such as gentamicin (0.12–16 mg/L), streptomycin (1–16 mg/L), ciprofloxacin (0.06–4 mg/L), tetracycline (0.25–16 mg/L), nalidixic acid (2–64 mg/L), chloramphenicol (2–32 mg/L) and erythromycin (0.5–32 mg/L), were determined in two different broth media: (i) modified Cation-Adjusted Mueller Hinton Broth (mCAMHB):

Cation-Adjusted Mueller Hinton Broth (Becton Dickinson, Heidelberg, Germany) supplemented with 2% PolyViteX Enrichment (BioMérieux, Marcy l'Etoile, France), and (ii) modified Mueller Hinton II (mMHII) broth: mCAMHB with 0.1% glucose, 63 mM $CaCl_2$, 53 mM $MgCl_2$ and 34 mM ferric pyrophosphate using custom 96-well Sensititre susceptibility plates (Trek Diagnostics Systems, East-Grinstead, England and MCS Diagnostics BV, JL Swalmen, the Netherlands), according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [26] and the informational supplement [27]. Antibiotics of the class of the beta-lactams were not tested because of the known natural resistance of *F. tularensis* strains to these antimicrobial substances [6,28]. The 96-well plates were incubated at 37 °C in 5% CO_2 atmosphere for 48 hours. The MIC values were defined as the lowest concentration exhibiting no visible growth. MICs were read after 24 and 48 hours incubation. For quality assurance, the reference strains of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were also tested by broth microdilution in mMHII broth and in mCAMHB.

Genetic characterisation of erythromycin resistance

The genetic characterisation of erythromycin resistance was carried out by PCR amplification and further sequencing of the genes encoding for the 23S rRNA (*rrl*), the L4 (*rplD*) and L22 (*rplV*) ribosomal proteins as previously described by Gestin and colleagues [29]. The sequences obtained were edited, aligned and compared in Sequencher (GeneCodes, Ann Arbor, United States) with the corresponding genes of the completely sequenced strains FTNF002-00 (group B.FTNF002-00, isolated in France [16]), F92 (group B.FTNF002-00, isolated in Germany [30]), FSC200 (group B.13, isolated in Sweden [31]) and LVS (group B.13, isolated in Russia) (NCBI/GenBank accession numbers: CP000803, CP003932, CP003862 and AM233362, respectively).

Results

Sixty-one strains were isolated between 1996 and 2013 from human and animal cases of tularaemia from a representative area of the Swiss territory (Table 1, Figure 2). Thirteen strains isolated before 2009 (JF3820, JF3821, JF3822, JF3824, JF3825, JF3826, JF3828, JF3829, JF3859, JF4092, JF4128, JF4212 and JF4242) had previously been characterised as *F. tularensis* subsp. *holarctica* belonging to group B.FTNF002-00 [17]. All other strains (n=48) were identified here as *F. tularensis* subsp. *holarctica*, and 43 of them were determined as group B.FTNF002-00 (Table 1) [17], while five strains JF4830, JF5340, JF5370, JF5405 and JF5468 did not harbour the deletions specific to the group B.FTNF002-00 in the RD23 and Ft-M24 markers. MLVA confirmed the clustering of the 56 strains (isolated between 1996 and 2013) belonging to group B.FTNF002-00 (data not shown). The highest variability among the markers used for MLVA was observed within markers Ft-M3 and Ft-M6 as previously reported for the group B.FTNF002-00 (Table 1) [15,17,18]. Concerning the strains not belonging to group B.FTNF002-00, the three strains, JF5340, JF5370 and JF5405 isolated from two human patients and one hare between 2012 and 2013, shared the same VNTR profile, while strains JF4830, isolated in 2010 from a patient returning from a vacation in eastern Europe (possibly corresponding to an imported case), and JF5468, isolated from a hare in 2013, revealed a distinct VNTR profile, with one variation in the Ft-M3 marker (Table 1). The four strains, JF5340, JF5370, JF5405 and JF5468 were isolated from a large geographical area of Switzerland extending from the central west to the east sides of the country (Figure 2).

CanSNP analyses were performed on a panel of 24 representative strains (shaded in grey in Table 1). All strains belonging to group B.FTNF002-00 and harbouring the specific deletions within markers RD23 and Ft-M24 showed the canSNP profile characteristic of the group B.FTNF002-00 (Table 1) [11,32]. All other strains (JF4830, JF5340, JF5370, JF5405 and JF5468) had a canSNP profile not corresponding to group B.FTNF002-00 (Table 1) [11]. In order to further characterise the strains not belonging to group B.FTNF002-00, the canSNP markers B.21, B.22 and B.23 were sequenced. They showed the SNP profile corresponding to group B.13 according to the genotypes described by Svensson et al. (Table 1) [11,32]. Moreover, a higher resolution of the genetic characterisation of the strains in group B.13 was obtained through analysis of the canSNP markers B.33 to B.38 [12]. Two subgroups were observed: the B.33/34 (JF5468 and JF5340) and the B.34/35 (JF4830, JF5370 and JF5405) according to the nomenclature described by Gyuranecz et al. [12] (Table 1 and Figure 1).

Antibiotic susceptibility profiles were determined for the panel of 24 representative strains (Table 2) by broth microdilution method using two broth media, mCAMHB and mMHII, for seven antibiotic drugs and read after 48 hours. The *F. tularensis* strains did not show any visible growth at the concentrations tested for nalidixic acid, chloramphenicol and ciprofloxacin in either broth medium, tested (Table 2). The MIC values for chloramphenicol and ciprofloxacin were below the breakpoint values provided in the CLSI guidelines, ≤8 µg/mL for chloramphenicol and ≤0.5 µg/mL for ciprofloxacin, while no breakpoint value is available for nalidixic acid [27]. MIC values for gentamicin ranged between 1 and 4 µg/mL in mMHII, but between ≤0.12 and 0.25 µg/mL in mCAMHB. The breakpoint value for gentamicin given in the CLSI guidelines for *F. tularensis* is ≤4 µg/mL [27]. The discrepancy in the MIC values observed for gentamicin between cultures of *F. tularensis* in mMHII and mCAMHB was confirmed for reference strains *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 (Table 2). MIC values for streptomycin ranged between 2 and 4 µg/mL in mMHII and were all at 4 µg/mL in mCAMHB (Table 2). The breakpoint value provided for streptomycin in the CLSI guidelines is ≤16 µg/mL when testing is performed in a CO_2 atmosphere [27]. MIC values for

Table 1a

Genetic characterisation of Swiss strains of *Francisella tularensis* subsp. *holarctica* by MLVA and SNPs, Switzerland, 1996–2013 (n=61)

Strain	Host, geographic origin, year of isolation	Subgroup according to [12] and [13]	MLVA						SNPs											
			Ft-M3	Ft-M6	Ft-M20	Ft-M21	Ft-M22	Ft-M24	B.11	B.12	B.20	B.21	B.22	B.23	B.33	B.34	B.35	B.36	B.37	B.38
JF3826	Monkey, Jura, 1996	B.FTNF002-00	342	311	255	396	254	464	T[d]	C[a]	A[a]	-	-	-	-	-	-	-	-	-
JF3821	Hare, Jura, 1997	B.FTNF002-00	297	311	255	396	254	464	T[d]	C[a]	A[a]	-	-	-	-	-	-	-	-	-
JF3820	Hare, Jura, 1998	B.FTNF002-00	333	311	255	396	254	464	T[d]	C[a]	A[a]	-	-	-	-	-	-	-	-	-
JF3822	Hare, Jura, 1998	B.FTNF002-00	297	311	255	396	254	464	T[d]	C[a]	A[a]	-	-	-	-	-	-	-	-	-
JF3859	Hare, eastern Switzerland, 1998	B.FTNF002-00	333	311	255	396	254	464	T[d]	C[a]	A[a]	-	-	-	-	-	-	-	-	-
JF3829	Monkey, Zurich, 2002	B.FTNF002-00	288	332	255	396	254	464	T[d]	C[a]	A[a]	-	-	-	-	-	-	-	-	-
JF3828	Human, Aargau, 2004	B.FTNF002-00	342	311	255	396	254	464	T[d]	C[a]	A[a]	-	-	-	-	-	-	-	-	-
JF3824	Human, Bern, 2005	B.FTNF002-00	333	311	255	396	254	464	T[d]	C[a]	A[a]	-	-	-	-	-	-	-	-	-
JF3825	Monkey, St. Gallen, 2006	B.FTNF002-00	297	311	255	396	254	464	T[d]	C[a]	A[a]	-	-	-	-	-	-	-	-	-
JF4092	Hare, Bern, 2007	B.FTNF002-00	297	311	255	396	254	464	T[d]	C[a]	A[a]	-	-	-	-	-	-	-	-	-
JF4128	Human, Lucerne, 2008	B.FTNF002-00	297	332	255	396	254	464	T[d]	C[a]	A[a]	-	-	-	-	-	-	-	-	-
JF4212	Human, Nidwald, 2008	B.FTNF002-00	297	353	255	396	254	464	T[d]	C[a]	A[a]	-	-	-	-	-	-	-	-	-
JF4242	Hare, Bern, 2008	B.FTNF002-00	306	311	255	396	254	464	T[d]	C[a]	A[a]	-	-	-	-	-	-	-	-	-
JF4429	Human, Jura, 2008	B.FTNF002-00	351	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF4455	Hare, Bern, 2008	B.FTNF002-00	333	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF4456	Human, Lucerne, 2008	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF4458	Hare, Bern, 2008	B.FTNF002-00	297	353	255	403	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF4496	Human, Basel, 2009	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF4515	Human, Aargau, 2008	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF4516	Human, Aargau, 2009	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF4517	Human, Aargau, 2009	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF4565	Hare, Bern, 2009	B.FTNF002-00	333	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF4628	Human, Zurich, 2009	B.FTNF002-00	297	353	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF4829	Human, Zurich, 2010	B.FTNF002-00	333	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF4830	**Human, Zurich, 2010**	**B.13**	**315**	**332**	**255**	**396**	**254**	**480**	**C[a]**	**A[d]**	**G[d]**	**G[a]**	**G[a]**	**G[a]**	**T[d]**	**A[d]**	**C[a]**	**C[a]**	**C[a]**	**T[a]**
JF4997	Hare, Graubünden, 2011	B.FTNF002-00	297	332	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5142	Human, Vaud, 2011	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5341	Mouse, Zurich, 2012	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5342	Mouse, Zurich, 2012	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5343	Mouse, Zurich, 2012	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5344	Mouse, Zurich, 2012	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5345	Mouse, Zurich, 2012	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-

–: not available; MLVA: multilocus variable-number tandem repeat analysis; SNPs: single nucleotide polymorphisms.

Strains selected for further analysis (canSNPs and antimicrobial agent susceptibilities, n=24) are shaded in grey. Strains belonging to the clade B.13 are highlighted in bold.

[a] Ancestral state.

[d] Derived state.

Table 1B

Genetic characterisation of Swiss strains of *Francisella tularensis* subsp. *holarctica* by MLVA and SNPs, Switzerland, 1996–2013 (n=61)

Strain	Host, geographic origin, year of isolation	Subgroup according to [12] and [13]	MLVA						SNPs											
			Ft-M3	Ft-M6	Ft-M20	Ft-M21	Ft-M22	Ft-M24	B.11	B.12	B.20	B.21	B.22	B.23	B.33	B.34	B.35	B.36	B.37	B.38
JF5346	Human, St. Gallen, 2012	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5349	Hare, Solothurn, 2012	B.FTNF002-00	297	311	255	396	254	464	T[d]	C[a]	A[a]	-	-	-	-	-	-	-	-	-
JF5350	Monkey, Bern, 2012	B.FTNF002-00	288	311	255	396	254	464	T[d]	C[a]	A[a]	-	-	-	-	-	-	-	-	-
JF5351	Hare, Solothurn, 2012	B.FTNF002-00	288	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5353	Marten, Aargau, 2012	B.FTNF002-00	306	311	255	396	254	464	T[d]	C[a]	A[a]	-	-	-	-	-	-	-	-	-
JF5355	Mouse, Zurich, 2012	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5356	Mouse, Zurich, 2012	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5357	Mouse, Zurich, 2012	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5368	Mouse, Zurich, 2012	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5369	Hare, Bern, 2012	B.FTNF002-00	297	353	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5372	Hare, Solothurn, 2012	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5373	Hare, Bern, 2012	B.FTNF002-00	297	311	255	396	254	464	T[d]	C[a]	A[a]	-	-	-	-	-	-	-	-	-
JF5374	Hare, Solothurn, 2012	B.FTNF002-00	288	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5375	Hare, Fribourg, 2012	B.FTNF002-00	324	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5379	Hare, Aargau, 2012	B.FTNF002-00	306	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5380	Hare, Jura, 2012	B.FTNF002-00	351	311	255	396	254	464	T[d]	C[a]	A[a]	-	-	-	-	-	-	-	-	-
JF5386	Hare, Bern, 2012	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5387	Hare, Jura, 2012	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5388	Hare, Jura, 2012	B.FTNF002-00	288	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5389	Hare, Bern, 2012	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5390	Human, Lucerne, 2012	B.FTNF002-00	306	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5393	Hare, Basel, 2012	B.FTNF002-00	306	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5394	Mouse, Zurich, 2012	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5409	Mouse, Zurich. 2012	B.FTNF002-00	297	311	255	396	254	464	T[d]	C[a]	A[a]	-	-	-	-	-	-	-	-	-
JF5410	Mouse, Zurich, 2012	B.FTNF002-00	297	311	255	396	254	464	-	-	-	-	-	-	-	-	-	-	-	-
JF5370	**Human, Bern, 2012**	**B.13**	**306**	**332**	**255**	**396**	**254**	**480**	**C[a]**	**A[d]**	**G[d]**	**G[a]**	**G[a]**	**G[a]**	**T[d]**	**A[d]**	**C[a]**	**C[a]**	**C[a]**	**T[a]**
JF5340	**Human, Lucerne, 2012**	**B.13**	**306**	**332**	**255**	**396**	**254**	**480**	**C[a]**	**A[d]**	**G[d]**	**G[a]**	**G[a]**	**G[a]**	**T[d]**	**G[a]**	**C[a]**	**C[a]**	**C[a]**	**T[a]**
JF5405	**Hare, Solothurn, 2012**	**B.13**	**306**	**332**	**255**	**396**	**254**	**480**	**C[a]**	**A[d]**	**G[d]**	**G[a]**	**G[a]**	**G[a]**	**T[d]**	**A[d]**	**C[a]**	**C[a]**	**C[a]**	**T[a]**
JF5468	**Hare, St. Gallen, 2013**	**B.13**	**315**	**332**	**255**	**396**	**254**	**480**	**C[a]**	**A[d]**	**G[d]**	**G[a]**	**G[a]**	**G[a]**	**T[d]**	**G[a]**	**C[a]**	**C[a]**	**C[a]**	**T[a]**

–: not available; MLVA: multilocus variable-number tandem repeat analysis; SNPs: single nucleotide polymorphisms.

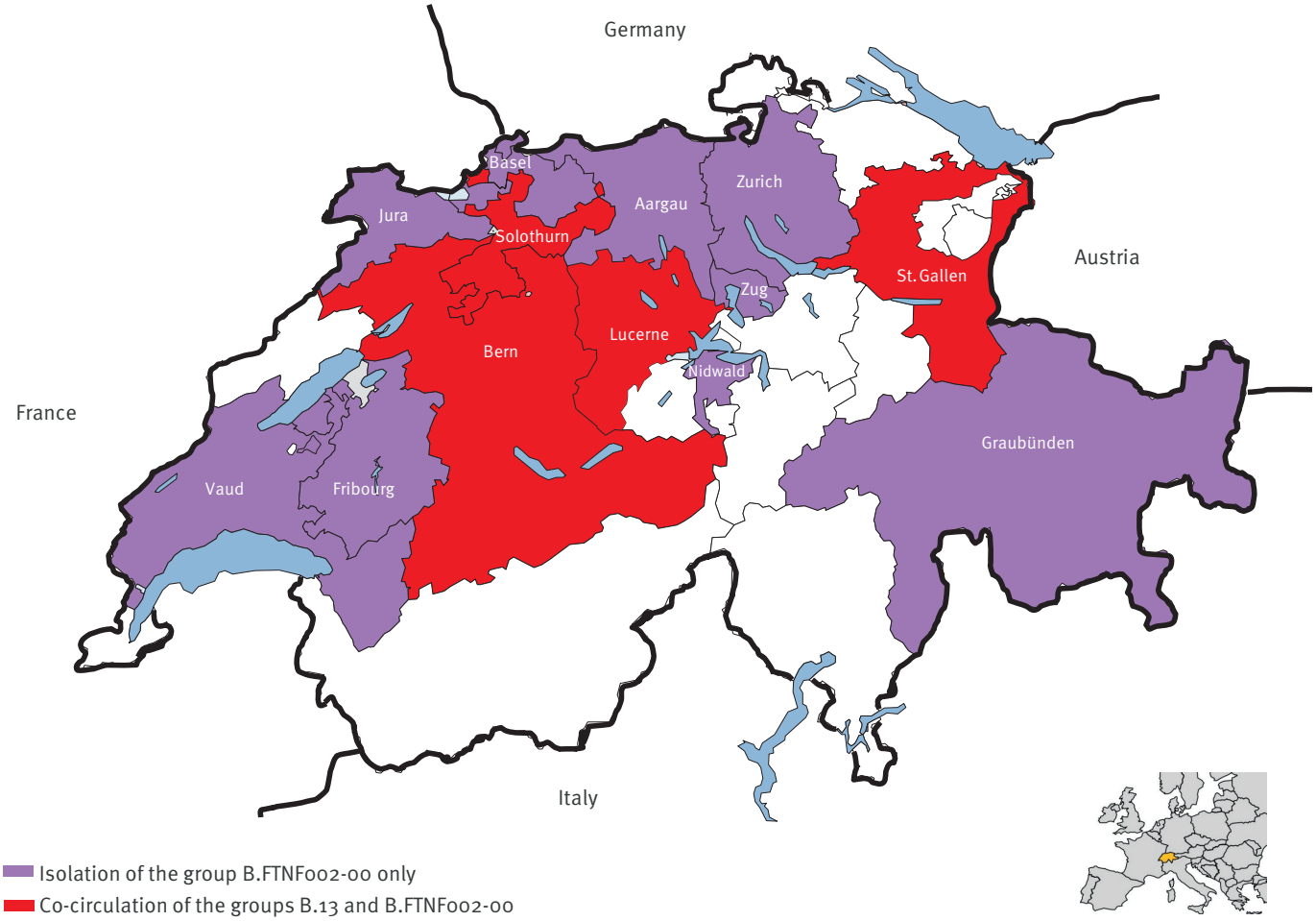
Strains selected for further analysis (canSNPs and antimicrobial agent susceptibilities, n=24) are shaded in grey. Strains belonging to the clade B.13 are highlighted in bold.

[a] Ancestral state.

[d] Derived state.

Figure 2

Francisella tularensis subsp. *holarctica* isolated in Switzerland, 1996–2013 (n=61)



Cantons where both groups B.FTNF002-00 and B.13 were circulating are coloured in red, while cantons where only the group B.FTNF002-00 was isolated are coloured in purple.

Map background downloaded from http://www.presentationmagazine.com/

tetracycline ranged between 2 and 8 µg/mL in mMHII, but were all ≤0.25 µg/mL in mCAMHB (Table 2). For this reason, MIC values for tetracycline were also tested by Etest in order to confirm the results obtained with mCAMHB. With this method, MIC values for tetracycline ranged between 0.19 and 0.38 µg/mL, which was similar to the ones measured by broth microdilution method with mCAMHB (Table 2). The breakpoint value given in the CLSI guidelines for tetracycline is ≤4 µg/mL [27]. Moreover, a difference in MIC values for tetracycline in mMHII and mCAMHB was also observed for reference strains *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 (Table 2). MIC values for erythromycin exhibited a bimodal distribution: Strains belonging to group B.13 exhibited erythromycin resistance with MIC values higher than 32 µg/mL, while all the strains belonging to group B.FTNF002-00 were sensitive to erythromycin, showing MIC values ranging from 1 to 8 µg/mL (Table 2). However, no breakpoint value for *F. tularensis* is provided for any macrolides in the CLSI guidelines [27].

Genetic characterisation of erythromycin resistance was performed by PCR amplification and sequencing of the three copies of the *rrl* gene and of the *rplD* and the *rplV* genes of the five strains showing phenotypic resistance to erythromycin and belonging to group B.13 (JF4830, JF5340, JF5370, JF5405 and JF5468). The sequences were compared to the corresponding genes of strains FTNF002-00 (group B.FTNF002-00), F92 (group B.FTNF002-00), FSC200 (group B.13) and LVS (group B.13). All five strains isolated in Switzerland, belonging to group B.13 and showing phenotypic resistance to erythromycin, had two mutations in all three copies of the *rrl* gene when compared to the available sequences of the strains FTNF002-00 and F92 of the group B.FTNF002-00. The first mutation was detected in domain I of the *rrl* gene, A453G (*E. coli* numbering), while the second mutation was observed in domain V of the *rrl* gene, A2059C (*E. coli* numbering). A silent mutation G to A at the third position of codon 181 (*E. coli* numbering) was found in the *rplD* gene encoding

Table 2

Antibiotic susceptibilities of *Francisella tularensis* subsp. *holarctica* strains after 48 hours in mMHII broth and mCAMH, Switzerland, 1996–2013 (n=24)

Strain	Subgroup according to [12] and [13]	mMHII broth MIC (µg/mL)							mCAMH broth MIC (µg/mL)						
		GEN	STR	CIP	TET	ERY	NAL	CHL	GEN	STR	CIP	TET	ERY	NAL	CHL
Escherichia coli ATCC 25922		8	16	≤0.06	>16	>32	≤2	4	0.5	>16	≤0.06	2	>32	≤2	4
Staphylococus aureus ATCC 29213		8	16	0.5	16	2	32	16	1	>16	0.5	1	2	32	16
JF3820	B.FTNF002-00	4	4	≤0.06	4	2	≤2	≤2	≤0.12	4	≤0.06	≤0.25	4	≤2	≤2
JF3821	B.FTNF002-00	2	4	≤0.06	2	1	≤2	≤2	≤0.12	4	≤0.06	≤0.25	4	≤2	≤2
JF3822	B.FTNF002-00	4	4	≤0.06	4	2	≤2	≤2	≤0.12	4	≤0.06	≤0.25	4	≤2	≤2
JF3824	B.FTNF002-00	4	4	≤0.06	8	4	≤2	≤2	≤0.12	4	≤0.06	≤0.25	4	≤2	≤2
JF3825	B.FTNF002-00	4	4	≤0.06	8	4	≤2	≤2	≤0.12	4	≤0.06	≤0.25	4	≤2	≤2
JF3826	B.FTNF002-00	2	4	≤0.06	4	2	≤2	≤2	≤0.12	4	≤0.06	≤0.25	4	≤2	≤2
JF3828	B.FTNF002-00	2	4	≤0.06	8	2	≤2	≤2	≤0.12	4	≤0.06	≤0.25	4	≤2	≤2
JF3829	B.FTNF002-00	2	4	≤0.06	4	2	≤2	≤2	≤0.12	4	≤0.06	≤0.25	4	≤2	≤2
JF3859	B.FTNF002-00	2	4	≤0.06	8	2	≤2	≤2	≤0.12	4	≤0.06	≤0.25	4	≤2	≤2
JF4092	B.FTNF002-00	2	2	≤0.06	4	2	≤2	≤2	≤0.12	4	≤0.06	≤0.25	4	≤2	≤2
JF4128	B.FTNF002-00	2	2	≤0.06	8	2	≤2	≤2	≤0.12	4	≤0.06	≤0.25	4	≤2	≤2
JF4212	B.FTNF002-00	1	2	≤0.06	4	1	≤2	≤2	≤0.12	4	≤0.06	≤0.25	4	≤2	≤2
JF4242	B.FTNF002-00	2	4	≤0.06	8	2	≤2	≤2	≤0.12	4	≤0.06	≤0.25	4	≤2	≤2
JF4830	B.13	2	2	≤0.06	4	>32	≤2	≤2	0.25	4	≤0.06	≤0.25	>32	≤2	≤2
JF5340	B.13	2	4	≤0.06	4	>32	≤2	≤2	≤0.12	4	≤0.06	≤0.25	>32	≤2	≤2
JF5349	B.FTNF002-00	2	4	≤0.06	4	8	≤2	≤2	0.25	4	≤0.06	≤0.25	4	≤2	≤2
JF5350	B.FTNF002-00	2	4	≤0.06	4	2	≤2	≤2	0.25	4	≤0.06	≤0.25	2	≤2	≤2
JF5353	B.FTNF002-00	2	4	≤0.06	4	1	≤2	≤2	0.25	4	≤0.06	≤0.25	2	≤2	≤2
JF5370	B.13	2	2	≤0.06	4	>32	≤2	≤2	≤0.12	4	≤0.06	≤0.25	>32	≤2	≤2
JF5373	B.FTNF002-00	2	2	≤0.06	4	2	≤2	≤2	0.25	4	≤0.06	≤0.25	2	≤2	≤2
JF5380	B.FTNF002-00	2	4	≤0.06	4	2	≤2	≤2	0.25	4	≤0.06	≤0.25	2	≤2	≤2
JF5405	B.13	2	4	≤0.06	4	>32	≤2	≤2	≤0.12	4	≤0.06	≤0.25	>32	≤2	≤2
JF5409	B.FTNF002-00	2	4	≤0.06	4	4	≤2	≤2	0.25	4	≤0.06	≤0.25	8	≤2	≤2
JF5468	B.13	2	4	≤0.06	4	>32	≤2	≤2	≤0.12	4	≤0.06	≤0.25	>32	≤2	≤2

CHL: chloramphenicol; CIP: ciprofloxacin; ERY: erythromycin; GEN: gentamicin; mCAMH: modified cation-adjusted Mueller Hinton broth; MIC: minimum inhibitory concentration; mMHII: modified Mueller Hinton II broth; NAL: nalidixic acid; STR: streptomycin; TET: tetracycline.

for the ribosomal protein L4, while no mutation was detected in the *rplV* gene encoding for the ribosomal protein L22. These mutations observed in Swiss strains belonging to group B.13 were exactly the same as those of the strain LVS and FSC200 belonging to group B.13.

The EMBL/GenBank accession numbers for the nucleotide sequences of the *rrl*, the *rplD* and the *rplV* genes are: KF712467, KF712466 and KF712465, respectively.

Discussion

This study describes the characterisation of *F. tularensis* strains isolated in Switzerland during the last 17 years. Until 2012, only strains belonging to group B.FTNF002-00 have been isolated in Switzerland from humans and animals. A single exception is strain JF4830 that was isolated from a human patient in 2010, who most probably acquired the infection travelling in eastern Europe. The new strains JF5340 and JF5370 were isolated from human patients with no history of travelling abroad for several months before the appearance of the first tularaemia-associated symptoms. The only exception is a stay at Lago Maggiore in Italy, at the border to Switzerland, a month before the initial symptoms, for the patient infected with strain JF5340. Strains JF5405 and JF5468 were isolated from the carcass of two wild hares in 2013. All four strains (JF5340, JF5370, JF5405 and JF5468) belonged to group B.13 and resolved with distinct MLVA profiles (Table 1). Moreover, the analysis of canSNP B.33 to B.38 led to identification of the subgroups B.33/34 and B. 34/35 previously described by Gyuranecz et al. [12]. Both subgroups were isolated in central and eastern Europe and from countries bordering Switzerland, such as Austria and Germany, but not from countries east of Romania [12]. Moreover, the subgroup B.33/34 is also known to be circulating in Sweden [12].

These findings reveal that group B.13 is currently circulating in Switzerland in the same areas as strains of group B.FTNF002-00 (Figure 2) and are affecting both human patients and free-ranging animals. This is in contrast to neighbouring Germany, where a strict separation between groups was described [18]. Strains isolated between 1996 and 2011 from humans, hares and captive non-human primates all belonged to group B.FTNF002-00. Because of the small number of strains isolated between 1996 and 2008, it is difficult to draw conclusions about a recent introduction of strains belonging to group B.13 or a long-lasting co-circulation of both groups following the expansion of group B.FTNF002-00 of *F. tularensis* subsp. holarctica.

Strains belonging to group B.FTNF002-00 are known to be sensitive to erythromycin [7,29,33]. Also the Swiss *F. tularensis* subsp. *holarctica* strains belonging to group B.FTNF002-00 are sensitive to erythromycin, while the new strains belonging to group B.13 are resistant (Table 2). Since strains resistant to erythromycin are actually circulating in Switzerland, macrolides are not recommended for the treatment of cases of tularaemia acquired in Switzerland and possibly also in neighbouring areas unless analysis of the infecting strains reveals sensitivity to this antibiotic. Because of the toxicity of recommended antibiotics against tularaemia for pregnant women and foetuses, Dentan et al. [21] proposed to treat it, in areas where the group B.FTNF002-00 is endemic, with a macrolide, more specifically with azithromycin. However, the spread to western Europe of strains resistant to macrolides poses serious concerns and needs to be carefully considered by the clinicians when facing a therapeutic choice in this context. Several studies suggest that strains of *F. tularensis* from western Europe are sensitive to macrolides [4,7,18,29,33,34].

Genetic analysis of the strains resistant to erythromycin revealed two mutations in the three copies of the *rrl* gene and a silent mutation in the *rlpD* gene encoding the ribosomal protein L4, compared with the strains belonging to group B.FTNF002-00: FTNF002-00 and F92. Interestingly, the same mutations are present in the strains LVS and FSC200, both belonging to group B.13. This finding may suggest that these mutations are shared among subgroups belonging to B.13 and may have appeared in a common ancestor. However, this hypothesis should be validated by testing a larger panel of strains.

Broth microdilution testing was performed in mMHII broth and in mCAMHB [4,7]. Results were compatible for all antibiotics tested in both media except for the MIC values recorded for gentamicin and tetracycline that showed higher values in mMHII broth than in mCAMHB (Table 2). This discrepancy was confirmed by testing the reference strains, *E. coli* ATCC 25922 and *S. aureus* ATCC 29213, which also showed higher MIC values for gentamicin and tetracycline in mMHII broth than the quality control ranges for broth microdilution method in mCAMHB reported in the CSLI guidelines [27], confirming that MIC values for these two antibiotics are higher using mMHII broth than mCAMHB (Table 2). High MIC values of reference strains for gentamicin and to a lesser extent for tetracycline tested in mMHII broth were previously described by Baker et al. [35]. They explained these results to be due to the addition of the bivalent cations Ca^{2+} and Mg^{2+} in the medium. MIC values for tetracycline were within the range considered clinically effective when tested with mCAMHB and by Etest. Nevertheless, given the high number of tularaemia cases for whom tetracycline-associated treatment failure has been described, including doxycycline [36-39], these antibiotics are not recommended in case of infection with *F. tularensis*.

MIC values for gentamicin, chloramphenicol, streptomycin and ciprofloxacin were within the range indicative of clinical efficacy in both media, although MIC values of four strains for gentamicin in mMHII were very close to the breakpoint value given in the CLSI guidelines [27]. Ciprofloxacin showed the lowest MIC values and prevented growth of all strains at 0.06 µg/

mL. The finding is consistent with previous reports on type A and type B tularaemia [5, 40-44] and supports the experience that ciprofloxacin may be an attractive treatment option for tularaemia [37].

Conclusion

In conclusion, at least two groups of *F. tularensis* subsp. *holarctica* are currently co-circulating in Switzerland. Of these, the group B.FTNF002-00 seems to be more prevalent and has been identified in tularaemia cases since 1996, while B.13 was less commonly isolated in Switzerland and not before 2012. Since strains belonging to the subgroups B.33/34 and B.34/35 are erythromycin-resistant, this antibiotic is not recommended to treat cases of tularaemia acquired in Switzerland without prior typing of the strains. These concerns should also apply in countries where the group B.FTNF002-00 seems to be prevalent given that the exact limits of the co-circulation areas are not known. The mutations resulting in erythromycin resistance in group B.13 strains are exactly the same as those present in the strain LVS belonging to the same group. Further investigations are warranted in order to understand if they are shared by all strains of the group B.13. In view of the in vitro results and of previous clinical observations, tetracyclines should not be a first choice of treatment for tularaemia, while ciprofloxacin appears suitable for tularaemia treatment. Moreover, because of the fastidious growth requirements of F. tularensis, supplements always need to be added to growth media to test antibiotic susceptibility [7,35]. Recently, Georgi et al. published the validation of a protocol for a broth microdilution method (medium not supplemented with divalent cations) for *F. tularensis* [4]. The use of a medium without supplemented divalent cations, mCAMH, has been compared with the previously described methods using mMHII broth (medium supplemented with divalent cations). Considering the discrepancy in the MIC values measured for gentamicin and tetracycline depending on the broth used, it is considered more appropriate the use of mCAMHB for antimicrobial testing of *F. tularensis* strains because it achieves results, at least relatively to some antimicrobial substances, that are less ambiguous than those achieved with the mMHII broth.

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Conflict of interest

None declared.

Authors' contributions

FCO: performed the experiments, analysed the data, drafted and revised the manuscript. JF: analysed the data and critically revised the manuscript. PP: designed the study, performed the experiments, analysed the data, drafted and revised the manuscript.

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Annex 3:

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CASE REPORT

Tularemia in the Southeastern Swiss Alps at 1,700 m above sea level

M. Ernst · P. Pilo · F. Fleisch · P. Glisenti

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Abstract A 37-year-old man presented with a 4-day history of nonbloody diarrhea, fever, chills, productive cough, vomiting, and more recent sore throat. He worked for the municipality in a village in the Swiss Alps near St. Moritz. Examination showed fever (40 °C), hypotension, tachycardia, tachypnea, decreased oxygen saturation (90 % at room air), and bibasilar crackles and wheezing. Chest radiography and computed tomography scan showed an infiltrate in the left upper lung lobe. He responded to empiric therapy with imipenem for 5 days. After the imipenem was stopped, the bacteriology laboratory reported that 2/2 blood cultures showed growth of *Francisella tularensis*. He had recurrence of fever and diarrhea. He was treated with ciprofloxacin (500 mg twice daily, oral, for 14 days) and symptoms resolved. Further testing confirmed that the isolate was *F. tularensis* (subspecies *holarctica*) belonging to the subclade B.FTNF002-00 (Western European cluster). This case may alert physicians that tularemia may occur in high-altitude regions such as the Swiss Alps.

Keywords *Francisella tularensis* · Infection · Pulmonary · Switzerland · Altitude

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Case report

A 37-year-old man presented with a 4-day history of nonbloody diarrhea, fever, chills, productive cough, vomiting, and more recent sore throat. He worked for the municipality in a village in the Swiss Alps near St. Moritz, which is situated in a valley at 1,700 m above sea level. He did not leave the local area during the previous 3 months. He did not eat any raw meat or milk. He had no close contact with domestic or wild animals, and he did not notice any tick bites. Past medical history included compensated congenital chronic hepatitis B infection. He had emigrated from Ethiopia to Switzerland 17 years ago, smoked 30 cigarettes per day, and had a history of chronic alcoholism that was treated with disulfiram for the previous 1.5 years.

Examination showed fever (40 °C), hypotension (80/60 mmHg), tachycardia (100 beats/min), tachypnea (20 breaths/min), decreased oxygen saturation (90 % on room air), and bibasilar crackles and wheezing. The abdomen was soft and had active bowel sounds. Skin examination and cardiac auscultation were normal. Laboratory studies showed white blood cell count 2.1×10^9/L (reference range, $3.0–9.0 \times 10^9$/L), neutrophils 1.29×10^9/L (reference range, $1.4–8.0 \times 10^9$/L), thrombocytes 69×10^9/L (reference range, $150–400 \times 10^9$/L), C-reactive protein 125 mg/L (reference range, <5 mg/L), alanine aminotransferase 77 U/L (reference range, <41 U/L), and aspartate aminotransferase 200 U/L (reference range, <40 U/L); the alanine and aspartate aminotransferase levels had been normal 2 weeks earlier. The human immunodeficiency virus and hepatitis C serology were negative. Urinalysis and abdominal ultrasonography were normal. Chest radiography showed marginal infiltrates in the left upper lung lobe. Chest computed tomography scan

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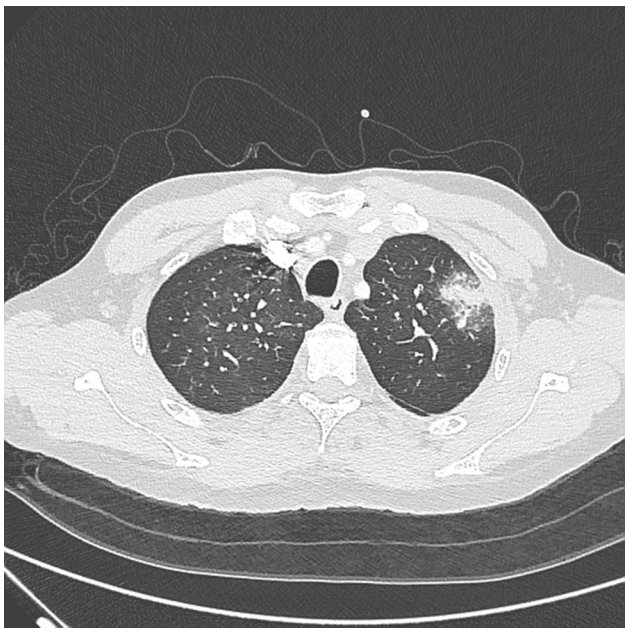

Fig. 1 A 37-year-old man who had diarrhea, fever, chills, cough, vomiting, and sore throat. Chest computed tomography scan showed an infiltrate in the left upper lung lobe

showed an infiltrate in the left upper lung lobe, enlarged lymph nodes in the left hilum, and normal upper liver, spleen, and adrenal glands (Fig. 1). Cytologic examination of bronchial brush biopsies showed inflammatory cells (mostly neutrophilic granulocytes) without malignancy.

The patient received empiric broad spectrum therapy with imipenem (2 g/d, intravenous). On day 5, all symptoms resolved, imipenem was stopped, and preliminary results of blood and stool cultures showed no bacterial growth. On day 7, the patient was asymptomatic and discharged from the hospital. At 2 days after discharge, the bacteriology laboratory reported that 2/2 blood cultures showed growth of *Francisella tularensis* after 7 days in the laboratory (minimal inhibitory concentration for ciprofloxacin, 0.032 mg/L). The patient was called by telephone and he reported recurrence of fever and diarrhea. He was started on ciprofloxacin (500 mg twice daily, oral, for 14 days), and symptoms resolved.

The isolate was further confirmed as *F. tularensis* (subspecies *holarctica*) by polymerase chain reaction tests that targeted the *fopA* gene and the region of difference one [1]. A supplementary polymerase chain reaction test that targeted the region of difference 23 identified the isolate as belonging to the subclade B.FTNF002-00 (Western European cluster) [1]. Sequence analysis of the single nucleotide polymorphism markers B.18 (derived state) and B.19 (ancestral state) confirmed the subclade B.FTNF002-00, which is endemic in Switzerland and Western Europe [2, 3]. In addition, the multiple-loci variable number of tandem repeats analysis profile corresponded to the profile of strains circulating in Western Europe and Switzerland (Fig. 2) [2–4].

Discussion

Tularemia is a rare but potentially severe zoonosis that is endemic in the northern hemisphere. It is caused by the Gram-negative coccobacillus *F. tularensis*, which initially was isolated in 1912 [5–7]. *F. tularensis* comprises three subspecies. The clinically relevant subspecies include subspecies *tularensis* (genotypes A1a, A1b, and A2) and subspecies *holarctica* (genotype B) and have different virulence and frequency of mortality [A1a, 4 %; A1b, 24 %; A2, 0 %; B, 7 %; (United States, 1964–2004)]. In Europe, only subspecies *holarctica* has been identified [8].

In humans, tularemia can cause several disease patterns, depending on the site of entry into the body. The most common form of the disease is ulceroglandular tularemia, which usually is caused by a bite from an infected arthropod vector or direct contact with a contaminated source [6]. After an incubation period (typically 3–6 days), the patient experiences a sudden onset of flu-like symptoms, especially chills, fever, headache, and generalized aches. Furthermore, ulceroglandular tularemia may present with an ulcer that may persist for several months. Glandular tularemia is a disease that has similar symptoms as ulceroglandular tularemia but without the appearance of an ulcer [7]. Oculoglandular tularemia may occur more rarely, and the bacterium is acquired by direct contamination of the conjunctiva [9].

The ingestion of infected foods or contaminated drinking water may cause oropharyngeal or gastrointestinal tularemia, depending on the site of colonization in host tissues [10]. Oropharyngeal tularemia may be associated with painful sore throat, enlargement of the tonsils, the formation of a yellow-white pseudomembrane, and swollen cervical lymph nodes [6]. Depending on the infecting dose, gastrointestinal tularemia may be mild and persistent or an acute fatal disease (typhoidal tularemia) associated with extensive bowel ulceration.

Primary pulmonary tularemia may be caused by the inhalation of bacteria, but most commonly may occur secondary to the septic spread of ulceroglandular, glandular, oculoglandular, or oropharyngeal tularemia [11]. The clinical and radiographic features of pulmonary tularemia are varied and the diagnosis may be difficult [11]. Pulmonary tularemia occasionally may occur without any overt signs of pneumonia. Inhalational tularemia may be caused by farming activities that involve the handling and generation of dust from hay that previously was the residence of infected rodents [6].

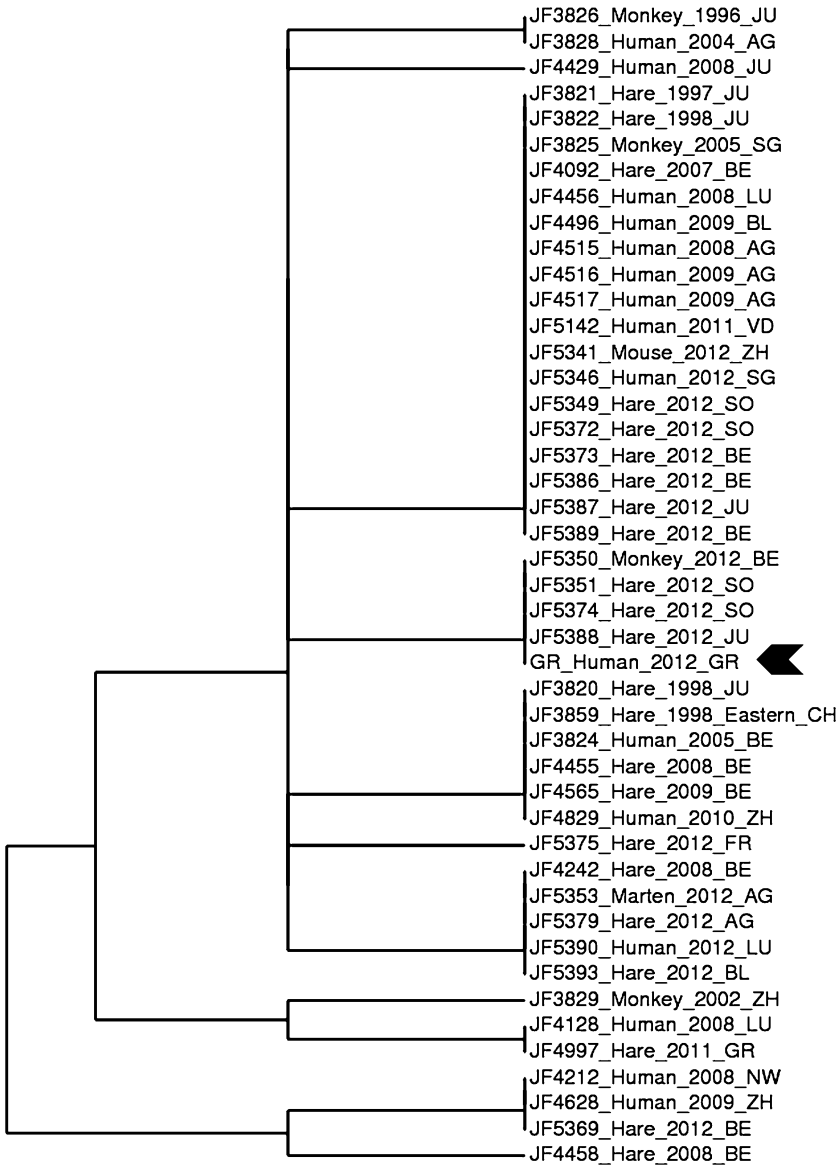

Fig. 2 Clustering dendrogram comparing Swiss strains of *Francisella tularensis* (subspecies *holarctica*) subclade B.FTNF002-00. Name of strain, host, year of isolation, and canton (Swiss state) are noted for each strain. The *arrowhead* shows the human strain isolated in Graubünden, Switzerland in 2012. This analysis was performed using six markers of multiple-loci variable number of tandem repeats analysis (Ft-M3, Ft-M6, Ft-M20, Ft-M21, Ft-M22, Ft-M24) and the program from the online software tool from the bank of multiple-loci variable number of tandem repeats analysis. The Newick string was imported into software (TreeDyn, GEMI Bioinformatics, Montpellier, France) and a dendrogram was drawn [4]. Abbreviations (Swiss cantons): *AG* Aargau, *BE* Bern, *BL* Basel Land, *FR* Freiburg, *GR* Graubünden, *JU* Jura, *LU* Luzern, *NW* Nidwald, *SG* Sankt-Gallen, *SO* Solothurn, *VD* Vaud, *ZH* Zürich

The diagnosis of tularemia may require a high index of clinical suspicion from the patient's presentation and epidemiologic factors. Diagnostic tests include serologic antibody test, direct polymerase chain reaction, and bacterial culture. Disadvantages of culture include the biological safety risk and risk of false negative results because of the fastidious growth requirements of the organism.

Effective antibiotic therapy for tularemia may include streptomycin, gentamicin, tetracyclines, chloramphenicol, and fluoroquinolones. Fluoroquinolones are highly effective against *F. tularensis* in vitro and have been used successfully to treat several patients in Europe that had the less virulent subspecies *holarctica*. In Europe, oral fluoroquinolones are the therapy of choice for mild-to-moderate disease because these drugs have fewer and less severe adverse events than aminoglycosides. However, fluoroquinolones may not be the treatment of choice in the United States because the virulence of strains may be greater in the United States than Europe [5, 12]. In the present patient, treatment with imipenem caused defervescence, even though *F. tularensis* subspecies *holarctica* may be resistant to imipenem [13]. This resistance may have caused the patient's relapse, which responded promptly to fluoroquinolone therapy. In pregnant women, ciprofloxacin and doxycycline are not approved by the United States Food and Drug Administration, and gentamicin or streptomycin may be recommended as alternative therapy [14].

Many hematophagous arthropod species worldwide, including ticks, tabanid flies (horseflies and deerflies), fleas, and mosquitoes, may transmit tularemia to mammalian hosts [15]. Arthropods such as ticks may be the

principal vectors of transmission in North America, as shown in 56 of 81 patients in Missouri. The average annual incidence of tularemia in Missouri in 2000–2007 was 40 cases per 100,000 people, but it is much less frequent in Europe [5]. In Switzerland, tularemia occurs sporadically and had an incidence of 0.04 cases per 100,000 people in 2004 (total, three cases), but the incidence in 2012 was 0.5 cases per 100,000 people (total, 41 cases). Tularemia has been a mandatory reportable disease in Switzerland since 2004 and all diagnosed cases should be registered [16]. In the canton of Graubünden in southeastern Switzerland, three cases of tularemia have been reported, and the present case is the first known case of typhoidal tularemia [16]. Only two other cases of typhoidal tularemia in Switzerland have been reported to the Swiss Federal Office of Public Health since 2004, one each from the Luzern and Aargau cantons, both located approximately 400 m above sea level [16]. Literature search showed no reported case of tularemia at higher elevation in the Swiss Alps, especially higher than 1,700 m above sea level.

The Swiss Federal Institute of Health has reported an increase in the incidence of tularemia since 2007 in Switzerland [16]. This may be attributed to improved diagnostic laboratory testing including the more frequent use of polymerase chain reaction, cysteine-enriched media such as chocolate agar, and prolonged culture. In addition, climate change caused by global warming may improve growth conditions for the most common vectors and may increase the incidence of tularemia, as shown in studies from Scandinavia, the Russian arctic region, and Turkey [17–19]. Comparable effects may occur in Switzerland because of climate change in regions of high altitude above sea level. An increase in winter temperature may improve survival of animal or bird hosts and insect vectors that may transmit infectious agents. Increased number of hot summer days may be associated with increased activity of infected mosquitoes, horseflies, and ticks and associated human exposure to pathogens carried by these insects and ticks [18].

The present case was remarkable because the presenting complaint was diarrhea, with a later onset of sore throat. Therefore, this patient may have had primary gastrointestinal tularemia and secondary septicemia. In addition, the patient was a council worker and may have acquired the infection from his job, working the most time outdoors. Numerous animals may be carriers of *F. tularensis* in the Upper Engadin region of Switzerland (Table 1) [20]. The patient denied any animal contact or other common sources of infection. The presenting symptom of diarrhea suggests that he may have ingested infected food or contaminated water. He had not left the valley for a minimum 3 months, which exceeds the typical incubation time (3–6 days) [7]. Therefore, he must have acquired the infection in the Upper Engadin region. As a council worker, the patient had frequent contact with fresh water that may have been contaminated, and this may be the most plausible route of exposure for this patient.

Table 1 Rodent and Lagomorph species that may be infected by *Francisella tularensis* in the Canton of Graubünden, Switzerland at elevation >1,500 m (modified from [20])

Species	Common name
Lepus timidus	Mountain hare
Lepus europaeus	Brown hare
Sciurus vulgaris	Red squirrel
Marmota marmota	Alpine marmot
Eliomys quercinus	Garden dormouse
Dryomys nitedula	Forest dormouse
Muscardinus avellanarius	Common dormouse (hazel dormouse)
Apodemus sylvaticus	Wood mouse
Apodemus flavicollis	Yellow-necked mouse
Apodemus alpicola	Alpine field mouse
Mus domesticus	House mouse
Clethrionomys glareolus	Bank vole
Arvicola terrestris	Water vole
Microtus subterraneus	European pine vole
Microtus arvalis	Common vole
Chionomys nivalis	European snow vole

Based on review of the literature and public health information, this may be the first reported case of tularemia in a high valley in the Swiss Alps >1,700 m above sea level. Climate change may increase the incidence of cases at high elevation in the future. This case report may alert physicians that tularemia may occur in high-altitude regions such as the Swiss Alps.

Conflict of interest The authors certify that they have no actual or potential conflict of interest in relation to this article.

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Annex 4:

Origgi, F.C., König, B., Lindholm, A.K., Mayor, D., Pilo, P. Tularemia among Free-Ranging Mice without Infection of Exposed Humans, Switzerland, 2012. **Emerg. Infect. Dis**. In Press

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Tularemia among Free-Ranging Mice without Infection of Exposed Humans, Switzerland, 2012

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The animals primarily infected by *Francisella tularensis* are rapidly consumed by scavengers, hindering ecologic investigation of the bacterium. We describe a 2012 natural tularemia epizootic among house mice in Switzerland and the assessment of infection of exposed humans. The humans were not infected, but the epizootic coincided with increased reports of human cases in the area.

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Although the house mouse (*Mus musculus domesticus*) is a common model for infection with *Francisella tularensis* (*1*), no recent and detailed data are available about natural tularemia outbreaks in this species. Tularemia mainly affects rodents and lagomorphs (*2*), but because these species are rapidly consumed by scavengers (*3*), it is challenging to conduct investigations of the biologic cycle of *F. tularensis* in the environment. Furthermore, the disease mostly occurs sporadically, although outbreaks have been reported in animals and humans (*2*). We describe a natural outbreak of tularemia among a population of free-ranging house mice; the epizootic occurred in Switzerland in 2012 and was associated with possible human exposure. The mouse study was approved by the Swiss Animal Experimentation Commission (Kantonales Veterinäramt Zürich; permit 51/2010).

The Study

At the edge of a forest in the Canton of Zurich, Switzerland, a population of house mice is housed in a 72-m^2 barn equipped with 40 nesting boxes. The population has been studied since 2002 to analyze the social structure and the population genetics of free-living house mice (*4*). The mice are monitored for research purposes every 2–3 days (*4*). Food, water, rodent bedding, and straw are provided ad libitum; mice are free to enter and exit the barn at any time. Larger animals are excluded from the barn, but other small mammals occasionally have been observed. In early June 2012, the mouse population in the barn was ≈360.

Starting in early June 2012, increased numbers of mice were found dead in the barn. During May 2012–June 2013, a total of 69 carcasses were collected and stored frozen until necropsy was performed, beginning in mid-July 2012, after the initial peak of the outbreak (Figure). Full pathologic analysis could be performed on samples from 35/69 mice, of which 15 were PCR-positive for *F. tularensis*. The primary organs were collected and processed for histologic analysis. Pathologic investigation showed the presence of macroscopic and histologic changes. Skin lesions consistent with bite and fight wounds were observed in 7 mice, 1 of which was PCR-positive for *F. tularensis*; only gram-positive cocci were detected in the associated skin lesions of this mouse by light microscopy. Splenomegaly was observed in 23 mice. In 12 of these mice, splenomegaly was secondary to tularemia, and in 8, it was associated with amyloidosis and was frequently multisystemic. In 3 mice, splenomegaly was associated with amyloidosis and *F.*

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tularensis infection. Red to dark red mottling of the lung was observed in several affected mice, but obvious lung hemorrhages were observed in only 2 mice. The main histologic finding was the presence of multiple foci of necrosis in spleen, liver, and lung. In addition, frequent prominent thrombi and emboli were seen in lungs in association with severe vascular inflammatory infiltration and necrosis.

Overall, lung lesions consistent with *F. tularensis* infection (necrotizing pneumonia) were seen in ~~67%~~10/15 of the *F. tularensis*–positive mice that were examined histologically; the lesions were observed throughout the outbreak and showed various degrees of size, extension, and severity. No similar lesions were observed in any of the *F. tularensis*–negative mice. A total of 69 samples were tested for *F. tularensis* by culture and direct PCR: 49 were spleen samples, including samples from mice not selected for pathologic investigation because of severe autolysis, and 20 were abdominal swab samples, which were used when the extreme grade of autolysis prevented the unambiguous identification of the spleen following dissection (*5*). Spleen and swab samples from 24/69 mice were positive for *F. tularensis* by PCR. Tularemia cases were observed during June–August 2012 (Figure). Eight isolates from the spleens of 8 mice were identified as *F. tularensis* subsp. *holarctica* belonging to the lineage B.FTNF002–00; these isolates shared a single multilocus variable number tandem repeat analysis profile (*6*,*7*).

During the epizootic, 11 researchers regularly entered the barn during June 1–August 31, 2012, and were considered to have been exposed to *F. tularensis* (Table). On May 30 and June 29, 2012, influenza-like symptoms developed in researchers 1 and 3, respectively. *F. tularensis* antibodies persist in the blood, and serology is a standard method for diagnosing tularemia in humans. Thus, in late November or mid-December 2012 (≈6 months after the epizootic began), we obtained blood samples from the 11 researchers for serologic testing (VIRapid tularemia test; Vircell, Granada, Spain) (*8*): 10 samples were negative. The sample from researcher 3 had a positive test reaction and was further tested by microagglutination (*8*); dilutions of 1:40–1:640 were tested, and results were negative at 1:40. A second blood sample was obtained from researcher 3 in mid-March 2013 and was still positive by the rapid test but again showed no agglutination. Serologic cross-reaction with *Brucella* spp. was assessed and excluded. We then used the whole antigen from an outbreak isolate to perform IgM and IgG Western blots on the first and second serum samples from researcher 3: results were negative.

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Conclusions

Data concerning natural outbreaks of tularemia are difficult to obtain, especially from house mice, whose carcasses rarely remain available for collection because of predators and scavengers (*3*). In this study, a large population of mice could be monitored under natural conditions, in the absence of antimicrobial drug treatment, during a tularemia outbreak. PCR confirmed that during the ≈3-month outbreak of tularemia, 7% of the mouse population died from the disease. This number is relatively low considering the described high sensitivity of this species to *F. tularensis* (*1*); however, the number of exposed mice is unknown, and not all dead mice were available for testing. The lesions observed were similar overall to those previously reported (*9*). However, in our investigation, lung lesions were occasionally as severe or more severe than those observed in other tissues. The lung lesions varied in size, severity, and extension but remained consistent overall, suggesting a possible single route of infection and/or systemic spread.

Cannibalism (*10*) might have favored the transmission of bacteria within the mouse population, but most of the carcasses with skin wounds tested negative for *F. tularensis*. Thus, transmission through cannibalism is not likely. Transmission through arthropods may be possible because the study population naturally harbors fleas and mites; ticks have not been observed.

Notification of *F. tularensis* outbreaks among rodents is essential, given the frequent presence of these animals in households and the consequent zoonotic potential of the pathogen (*11*). A unique aspect of this investigation is that we were able to evaluate humans with known exposure to infected animals. Eleven researchers entered the barn inhabited by house mice and monitored/handled the animals every 2–3 days without the use of specific personal protective equipment, except for disposable gloves; some of the mice were later found to be infected with *F. tularensis* (for more details about the monitoring/handling of animals, see [*4*]). The barn is a closed environment filled with bedding; mouse excrement is present on all surfaces and has the potential for aerosolization. Nevertheless, seroconversion was not detected in any of the researchers, bringing to question whether shedding of *F. tularensis* in urine and feces of mice is a key source of *F. tularensis* transmission for humans.

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Thus far, reports about *F. tularensis* shedding in rodents have had inconsistent findings (*12–14*). However, this is a crucial point to investigate because *F. tularensis* shedding through urine and feces would not only affect outdoor environments but also household environments via rodent infestation. Moreover, in Switzerland 150% more human tularemia cases were reported in 2012 than in 2011; the increase was mostly due to cases in the same area where the barn in this study is located (*15*), confirming the importance of monitoring sentinel animals for tularemia to better understand the ecology of *F. tularensis*.

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Dr Origgi, a veterinary pathologist and microbiologist, is responsible for the wildlife diagnostic service of the Centre for Fish and Wildlife Health at the University of Bern. His main interests are host–pathogen interactions and lower vertebrate pathology and immunology.

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Table. Estimated time 11 researchers spent in a barn inhabited by *Francisella tularensis*–infected house mice, Switzerland, June 1–August 31, 2012

Researcher	Total time in barn	
no.	Hours	Days
1	78	22
2	49	15
3	32	4
4	29	4
5	21	4
6	18	2
7	9	1
8	9	1
9	9	1
10	9	1
11	9	1

Figure. Monthly distribution of the number carcasses of free-ranging house mice collected from a barn and the number positive for *F. tularensis*, Switzerland, May 2012–June 2013.

Annex 5:

Origgi, F.C., Pilo, P. Pathology, bacteriology and proposed pathogenesis of Tularemia in European Brown Hares (*Lepus europaeus*): a model for human tularemia. In preparation

Annex 6:

Dobay, A., Pilo, P., Lindholm, A.K., Origgi, F.C., Bagheri, H.C., König, B. Epizootic of tularemia in a closely-monitored free-roaming population of wild house mice. In preparation

Annex 7:

Origgi, F.C., Pilo P. Role of ticks (*Ixodes ricinus*) in transmission of *Francisella tularensis* subsp. *holarctica* subgroup B.FTNF002-00 in European Brown Hares (*Lepus europaeus*). In preparation