Experimental infection of voles with francisella tularensis indicates their amplification role in tularemia outbreaks

Rossow, Heidi; Forbes, Kristian M; Tarkka, Eveliina; Kinnunen, Paula M; Hemmilä, Heidi; Huitu, Otso; Nikkari, Simo; Henttonen, Heikki; Kipar, Anja; Vapalahti, Olli

Abstract: Tularemia outbreaks in humans have been linked to fluctuations in rodent population density, but the mode of bacterial maintenance in nature is unclear. Here we report on an experiment to investigate the pathogenesis of Francisella tularensis infection in wild rodents, and thereby assess their potential to spread the bacterium. We infected 20 field voles (Microtus agrestis) and 12 bank voles (Myodes glareolus) with a strain of F. tularensis ssp. holarctica isolated from a human patient. Upon euthanasia or death, voles were necropsied and specimens collected for histological assessment and identification of bacteria by immunohistology and PCR. Bacterial excretion and a rapid lethal clinical course with pathological changes consistent with bacteremia and tissue necrosis were observed in infected animals. The results support a role for voles as an amplification host of F. tularensis, as excreta and, in particular, carcasses with high bacterial burden could serve as a source for environmental contamination.

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Abstract:
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Response to Reviewers:

Helsinki, July 23, 2014

Response to the decision letter
PONE-D-14-20237
Experimental infection of voles with Francisella tularensis indicates their amplification role in tularemia outbreaks

Dear Prof. Dumler,

Attached is a revised version of our manuscript “Experimental infection of voles with Francisella tularensis indicates their amplification role in tularemia outbreaks”. Thank you for the opportunity to improve and resubmit our work and to the reviewers for their thorough work and helpful comments. We have revised the manuscript according to their suggestions and replied to each comment in detail. We used the track changes mode to mark the changes in the manuscript. We hope that the revised manuscript is now suitable for publication in PLOS ONE.

Sincerely,

Heidi Rossow

Journal requirements:

1. We note that you stated “data are available upon request” at submission. Could you please confirm that all data underlying the findings in your study are freely available in the manuscript, supplemental files, or in a public repository?

RESPONSE: All data underlying the findings in our study are freely available in the manuscript.

Comprehensive data concerning materials, methods and results can be found summarized in the manuscript. In addition, we have residual formalin fixed and frozen tissue samples, histological slides from each individual and detailed PCR-results which are overly numerous and detailed and not of interest for the reader and technically impossible due to space and text limitations to attach to the manuscript. Obviously, this is thy type of detailed individual data that is “normally” not included in a manuscript. However if for some reason this is requested, it will be accessible from the authors.

Re: PONE-D-14-20237, Rossow et al.
Title: “Experimental infection of voles with Francisella tularensis indicates their amplification role in tularemia outbreaks”

Responses to Reviewer:

REVIEWER 1:

REVIEWER: 1. In own studies on rodents it could be observed that critically ill mice do not or rarely release droplets in the pre-final phase of the infection. Was stool and urine collected after natural release or collected during necropsy of the animals? The latter could be assumed, but would this be of relevance for interpretation as source of infection?

1.1. RESPONSE: All sampling was done immediately after the death of the animals, i.e. during the necropsy. It is true that the amounts of F. tularensis in droplets were low, but as F. tularensis has been previously isolated from rodent faeces released under natural conditions (Dahlstrand et al 1971, Grunow et al 2012), we believe that it could be a source of infection although surely of less relevance than the carcasses themselves. The course of infection could also be different under natural conditions.
and chronic shedding of *F. tularensis* has been reported (Bell et al 1975). We have attempted to clarify this point in the discussion section (lines 280-3).

REVIEWER: 2. Did the authors study any kinetics how long living bacteria could be isolated from stool and urine after collecting this material? This would indicate a time frame in which this contaminated material could be infectious.

1.2. RESPONSE: Unfortunately not. The sample sizes were quite small (especially for urine) and urine samples could not be obtained from all individuals. Thus we had samples for only one analysis.

REVIEWER: 3. If *F. tularensis* survives in stool and urine, what environmental conditions like temperature and humidity would conserve the viability of the pathogen?

1.3. RESPONSE: This would be a very interesting issue for future studies, but we could not address this experimentally in this study. We found one very thorough study on this (Pomanskala 1956) where *F. tularensis* has been shown to survive longest under winter conditions. This information can also be found in our manuscript in the discussion section (lines 296-300).

REVIEWER 2:

REVIEWER: 1. The authors do need to take care with their conclusion that long-term infection with Francisellia t.h. is unlikely from their experiments. There is a whole host of literature that suggest that the route of infection is critical to the virulence in voles and that ingesting the bacteria can create a less-virulent and long-term infection.

2.1. RESPONSE: Thank you for the suggestion. We have now added this point in the discussion section and added some of the suggested references (lines 280-3).

REVIEWER: 2. Due to the limited scope of the manuscript and the fact that it is well known that Francisellia t.h. kills voles, I would recommend that this paper be shortened to a short report or something similar.

2.2. RESPONSE: As none of the other reviewers nor the academic editor proposed shortening the paper, we interpreted that it is possible to keep the length as it is.

REVIEWER 3:

REVIEWER: 1. But if voles have “massive bacteremia” (page 14, line 323), could mosquitos not also become infected by feeding on voles and then transmit to humans?

3.1. RESPONSE: Thank you for pointing this. There are few species of mosquitoes that, as adults, first take a blood meal from a vole and then human. In theory, if that would happen, it could be possible that they transmit tularemia directly from voles to humans. However, we did not find evidence for that. Instead, there is evidence for developing mosquitoes picking *F. tularensis* up from the water. This is definitely an issue that should be addressed in future studies. We now have added this point to the discussion section (lines 326-8).

REVIEWER 2: The authors could also mention a bit more explicitly the possibility that environmental contamination of water/grass may -- through ingestion -- propagate the outbreak among voles.

3.2. RESPONSE: We have now added this point in the discussion section (lines 330-1).

REVIEWER 3: Figure 4 legend – I think the last two panels may be mislabeled (second “G” should be “H” and following “B” should be “I”?)

3.3. RESPONSE: This has been corrected.
| Question                      | Response                                                                                                                                                                                                 |
|-------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Financial Disclosure         | The study was partially funded by the Hospital district of Helsinki and Uusimaa (EVO TYH20113 to OV, http://www.hus.fi/Sivut/default.aspx), the Academy of Finland (grants no. 251836 to OV and 133495 to OH, http://www.aka.fi/fi/A/), and the Finnish Foundation of Veterinary Research (to HR in 2013, http://www.sels.fi/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. |
| Competing Interests          | The authors have declared that no competing interests exist.                                                                                                                                              |
If no authors have any competing interests to declare, please enter this statement in the box: "The authors have declared that no competing interests exist."

Ethics Statement

You must provide an ethics statement if your study involved human participants, specimens or tissue samples, or vertebrate animals, embryos or tissues. All information entered here should also be included in the Methods section of your manuscript. Please write "N/A" if your study does not require an ethics statement.

Human Subject Research (involved human participants and/or tissue)

All research involving human participants must have been approved by the authors' Institutional Review Board (IRB) or an equivalent committee, and all clinical investigation must have been conducted according to the principles expressed in the Declaration of Helsinki. Informed consent, written or oral, should also have been obtained from the participants. If no consent was given, the reason must be explained (e.g. the data were analyzed anonymously) and reported. The form of consent (written/oral), or reason for lack of consent, should be indicated in the Methods section of your manuscript.

Please enter the name of the IRB or Ethics Committee that approved this study in the space below. Include the approval number and/or a statement indicating approval of this research.

Animal Research (involved vertebrate animals, embryos or tissues)

All animal work must have been conducted according to relevant national and international guidelines. If your study involved non-human primates, you must provide details regarding animal welfare and steps taken to ameliorate suffering; this is in accordance with the

Experimental procedures and facilities were approved by the Finnish Animal Experiment Board (Permit ESAVI/6162/04.10.03/2012), which followed the Finnish legislation for animal experiments. Rodents in this experiment were euthanized via cervical dislocation under isoflurane anesthesia. All efforts were made to minimize animal suffering.
recommendations of the Weatherall report, "The use of non-human primates in research." The relevant guidelines followed and the committee that approved the study should be identified in the ethics statement.

If anesthesia, euthanasia or any kind of animal sacrifice is part of the study, please include briefly in your statement which substances and/or methods were applied.

Please enter the name of your Institutional Animal Care and Use Committee (IACUC) or other relevant ethics board, and indicate whether they approved this research or granted a formal waiver of ethical approval. Also include an approval number if one was obtained.

**Field Permit**

Please indicate the name of the institution or the relevant body that granted permission.

**Data Availability**

PLOS journals require authors to make all data underlying the findings described in their manuscript fully available, without restriction and from the time of publication, with only rare exceptions to address legal and ethical concerns (see the PLOS Data Policy and FAQ for further details). When submitting a manuscript, authors must provide a Data Availability Statement that describes where the data underlying their manuscript can be found.

Your answers to the following constitute your statement about data availability and will be included with the article in the event of publication. **Please note that simply stating ‘data available on request from the author’ is not acceptable. If, however, your data are only available upon request from the author(s), you must answer "No" to the first question below, and explain your exceptional situation in the text box provided.**

Do the authors confirm that all data underlying the findings described in their manuscript are fully available without restriction?

| Yes - all data are fully available without restriction | All data underlying the findings in your study are freely available in the manuscript |
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If your data are all contained within the paper and/or Supporting Information files, please state this in your answer below. For example, “All relevant data are within the paper and its Supporting Information files.”

If your data are held or will be held in a public repository, include URLs, accession numbers or DOIs. For example, “All XXX files are available from the XXX database (accession number(s) XXX, XXX).” If this information will only be available after acceptance, please indicate this by ticking the box below. If neither of these applies but you are able to provide details of access elsewhere, with or without limitations, please do so in the box below. For example:

“Data are available from the XXX Institutional Data Access / Ethics Committee for researchers who meet the criteria for access to confidential data.”

“Data are from the XXX study whose authors may be contacted at XXX.”

* typeset

Additional data availability information:
Helsinki, 6 Mai 2014

Dear Editor,

Our original research article reports an experimental infection study to evaluate the clinical course and the pathogenesis of infection, as well as the pathogenicity of *Francisella tularensis*, the causative agent of human tularemia, in wild rodents.

Tularemia is a severe zoonotic disease, which is endemic in large regions of Northern Europe. Finland, in particular, has the highest incidence of human tularemia of all EU countries. In other parts of Europe, it is considered emerging, and several recent human disease outbreaks have been reported.

High rodent densities have been linked to tularemia outbreaks in humans, and exposure to rodents or their droppings is suspected as the source of infection for large outbreaks. However, the precise role of rodents in bacterial maintenance, and the nature of their association with human disease - transmitted mainly through mosquito bites in Fennoscandia - remain unclear. The purpose of our experiment was to study the infection in the dominant wild rodent species in Finland, field voles and bank voles, as a means for understanding their association with human disease.

The current experiment demonstrates that both field voles and bank voles are highly susceptible to the bacterium. Bacterial excretion and a rapid lethal clinical course with pathological changes consistent with bacteremia and severe tissue necrosis were observed in infected animals. These findings indicate that naturally infected dead voles can lead to heavy contamination of the environment and provide an explanation for the common association between rodent density and human tularemia incidence.

Despite the substantial public health importance of tularemia, limited data is available on the ecology of *Francisella tularensis*. As such, our results provide novel insights into the maintenance of *Francisella tularensis* in nature and the processes leading to human outbreaks.

Experts in the field who could potentially serve as reviewers of this work include:

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PLOS ONE Academic Editors to handle this manuscript could be: Martin Beer, Lloyd Vaughan and Yousef Abu-Kwaik. The abstract is 150 words, the main manuscript 3364 words and there are 40 references. All authors have approved the final version of the manuscript. On behalf of all authors, I would like to thank you for your considering our manuscript for publication in PLOS ONE.

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Experimental infection of voles with *Francisella tularensis* indicates their amplification role in tularemia outbreaks

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**Running title:** Experimental tularemia in voles

**Key words:** Experimental infection—*Francisella tularensis*—Pathology—Rodents—Tularemia—Voles

**Article Summary Line:** We used experimental infection of wild voles with *Francisella tularensis* to demonstrate their likely role as amplification hosts in human tularemia outbreaks.

**Abstract**

Tularemia outbreaks in humans have been linked to fluctuations in rodent population density, but the mode of bacterial maintenance in nature is unclear. Here we report on an experiment to investigate the pathogenesis of *Francisella tularensis* infection in wild rodents, and thereby assess their potential to spread the bacterium. We infected 20 field voles (*Microtus agrestis*) and 12 bank voles (*Myodes glareolus*) with a strain of *F. tularensis* ssp. *holarctica* isolated from a human patient. Upon euthanasia or death, voles were necropsied and specimens collected for histological assessment and identification of bacteria by immunohistology and PCR. Bacterial excretion and a rapid lethal clinical course with pathological changes consistent with bacteremia and tissue necrosis were observed in infected animals. The results support a role for voles as an amplification host of *F. tularensis.*
as excreta and, in particular, carcasses with high bacterial burden could serve as a source for environmental contamination.

**Introduction**

*Francisella tularensis* is a zoonotic intracellular bacterium that belongs to the $\gamma$-subclass of *Proteobacteria* (1, 2). Two *F. tularensis* subspecies cause clinical infections in humans: *F. tularensis* subsp. *tularensis* (type A), which is almost exclusively found in North America, and *F. tularensis* subsp. *holarctica* (type B), which occurs throughout the Holarctic region (3). In Finland, dozens to several hundreds of human tularemia cases are registered each year, and incidence rates show marked geographical variation between districts (4). From 1996 to 2004, the cumulative incidence of human tularemia in Finland was over 37 cases/100,000 inhabitants, which is the highest of all EU member states (5). Meanwhile, a series of outbreaks has demonstrated the re-emergence of this disease in other European countries (6-8).

*F. tularensis* is renowned for its high infectivity and wide host range. The infectious dose for humans can be as low as 10 bacteria (9), and the bacterium has been isolated from numerous mammalian species, including rabbits, hares, voles and other rodents (10-13), and detected from natural waters and mud, and from mosquito larvae collected in endemic areas (14, 15). It is very likely that *F. tularensis* persists in natural waters, possibly in aquatic protozoa (16).

Humans become infected with *F. tularensis* through arthropod bites, direct contact with infected animals, inhalation of infective aerosols, or ingestion of contaminated food or water (4, 9). Clinical manifestations depend mainly on the infection route, and the
disease severity depends on the infecting subspecies and strain (17). After an incubation period of approximately 3-5 days (range: 1-14 days), non-specific influenza-like symptoms, especially fever, chills and headache, arise usually with rapid onset (2, 9, 17, 18). Infection through the skin results in ulceroglandular tularemia, while infection via the mucous membranes induces ulceroglandular, glandular, oculoglandular, or oropharyngeal tularemia (2, 17). In Fennoscandia, where the bacterium is transmitted mainly through mosquito bites (4, 19), the ulceroglandular form is most common (4). Inhalation of aerosolized *F. tularensis* causes pulmonary tularemia, the most severe form of the disease (20-22).

Tularemia outbreaks in humans have been linked to high rodent densities (7, 18, 23-25), and exposure to rodents or their droppings is suspected as the infection source in a large outbreak in Kosovo (24, 25). However, the precise role of rodents in bacterial maintenance, and the nature of their association with human disease has remained unclear. In Finland, the field vole (*Microtus agrestis*) and bank vole (*Myodes glareolus*) are the dominant rodent species (26), and hence the most plausible hosts for *F. tularensis*. Indeed, we have recently detected the bacterium in screening of wild field voles in Finland (27). Here we report on an experiment to evaluate the pathogenicity of *F. tularensis* for these species, in order to further elucidate factors affecting their association with human disease outbreaks.

**Materials and Methods**

**Ethics**

Experimental procedures and facilities were approved by the Finnish Animal Experiment Board (Permit ESAVI/6162/04.10.03/2012), which followed the Finnish legislation for animal experiments. All efforts were made to minimize animal suffering.
Naturally infected animals

Three naturally *F. tularensis*-infected, PCR-positive adult field voles, trapped as part of a screening project in the Konnevesi area in Central Finland (27), were evaluated for the presence of bacteria in tissues and associated pathological changes as a reference for the experimental infection study. Tissue specimens from lungs, liver and kidneys were collected from these animals and frozen at -20°C. Samples were later thawed and fixed in 10% buffered formalin for histopathological and immunohistological examination.

Animals for experimental infections

The experimental infections were conducted on visibly healthy adult (> 8 weeks of age) field and bank voles. These animals were laboratory-born at the Finnish Forest Research Institute, Suonenjoki station, and were the progeny of wild voles captured in the surrounding area.

For the experimental infections, voles were transferred to the biosafety level 3 laboratory of the Faculty of Veterinary Medicine, University of Helsinki, Finland, where they were housed in individually ventilated and HEPA-filtered isolation cages (Isocage Unit, Tecniplast, Italy). Wood shavings covered the cage floor, and a cardboard roll was supplied for additional cover. Water and rodent pellets (22.5 % crude protein, 5 % crude fat, 4.5 % crude fiber and 6.5 % crude ash) were supplied *ad libitum*, and voles were given a slice of fresh apple every 1-2 days. Voles were placed into the cages three days prior to experimental infections.
Bacteriology

A strain of *F. tularensis*, which had originally been isolated from a cutaneous ulcer of a 49-year-old woman, identified as ssp. *holarctica* by 16S rRNA gene sequencing, was used for the experimental infections. Bacteria were cultured on chocolate agar plates and incubated at +35 °C in 5% CO₂ for five days. MacFarland 1.0 suspension was prepared in sterile isotonic saline and diluted in ten-fold series to approximately 1000 colony-forming units (cfu)/ml. The actual concentration was determined by plate counting in each experiment. The diluted suspension was kept on ice and used for inoculations within 1-2 h of preparation. The viable count of *F. tularensis* in the remaining dilution was similar to that of the fresh dilution.

Experimental infections

Pilot study

A pilot study was conducted to identify a bacterial delivery route and dose that best mimics natural infections in voles, and to gather information on the incubation period and clinical course of infection. For this, two field voles were allocated to each of 4 dose/route combinations (total n = 8): either 120 (low dose) or 1,200 (high dose) cfu of *Francisella tularensis* ssp. *holarctica* (diluted in 100 µl of sterile isotonic saline), and either intranasal (i.n.) or subcutaneous (s.c.) delivery route. Experimental infections were conducted under brief isoflurane anesthesia, and s.c. injections were delivered between the shoulder blades. One further vole served as an uninfected control. The animals were checked twice daily for signs of illness or death, and immediately euthanized if they exhibited signs of illness. After 9 days, all remaining voles were euthanized via cervical dislocation under isoflurane.
anesthesia. A full post mortem examination was performed immediately after death or when the voles were found dead, and samples from the spleen, lung, liver, and kidney were aseptically collected and frozen at -80°C for PCR analysis. In addition, samples of heart, lungs, liver, kidneys, spleen, mesenteric and mediastinal lymph nodes, brain, and inoculation sites (skin, nose) were fixed in 10% buffered formalin, for histological and immunohistological assessment.

Main study

For the main experiment, 12 field voles and 12 bank voles were injected s.c. with a 100 µl suspension containing 70 cfu of F. tularensis ssp. holarctica in sterile isotonic saline. Three randomly selected animals of each species served as non-infected controls and were injected with 100 µl sterile isotonic saline alone. Voles were checked twice daily for signs of illness and death. Three infected voles of each species were electively euthanized on days 1 and 3 post infection (p.i). The remaining voles were euthanized by cervical dislocation under isoflurane anesthesia if symptomatic. Animals were necropsied immediately after death, and urine, feces, spleen, and kidney samples were aseptically collected and frozen at -80°C for PCR analysis. Tissue specimens from lungs, liver, spleen, bone marrow, kidneys, stomach, duodenum, jejunum, colon, and the inoculation site were fixed in 10% buffered formalin for histological and immunohistological assessment.

Histology and immunohistology

Fornalin-fixed tissue specimens from all animals were trimmed and routinely paraffin wax embedded. Sections (3-5 µm) were prepared and stained with hematoxylin-eosin (HE) or used for immunohistology (IH). IH was performed using a mouse monoclonal
antibody against *F. tularensis* LPS (clone T14; Meridian Life Sciences, Memphis, USA) and
the horseradish peroxidase method (Envision; Dako, Glostrup, Denmark) with
diaminobenzidine as chromogen, after antigen retrieval with citrate buffer (pH 6.0)
microwave pretreatment.

**DNA extraction and PCR analyses**

DNA was extracted from vole tissue samples and excreta using commercial kits. The Wizard Genomic DNA Purification Kit (Promega, Madison, USA) was used for spleen and kidney samples, following the protocol for animal tissue. The QIAamp DNA Stool kit (Qiagen, Hilden, Germany) was employed for fecal samples (20 mg feces + 160 µl phosphate-buffered saline). From urine samples (24.5 -140 µl), DNA was extracted with the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany), using the protocol for purification of cellular, bacterial or viral DNA from urine. Each sample batch contained water as a negative control. DNA concentration and purity were determined with the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

The DNA samples were subjected to a modified semi-quantitative real-time PCR assay (qPCR) targeting the 23kDa gene of *F. tularensis* (27, 28). All PCRs were run in duplicate with an ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA). DNA from tissue samples was analyzed using 1:100 dilutions, and for urine and fecal samples, three 10-fold (undiluted, 1:10, 1:100) dilutions were examined. The PCR assay included an internal positive inhibition control, water as negative non-template control, and *F. tularensis* LVS control strain DNA as positive control. The amount of *F. tularensis* bacteria in each sample was estimated based on genomic equivalents (GE). To enable comparison of *F.
tularensis amounts in tissues of experimentally versus naturally infected voles, we also calculated the GE amount in relation to the estimated number of cells in tissue samples (27).

Results

Naturally infected wild field voles

In the three F. tularensis-infected wild field voles (27), bacteremia was confirmed by histology and immunohistology. Bacteria were found as aggregates within vessels and capillaries, specifically also in liver sinusoids and renal glomerular capillaries. They were abundant in the splenic red pulp where they were associated with extensive necrosis (Figure 1). In addition, bacteria were identified within macrophages in the liver (i.e. Kupffer cells: Figure 1B) and the splenic red pulp. In the livers, individual necrotic hepatocytes were also seen.

Pilot study in field voles

A pilot study was conducted on field voles to evaluate different infection routes (s.c. and i.n.) and doses (high and low dose). All voles remained asymptomatic during the first four days after infection. On day 5 p.i., four infected voles (two low dose s.c., one high dose i.n., and one high dose s.c.) were found dead, and another animal (high dose s.c.) was euthanized due to general malaise. On day 6 p.i., one symptomatic vole (high dose i.n.) was euthanized. Both low dose i.n. infected voles survived until day 9 p.i., when one was found dead and the other, which had remained asymptomatic, as well as the uninfected control animal, were electively euthanized at the scheduled end of the experiment.

The post mortem examination did not reveal any significant gross changes. Histology confirmed severe bacteremia in all but the electively euthanized low dose i.n.
infected vole and the control animal, with bacterial aggregates in vessels in all examined organs and in the cardiac chambers. The pathological changes were very similar to those seen in the naturally infected voles and are typical for tularemia in other species (10, 13), such as extensive splenic and lymph node necrosis with abundant cell-free bacteria (Figure 2A-D).

Two i.n. infected voles (one high dose and one low dose) also showed a multifocal extensive necrotizing pneumonia with abundant bacteria both cell-free and in macrophages (Figure 2E, F), features not seen in the naturally infected voles. This indicates direct aerosol infection of the lung and subsequent bacteremia, in particular since the animals exhibited neither histological changes nor bacteria in the nasal cavity. Bacterial loads in organs did not substantially vary in relation to the route and dose of infection and were generally high in all tissues of symptomatic animals. In the infected vole that had remained asymptomatic, systemic infection was confirmed by PCR, albeit with low bacterial organ loads and without any histological changes or IH evidence of bacteria in any examined tissue.

Main experimental study in field and bank voles

For the main study, a low dose delivered via s.c. injection was chosen, as the pilot study demonstrated it to best mimic the natural infection in voles. All animals that were sacrificed on days 1 and 3 p.i. (three field voles and three bank voles at each time point) had been asymptomatic and did not exhibit any significant gross changes. On day 1 p.i., PCR did not detect *F. tularensis* DNA in spleen, kidney, feces or urine (Figure 3), and IH did not identify bacteria in any tissue (Table 1). Histological changes were restricted to the injection site, where focal interstitial hemorrhage was generally seen. In one bank vole a focal macrophage aggregate was found in the adipose tissue of the inoculation site, and IH identified a few bacteria within the macrophages.
On day 3 p.i., a neutrophil-dominated inflammatory reaction with intracellular (macrophages, neutrophils) and cell-free bacteria was often seen at the inoculation site (Figure 4A). The spleen of all animals tested positive for *F. tularensis* DNA (Figure 3), and in all but one weakly PCR-positive spleen, IH identified variable amounts of bacteria within macrophages in the red pulp (Figure 4B, Table 1), confirming cell associated bacteremia. This was not associated with distinct histological changes in the spleen. In two bank voles, the kidney was weakly PCR-positive, and IH identified some bacteria in glomerular capillaries, without other histological changes. The urine of both these animals was PCR-negative. IH also identified bacteria in the livers, as individual cells in sinuses, and identified patches of reactive hepatocytes. Some bacteria were found in capillaries in the lungs of the three bank voles, again without distinct histopathologic changes.

On day 4 p.i., one field vole displayed general malaise and was euthanized, and on day 5 p.i., the remaining 5 field voles and 6 bank voles died or were visibly symptomatic and euthanized. PCR demonstrated *F. tularensis* DNA in the urine and high *F. tularensis* loads in the spleens and kidneys of all animals (Figure 3); in voles euthanized on day 5 p.i., *F. tularensis* was also identified in feces (Figure 3, Table). Histology and IH confirmed these results and revealed features similar to those in the pilot study. The findings were similar in both species. In general, large bacterial aggregates were seen in the splenic red pulp and in capillaries in all examined organs. In the kidneys, bacteria were found in both glomerular and interstitial capillaries (Figure 4C). Apart from disseminated bacterial aggregates between hepatic cords, the liver carried bacteria within Kupffer cells and exhibited multifocal random hepatocellular necrosis (Figure 4D).

In the spleen, the red pulp was almost completely effaced due to necrosis (and loss) of cells, and the white pulp was markedly reduced, with extensive (follicular) apoptosis/necrosis and replacement by bacteria. Lymph nodes exhibited focal areas of
necrosis with abundant bacteria (Figure 4E). In the bone marrow, bacteria were found within mononuclear cells (most consistent with macrophages) and sometimes cell free (Figure 4F), and there was extensive necrosis/apoptosis of myelopoietic cells. Examination of the gastrointestinal tract identified bacteria within capillaries in all compartments, within Peyer’s patches (Figure 4G) and occasionally also in intestinal epithelial cells in both the small and large intestine (Figure 4H, I). Some small macrophage aggregates with bacteria were found in the lamina propria mucosae. More extensive inflammatory infiltrates were restricted to inoculation sites, where variably extensive necrosis and neutrophil infiltration with masses of cell free bacteria was seen.

Control voles remained asymptomatic and were euthanized on day 9, at the scheduled end of the experiment. They were negative for *F. tularensis* by PCR and IH and did not exhibit any histological changes.

**Discussion**

The current study presents an experimental model that mimics natural *F. tularensis* ssp. *holarctica* infection of wild voles and demonstrates that both field voles and bank voles are highly susceptible to the bacterium. Infected animals died with bacteremia, following a rapid clinical course and generally with very high bacterial loads in organs. We showed that infected voles excrete *F. tularensis* in their urine and feces around the time of death. The bacterial burden in excreta was relatively low compared to the bacterial load in tissues, but since only a low dose is generally required for infection (9), feces and urine might be infective for other animals and humans. Furthermore, the course of infection could be different under natural conditions. Long-term infections and shedding of *F. tularensis* have been reported after oral infection (29, 30) and the oral route of infection should be studied in future. The presence of bacterial aggregates within the glomerular tufts in the kidneys and
within mucosal vessels and between epithelial cells in the intestinal mucosa of animals by day 5 p.i. also indicates that *F. tularensis* is excreted in urine and feces at this stage. Excretion of *F. tularensis*, in addition to contamination from dead animals, might serve to transfer the bacteria into the environment, which could also include mosquito breeding sites. In support of this premise, *F. tularensis* has been demonstrated to survive in water for several weeks (31, 32). The survival is supported by protozoa, which are commonly found in natural aquatic systems as part of their normal biofilms (16).

Outbreaks of airborne tularemia in humans are mainly linked to farm work and other outdoor activities (6, 8, 12, 22, 33, 34), for example exposure to hay dust has been associated with pneumonic tularemia (4). This might be due to bacteria-containing aerosols originating from animal carcasses or excreta made airborne by agricultural machines. Similarly, Puumala hantavirus infection is acquired by inhalation from rodent excreta, and considerably more often by farmers (35). *F. tularensis* has been shown to survive up to 192 days in the environment on straw and grain depending on the temperature of the surrounding air (36). Survival is longest in winter conditions, as the amount of viable bacteria decreases with rising temperatures (36). The enhanced survival of *F. tularensis* in cool temperatures might be one factor contributing to the high tularemia incidence in Fennoscandia.

Our analysis of the pathogenesis of tularemia indicates that the bacteria are taken up locally (i.e. at the inoculation site) by macrophages and neutrophils and then distributed throughout the body, to eventually accumulate in the blood. Accordingly, they were found both within monocytes and cell free in vessels of almost all organs, and led to necrosis of infected cells, resulting in extensive necrosis particularly in the lymphatic tissues (i.e. spleen and lymph nodes). Interestingly, apart from the inoculation site, this was not associated with an overt inflammatory response. Similar changes have been reported in hares, in which tularemia is mainly characterized by acute focal necrosis without cellular reaction in liver,
spleen, and bone marrow (10). Recently, *F. tularensis* infection even without lesions has been described in squirrels (37). In our pilot study, two intranasally infected voles exhibited a necrotising to granulomatous pneumonia, indicating direct infection of the lung (not via bacteremia). This kind of prominent change is typical for inhalational tularemia; severe necrotizing pneumonia has been demonstrated in monkeys (38) and mice (39) after *F. tularensis* spp. *tularensis* aerosol exposure. Necrotizing granulomatous inflammation is also seen in lung biopsies of human patients with pulmonary tularemia (40, 41).

In Fennoscandia, tularemia is primarily mosquito-transmitted, and large human outbreaks occur regularly (4, 19). Mosquitoes have been shown experimentally to become persistently infected already as larvae and then transtadiably through the developmental stages to adults, without however evidence of *F. tularensis* replication (42). It has been shown that *F. tularensis* multiplies in protozoa (16), but mammals are probably also needed, as local amplifiers to facilitate the spread of the disease (42) e.g. through contaminated water and subsequently mosquitoes. In Sweden, a temporal link between outbreaks in humans and rodent density cycles has been reported during 1960s and 1970s (23). Moreover, our recent survey of wild rodent species identified *F. tularensis* in wild field voles (27), and we show here that the massive bacteremia and pathological lesions after experimental infection are identical to those in naturally infected animals. Mosquitoes might also become infected by feeding on bacteremic voles and then perhaps directly transmit *F. tularensis* to humans – however, there is no data to support this. It is also possible that *F. tularensis*, amongst other things, contributes to the density crash of vole populations in certain areas, at which stage *F. tularensis* is released into the environment. This environmental contamination presumably also propagates the outbreak among voles. As our results show, infected dead voles can lead to heavy contamination of the environment and provide an explanation for the common association between rodent density and human tularemia incidence.
In summary, the fact that voles readily developed lethal tularemia, together with the severity and similarity of the lesions in both experimentally and naturally infected animals, suggest that long-term or latent infection of these species is unlikely, yet some reservation concerning the infection routes may be warranted. Instead, voles are likely to play a role as amplification hosts and lead to bacterial contamination of the local environment, and by this mechanism contribute to the incidence of human tularemia.

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**Figure Legends**

**Figure 1.** Naturally *F. tularensis* sp. *holartica* infected field vole that had been trapped and euthanized (27). A. Lung with bacterial clumps in vessel lumina (arrow) and bacterial aggregates in capillaries (arrowhead). B. Liver with bacterial clumps in sinusoids (arrow) and smaller aggregates within Kupffer cells (arrowheads). C. Kidney with bacterial aggregates in
larger vessels (arrow) and glomerular capillaries (arrowheads). **D.** Spleen with abundant bacterial clumps, in association with necrosis (N), in the red pulp. Horseradish peroxidase method, Papanicolaou’s hematoxylin counterstain. Bars = 20 µm.

**Figure 2.** Pilot study, field voles that had died from or were euthanized after experimental *F. tularensis sp. holartica* infection. **A, B.** Spleen from an animal found dead on day 5 after subcutaneous (s.c.) infection with a low dose (LD). Clumps of bacteria are found cell-free and within (degenerating) macrophages in the red pulp, often surrounding follicles (F). Numerous lymphocytes in follicles undergo apoptosis (A: arrowhead). **C, D.** Mesenteric lymph node from an animal found dead on day 5 after s.c. infection with a high dose (HD). **C.** Cortex with focal area of necrosis (arrow). **D.** Clumps of bacteria are present within the sinuses (arrow) and in areas of necrosis (arrowhead). **E, F.** Lung from a vole found dead on day 9 after HD intranasal infection. Focal area of extensive necrosis with large aggregates of bacteria (arrows). Inset in F: Lung from a vole that was euthanized on day 5 after HD s.c. infection. Bacteria are found within circulating leukocytes in lung capillaries. **A, C, E.** HE stain, B, D, F. Horseradish peroxidase method, Papanicolaou’s hematoxylin counterstain. Bars = 20 µm (A-D, F), 50 µm (E), 10 µm (Inset F).

**Figure 3.** Quantification of *F. tularensis* DNA in spleen, kidney, faces and urine of infected voles by day post infection determined using real-time PCR targeting the bacterial 23 kDa gene of *F. tularensis* (27, 28). Samples were collected at the following time points: day 1 post infection (p.i.), day 3 p.i. and days 4-5 p.i.
Figure 4. Main study, voles after subcutaneous infection with *F. tularensis* sp. holartica. A, B. Day 3 post infection (p.i.). A. Field vole, injection site in the subcutis with moderate pyogranulomatous inflammation. Bacteria are present within leukocytes (arrows) and as cell free aggregates (arrowhead). B. Bank vole, spleen. In the red pulp, in particular surrounding follicles (F) and T cell zones, are aggregates of macrophages with intracellular bacteria (arrows). C-I. Day 5 post infection. C. Field vole, kidney. Bacteria form clumps in the lumen of interstitial veins (arrows) and glomerular tufts (arrowhead). D. Field vole, liver. Bacteria form aggregates within sinusoids (arrows) and are present within Kupffer cells (arrowheads). E. Field vole, mesenteric lymph node and large artery (A). Cell free bacteria fill the lumen of the artery and are present within necrotic areas in the lymph node (arrow). F. Bank vole, bone marrow. Bacteria are mainly found within mononuclear (myeloid) cells (arrowhead). G. Field vole, duodenum with Peyer’s patch, exhibiting bacteria within cells and cell-free, also towards the mucosal surface (arrowhead). H. Field vole, jejunum. Bacterial aggregates fill capillaries (arrow) and are present within cells, also in the lamina epithelialis mucosae (arrowhead). I. Bank vole, colon. Bacterial aggregates fill capillaries (arrow) and are present within cells, also in the lamina epithelialis mucosae (arrowhead). Horseradish peroxidase method, Papanicolaou’s hematoxylin counterstain. Bars = 20 µm (A-D, G), 50 µm (E), 10 µm (F, H, I).

Table Detection of *Francisella tularensis* in organs and excretions of experimentally infected voles

| Species and specimens | Day 1 | Day 3 | Day 4 | Day 5 |
|-----------------------|-------|-------|-------|-------|
|                       | PCR   | IH    | PCR   | IH    |
|                       | PCR   | IH    | PCR   | IH    |

No. PCR\(^a\)- and IH\(^b\)-positive/ No. examined
**Microtus agrestis**, field vole

|                | Spleen | Kidney | Feces | Urine |
|----------------|--------|--------|-------|-------|
|                | 0/3    | 0/3    | 0/3   | NA    |
|                | 3/3    | 0/3    | 0/1   | NA    |
|                | 1/2    | 1/1    | 5/5   |       |
|                | 1/1    | 1/1    | 5/5   | 5/5   |

**Myodes glareolus**, bank vole

|                | Spleen | Kidney | Feces | Urine |
|----------------|--------|--------|-------|-------|
|                | 0/3    | 0/3    | 0/3   | NA    |
|                | 3/3    | 2/3    | 0/3   | NA    |
|                | 3/3    | 2/3    | 6/6   |       |
|                | 6/6    | 6/6    |       | 6/6   |

\(^a\)real-time PCR targeting the bacterial 23 kDa gene of *F. tularensis* (27, 28)

\(^b\)IH, Immunohistology using a mouse monoclonal antibody against *F. tularensis* lipopolysaccharide (clone T14; IgG3)

No, number; PCR, polymerase chain reaction; IH, immunohistology; NA, not available

Denominators represent the total amount of screened animals.
Figure 3
Click here to download Figure: Figure_main_experiment_PCR.pdf

Average value (GE/mg tissue or mg stool or µl urine)

Field vole
Bank vole
Day pi 1
Day pi 3
Day pi 4 - 5
Experimental infection of voles with *Francisella tularensis* indicates their amplification role in tularemia outbreaks

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Running title: Experimental tularemia in voles

Key words: Experimental infection—Francisella tularensis—Pathology—Rodents—Tularemia—Voles

Article Summary Line: We used experimental infection of wild voles with Francisella tularensis to demonstrate their likely role as amplification hosts in human tularemia outbreaks.

Abstract

Tularemia outbreaks in humans have been linked to fluctuations in rodent population density, but the mode of bacterial maintenance in nature is unclear. Here we report on an experiment to investigate the pathogenesis of Francisella tularensis infection in wild rodents, and thereby assess their potential to spread the bacterium. We infected 20 field voles (Microtus agrestis) and 12 bank voles (Myodes glareolus) with a strain of F. tularensis ssp. holarctica isolated from a human patient. Upon euthanasia or death, voles were necropsied and specimens collected for histological assessment and identification of bacteria by immunohistology and PCR. Bacterial excretion and a rapid lethal clinical course with pathological changes consistent with bacteremia and tissue necrosis were observed in infected animals. The results support a role for voles as an amplification host of F. tularensis.
as excreta and, in particular, carcasses with high bacterial burden could serve as a source for environmental contamination.

Introduction

*Francisella tularensis* is a zoonotic intracellular bacterium that belongs to the \( \gamma \)-subclass of *Proteobacteria* (1, 2). Two *F. tularensis* subspecies cause clinical infections in humans: *F. tularensis* subsp. *tularensis* (type A), which is almost exclusively found in North America, and *F. tularensis* subsp. *holarctica* (type B), which occurs throughout the Holarctic region (3). In Finland, dozens to several hundreds of human tularemia cases are registered each year, and incidence rates show marked geographical variation between districts (4). From 1996 to 2004, the cumulative incidence of human tularemia in Finland was over 37 cases/100,000 inhabitants, which is the highest of all EU member states (5). Meanwhile, a series of outbreaks has demonstrated the re-emergence of this disease in other European countries (6-8).

*F. tularensis* is renowned for its high infectivity and wide host range. The infectious dose for humans can be as low as 10 bacteria (9), and the bacterium has been isolated from numerous mammalian species, including rabbits, hares, voles and other rodents (10-13), and detected from natural waters and mud, and from mosquito larvae collected in endemic areas (14, 15). It is very likely that *F. tularensis* persists in natural waters, possibly in aquatic protozoa (16).

Humans become infected with *F. tularensis* through arthropod bites, direct contact with infected animals, inhalation of infective aerosols, or ingestion of contaminated food or water (4, 9). Clinical manifestations depend mainly on the infection route, and the
disease severity depends on the infecting subspecies and strain (17). After an incubation period of approximately 3-5 days (range: 1-14 days), non-specific influenza-like symptoms, especially fever, chills and headache, arise usually with rapid onset (2, 9, 17, 18). Infection through the skin results in ulceroglandular tularemia, while infection via the mucous membranes induces ulceroglandular, glandular, oculoglandular, or oropharyngeal tularemia (2, 17). In Fennoscandia, where the bacterium is transmitted mainly through mosquito bites (4, 19), the ulceroglandular form is most common (4). Inhalation of aerosolized *F. tularensis* causes pulmonary tularemia, the most severe form of the disease (20-22).

Tularemia outbreaks in humans have been linked to high rodent densities (7, 18, 23-25), and exposure to rodents or their droppings is suspected as the infection source in a large outbreak in Kosovo (24, 25). However, the precise role of rodents in bacterial maintenance, and the nature of their association with human disease has remained unclear. In Finland, the field vole (*Microtus agrestis*) and bank vole (*Myodes glareolus*) are the dominant rodent species (26), and hence the most plausible hosts for *F. tularensis*. Indeed, we have recently detected the bacterium in screening of wild field voles in Finland (27). Here we report on an experiment to evaluate the pathogenicity of *F. tularensis* for these species, in order to further elucidate factors affecting their association with human disease outbreaks.

**Materials and Methods**

**Ethics**

Experimental procedures and facilities were approved by the Finnish Animal Experiment Board (Permit ESAVI/6162/04.10.03/2012), which followed the Finnish legislation for animal experiments. All efforts were made to minimize animal suffering.
Naturally infected animals

Three naturally F. tularensis-infected, PCR-positive adult field voles, trapped as part of a screening project in the Konnevesi area in Central Finland (27), were evaluated for the presence of bacteria in tissues and associated pathological changes as a reference for the experimental infection study. Tissue specimens from lungs, liver and kidneys were collected from these animals and frozen at -20°C. Samples were later thawed and fixed in 10% buffered formalin for histopathological and immunohistological examination.

Animals for experimental infections

The experimental infections were conducted on visibly healthy adult (> 8 weeks of age) field and bank voles. These animals were laboratory-born at the Finnish Forest Research Institute, Suonenjoki station, and were the progeny of wild voles captured in the surrounding area.

For the experimental infections, voles were transferred to the biosafety level 3 laboratory of the Faculty of Veterinary Medicine, University of Helsinki, Finland, where they were housed in individually ventilated and HEPA-filtered isolation cages (Isocage Unit, Tecniplast, Italy). Wood shavings covered the cage floor, and a cardboard roll was supplied for additional cover. Water and rodent pellets (22.5 % crude protein, 5 % crude fat, 4.5 % crude fiber and 6.5 % crude ash) were supplied ad libitum, and voles were given a slice of fresh apple every 1-2 days. Voles were placed into the cages three days prior to experimental infections.
Bacteriology

A strain of *F. tularensis*, which had originally been isolated from a cutaneous ulcer of a 49-year-old woman, identified as ssp. *holarctica* by 16S rRNA gene sequencing, was used for the experimental infections. Bacteria were cultured on chocolate agar plates and incubated at +35°C in 5% CO₂ for five days. MacFarland 1.0 suspension was prepared in sterile isotonic saline and diluted in ten-fold series to approximately 1000 colony-forming units (cfu)/ml. The actual concentration was determined by plate counting in each experiment. The diluted suspension was kept on ice and used for inoculations within 1-2 h of preparation. The viable count of *F. tularensis* in the remaining dilution was similar to that of the fresh dilution.

Experimental infections

Pilot study

A pilot study was conducted to identify a bacterial delivery route and dose that best mimics natural infections in voles, and to gather information on the incubation period and clinical course of infection. For this, two field voles were allocated to each of 4 dose/route combinations (total n = 8): either 120 (low dose) or 1,200 (high dose) cfu of *Francisella tularensis* ssp. *holarctica* (diluted in 100 µl of sterile isotonic saline), and either intranasal (i.n.) or subcutaneous (s.c.) delivery route. Experimental infections were conducted under brief isoflurane anesthesia, and s.c. injections were delivered between the shoulder blades. One further vole served as an uninfected control. The animals were checked twice daily for signs of illness or death, and immediately euthanized if they exhibited signs of illness. After 9 days, all remaining voles were euthanized via cervical dislocation under isoflurane
anesthesia. A full post mortem examination was performed immediately after death or when the voles were found dead, and samples from the spleen, lung, liver, and kidney were aseptically collected and frozen at -80°C for PCR analysis. In addition, samples of heart, lungs, liver, kidneys, spleen, mesenteric and mediastinal lymph nodes, brain, and inoculation sites (skin, nose) were fixed in 10% buffered formalin, for histological and immunohistological assessment.

Main study

For the main experiment, 12 field voles and 12 bank voles were injected s.c. with a 100 µl suspension containing 70 cfu of *F. tularensis* ssp. *holarctica* in sterile isotonic saline. Three randomly selected animals of each species served as non-infected controls and were injected with 100 µl sterile isotonic saline alone. Voles were checked twice daily for signs of illness and death. Three infected voles of each species were electively euthanized on days 1 and 3 post infection (p.i). The remaining voles were euthanized by cervical dislocation under isoflurane anesthesia if symptomatic. Animals were necropsied immediately after death, and urine, feces, spleen, and kidney samples were aseptically collected and frozen at -80°C for PCR analysis. Tissue specimens from lungs, liver, spleen, bone marrow, kidneys, stomach, duodenum, jejunum, colon, and the inoculation site were fixed in 10% buffered formalin for histological and immunohistological assessment.

Histology and immunohistology

Formalin-fixed tissue specimens from all animals were trimmed and routinely paraffin wax embedded. Sections (3-5 µm) were prepared and stained with hematoxylin-eosin (HE) or used for immunohistology (IH). IH was performed using a mouse monoclonal
antibody against *F. tularensis* LPS (clone T14; Meridian Life Sciences, Memphis, USA) and
the horseradish peroxidase method (Envision; Dako, Glostrup, Denmark) with
diaminobenzidine as chromogen, after antigen retrieval with citrate buffer (pH 6.0)
microwave pretreatment.

**DNA extraction and PCR analyses**

DNA was extracted from vole tissue samples and excreta using commercial kits.
The Wizard Genomic DNA Purification Kit (Promega, Madison, USA) was used for spleen
and kidney samples, following the protocol for animal tissue. The QIAamp DNA Stool kit
(Qiagen, Hilden, Germany) was employed for fecal samples (20 mg feces + 160 µl
phosphate-buffered saline). From urine samples (24.5 -140 µl), DNA was extracted with the
QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany), using the protocol for purification
of cellular, bacterial or viral DNA from urine. Each sample batch contained water as a
negative control. DNA concentration and purity were determined with the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

The DNA samples were subjected to a modified semi-quantitative real-time PCR
assay (qPCR) targeting the 23kDa gene of *F. tularensis* (27, 28). All PCRs were run in
duplicate with an ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA). DNA
from tissue samples was analyzed using 1:100 dilutions, and for urine and fecal samples,
three 10-fold (undiluted, 1:10, 1:100) dilutions were examined. The PCR assay included an
internal positive inhibition control, water as negative non-template control, and *F. tularensis
LVS* control strain DNA as positive control. The amount of *F. tularensis* bacteria in each
sample was estimated based on genomic equivalents (GE). To enable comparison of *F.*
tularensis amounts in tissues of experimentally versus naturally infected voles, we also calculated the GE amount in relation to the estimated number of cells in tissue samples (27).

Results

Naturally infected wild field voles

In the three *F. tularensis*-infected wild field voles (27), bacteremia was confirmed by histology and immunohistology. Bacteria were found as aggregates within vessels and capillaries, specifically also in liver sinusoids and renal glomerular capillaries. They were abundant in the splenic red pulp where they were associated with extensive necrosis (Figure 1). In addition, bacteria were identified within macrophages in the liver (i.e. Kupffer cells: Figure 1B) and the splenic red pulp. In the livers, individual necrotic hepatocytes were also seen.

Pilot study in field voles

A pilot study was conducted on field voles to evaluate different infection routes (s.c. and i.n.) and doses (high and low dose). All voles remained asymptomatic during the first four days after infection. On day 5 p.i., four infected voles (two low dose s.c., one high dose i.n., and one high dose s.c.) were found dead, and another animal (high dose s.c.) was euthanized due to general malaise. On day 6 p.i., one symptomatic vole (high dose i.n.) was euthanized. Both low dose i.n. infected voles survived until day 9 p.i., when one was found dead and the other, which had remained asymptomatic, as well as the uninfected control animal, were electively euthanized at the scheduled end of the experiment.

The *post mortem* examination did not reveal any significant gross changes. Histology confirmed severe bacteremia in all but the electively euthanized low dose i.n.
infected vole and the control animal, with bacterial aggregates in vessels in all examined organs and in the cardiac chambers. The pathological changes were very similar to those seen in the naturally infected voles and are typical for tularemia in other species (10, 13), such as extensive splenic and lymph node necrosis with abundant cell-free bacteria (Figure 2A-D).

Two i.n. infected voles (one high dose and one low dose) also showed a multifocal extensive necrotizing pneumonia with abundant bacteria both cell-free and in macrophages (Figure 2E, F), features not seen in the naturally infected voles. This indicates direct aerosol infection of the lung and subsequent bacteremia, in particular since the animals exhibited neither histological changes nor bacteria in the nasal cavity. Bacterial loads in organs did not substantially vary in relation to the route and dose of infection and were generally high in all tissues of symptomatic animals. In the infected vole that had remained asymptomatic, systemic infection was confirmed by PCR, albeit with low bacterial organ loads and without any histological changes or IH evidence of bacteria in any examined tissue.

**Main experimental study in field and bank voles**

For the main study, a low dose delivered via s.c. injection was chosen, as the pilot study demonstrated it to best mimic the natural infection in voles. All animals that were sacrificed on days 1 and 3 p.i. (three field voles and three bank voles at each time point) had been asymptomatic and did not exhibit any significant gross changes. On day 1 p.i., PCR did not detect *F. tularensis* DNA in spleen, kidney, feces or urine (Figure 3), and IH did not identify bacteria in any tissue (Table 1). Histological changes were restricted to the injection site, where focal interstitial hemorrhage was generally seen. In one bank vole a focal macrophage aggregate was found in the adipose tissue of the inoculation site, and IH identified a few bacteria within the macrophages.
On day 3 p.i., a neutrophil-dominated inflammatory reaction with intracellular (macrophages, neutrophils) and cell-free bacteria was often seen at the inoculation site (Figure 4A). The spleen of all animals tested positive for *F. tularensis* DNA (Figure 3), and in all but one weakly PCR-positive spleen, IH identified variable amounts of bacteria within macrophages in the red pulp (Figure 4B, Table 1), confirming cell associated bacteremia. This was not associated with distinct histological changes in the spleen. In two bank voles, the kidney was weakly PCR-positive, and IH identified some bacteria in glomerular capillaries, without other histological changes. The urine of both these animals was PCR-negative. IH also identified bacteria in the livers, as individual cells in sinuses, and identified patches of reactive hepatocytes. Some bacteria were found in capillaries in the lungs of the three bank voles, again without distinct histopathologic changes.

On day 4 p.i., one field vole displayed general malaise and was euthanized, and on day 5 p.i., the remaining 5 field voles and 6 bank voles died or were visibly symptomatic and euthanized. PCR demonstrated *F. tularensis* DNA in the urine and high *F. tularensis* loads in the spleens and kidneys of all animals (Figure 3); in voles euthanized on day 5 p.i., *F. tularensis* was also identified in feces (Figure 3, Table). Histology and IH confirmed these results and revealed features similar to those in the pilot study. The findings were similar in both species. In general, large bacterial aggregates were seen in the splenic red pulp and in capillaries in all examined organs. In the kidneys, bacteria were found in both glomerular and interstitial capillaries (Figure 4C). Apart from disseminated bacterial aggregates between hepatic cords, the liver carried bacteria within Kupffer cells and exhibited multifocal random hepatocellular necrosis (Figure 4D).

In the spleen, the red pulp was almost completely effaced due to necrosis (and loss) of cells, and the white pulp was markedly reduced, with extensive (follicular) apoptosis/necrosis and replacement by bacteria. Lymph nodes exhibited focal areas of
necrosis with abundant bacteria (Figure 4E). In the bone marrow, bacteria were found within mononuclear cells (most consistent with macrophages) and sometimes cell free (Figure 4F), and there was extensive necrosis/apoptosis of myelopoietic cells. Examination of the gastrointestinal tract identified bacteria within capillaries in all compartments, within Peyer’s patches (Figure 4G) and occasionally also in intestinal epithelial cells in both the small and large intestine (Figure 4H, I). Some small macrophage aggregates with bacteria were found in the lamina propria mucosae. More extensive inflammatory infiltrates were restricted to inoculation sites, where variably extensive necrosis and neutrophil infiltration with masses of cell free bacteria was seen.

Control voles remained asymptomatic and were euthanized on day 9, at the scheduled end of the experiment. They were negative for *F. tularensis* by PCR and IH and did not exhibit any histological changes.

### Discussion

The current study presents an experimental model that mimics natural *F. tularensis* ssp. *holarctica* infection of wild voles and demonstrates that both field voles and bank voles are highly susceptible to the bacterium. Infected animals died with bacteremia, following a rapid clinical course and generally with very high bacterial loads in organs. We showed that infected voles excrete *F. tularensis* in their urine and feces around the time of death. The bacterial burden in excreta was relatively low compared to the bacterial load in tissues, but since only a low dose is generally required for infection (9), feces and urine might be infective are realistic sources of infections for other animals and humans. Furthermore, the course of infection could be different under natural conditions. Long-term infections and shedding of *F. tularensis* have been reported after oral infection (29, 30) and the oral route of infection should be studied in future. The presence of bacterial aggregates within the
glomerular tufts in the kidneys and within mucosal vessels and between epithelial cells in the
intestinal mucosa of animals by day 5 p.i. also indicates that *F. tularensis* is excreted in urine
and feces at this stage. Excretion of *F. tularensis*, in addition to contamination from dead
animals, might serve to transfer the bacteria into the environment, which could also include
mosquito breeding sites. In support of this premise, *F. tularensis* has been demonstrated to
survive in water for several weeks (29, 30, 31, 32). The survival is supported by protozoa,
which are commonly found in natural aquatic systems as part of their normal biofilms (16).

Outbreaks of airborne tularemia in humans are mainly linked to farm work and
other outdoor activities (6, 8, 12, 22, 33, 34), for example exposure to hay dust has been
associated with pneumonic tularemia (4). This might be due to bacteria-containing aerosols
originating from animal carcasses or excreta made airborne by agricultural machines.
Similarly, Puumala hantavirus infection is acquired by inhalation from rodent excreta, and
considerably more often by farmers (35). *F. tularensis* has been shown to survive up to 192
days in the environment on straw and grain depending on the temperature of the surrounding
air (36). Survival is longest in winter conditions, as the amount of viable bacteria decreases
with rising temperatures (36). The enhanced survival of *F. tularensis* in cool temperatures
might be one factor contributing to the high tularemia incidence in Fennoscandia.

Our analysis of the pathogenesis of tularemia indicates that the bacteria are taken up
locally (i.e. at the inoculation site) by macrophages and neutrophils and then distributed
throughout the body, to eventually accumulate in the blood. Accordingly, they were found
both within monocytes and cell free in vessels of almost all organs, and led to necrosis of
infected cells, resulting in extensive necrosis particularly in the lymphatic tissues (i.e. spleen
and lymph nodes). Interestingly, apart from the inoculation site, this was not associated with
an overt inflammatory response. Similar changes have been reported in hares, in which
tularemia is mainly characterized by acute focal necrosis without cellular reaction in liver,
spleen, and bone marrow (10). Recently, *F. tularensis* infection even without lesions has been described in squirrels (375). In our pilot study, two intranasally infected voles exhibited a necrotising to granulomatous pneumonia, indicating direct infection of the lung (not via bacteremia). This kind of prominent change is typical for inhalational tularemia; severe necrotizing pneumonia has been demonstrated in monkeys (386) and mice (392) after *F. tularensis* spp. *tularensis* aerosol exposure. Necrotizing granulomatous inflammation is also seen in lung biopsies of human patients with pulmonary tularemia (4038, 4139).

In Fennoscandia, tularemia is primarily mosquito-transmitted, and large human outbreaks occur regularly (4, 19). Mosquitoes have been shown experimentally to become persistently infected already as larvae and then transtationally through the developmental stages to adults, without however evidence of *F. tularensis* replication (420). It has been shown that *F. tularensis* multiplies in protozoa (16), but mammals are probably also needed, as local amplifiers to facilitate the spread of the disease (429) e.g. through contaminated water and subsequently mosquitoes. In Sweden, a temporal link between outbreaks in humans and rodent density cycles has been reported during 1960s and 1970s (23). Moreover, our recent survey of wild rodent species identified *F. tularensis* in wild field voles (27), and we show here that the massive bacteremia and pathological lesions after experimental infection are identical to those in naturally infected animals. Mosquitoes might also become infected by feeding on bacteremic voles and then perhaps directly transmit *F. tularensis* to humans – however, there is no data to support this. It is also possible that *F. tularensis*, amongst other things, contributes to the density crash of vole populations in certain areas, at which stage *F. tularensis* is released into the environment. This environmental contamination presumably also propagates the outbreak among voles. As our results show, infected dead voles can lead to heavy contamination of the environment and provide an explanation for the common association between rodent density and human tularemia incidence.
In summary, the fact that voles readily developed lethal tularemia, together with the severity and similarity of the lesions in both experimentally and naturally infected animals, suggest that long-term or latent infection of these species is unlikely, yet some reservation concerning the infection routes may be warranted. Instead, voles are likely to play a role as amplification hosts and lead to bacterial contamination of the local environment, and by this mechanism contribute to the incidence of human tularemia.

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Biographical Sketch

DVM Heidi Rossow is a doctoral student and researcher at the Department of Veterinary Biosciences, Faculty of Veterinary Medicine, University of Helsinki, Finland. Her primary research interests are the epidemiology, ecology and pathogenesis of tularemia.

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Figure Legends

Figure 1. Naturally *F. tularensis sp. holartica* infected field vole that had been trapped and euthanized (27). **A.** Lung with bacterial clumps in vessel lumina (arrow) and bacterial aggregates in capillaries (arrowhead). **B.** Liver with bacterial clumps in sinusoids (arrow) and smaller aggregates within Kupffer cells (arrowheads). **C.** Kidney with bacterial aggregates in larger vessels (arrow) and glomerular capillaries (arrowheads). **D.** Spleen with abundant bacterial clumps, in association with necrosis (N), in the red pulp. Horseradish peroxidase method, Papanicolaou’s hematoxylin counterstain. Bars = 20 µm.

Figure 2. Pilot study, field voles that had died from or were euthanized after experimental *F. tularensis sp. holartica* infection. **A, B.** Spleen from an animal found dead on day 5 after subcutaneous (s.c.) infection with a low dose (LD). Clumps of bacteria are found cell-free and within (degenerating) macrophages in the red pulp, often surrounding follicles (F). Numerous lymphocytes in follicles undergo apoptosis (A: arrowhead). **C, D.** Mesenteric lymph node from an animal found dead on day 5 after s.c. infection with a high dose (HD). **C.** Cortex with focal area of necrosis (arrow). **D.** Clumps of bacteria are present within the sinuses (arrow) and in areas of necrosis (arrowhead). **E, F.** Lung from a vole found dead on day 9 after HD intranasal infection. Focal area of extensive necrosis with large aggregates of bacteria (arrows). Inset in F: Lung from a vole that was euthanized on day 5 after HD s.c. infection. Bacteria are found within circulating leukocytes in lung capillaries. **A, C, E.** HE stain, **B, D, F.** Horseradish peroxidase method, Papanicolaou’s hematoxylin counterstain. Bars = 20 µm (**A-D, F**), 50 µm (**E**), 10 µm (**Inset F**).
Figure 3. Quantification of *F. tularensis* DNA in spleen, kidney, faces and urine of infected voles by day post infection determined using real-time PCR targeting the bacterial 23 kDa gene of *F. tularensis* (27, 28). Samples were collected at the following time points: day 1 post infection (p.i.), day 3 p.i. and days 4-5 p.i.

Figure 4. Main study, voles after subcutaneous infection with *F. tularensis* sp. holartica. A, B. Day 3 post infection (p.i.). A. Field vole, injection site in the subcutis with moderate pyogranulomatous inflammation. Bacteria are present within leukocytes (arrows) and as cell free aggregates (arrowhead). B. Bank vole, spleen. In the red pulp, in particular surrounding follicles (F) and T cell zones, are aggregates of macrophages with intracellular bacteria (arrows). C-I. Day 5 post infection. C. Field vole, kidney. Bacteria form clumps in the lumen of interstitial veins (arrows) and glomerular tufts (arrowhead). D. Field vole, liver. Bacteria form aggregates within sinusoids (arrows) and are present within Kupffer cells (arrowheads). E. Field vole, mesenteric lymph node and large artery (A). Cell free bacteria fill the lumen of the artery and are present within necrotic areas in the lymph node (arrow). F. Bank vole, bone marrow. Bacteria are mainly found within mononuclear (myeloid) cells (arrowhead). G. Field vole, duodenum with Peyer’s patch, exhibiting bacteria within cells and cell-free, also towards the mucosal surface (arrowhead). H. Field vole, jejunum. Bacterial aggregates fill capillaries (arrow) and are present within cells, also in the lamina epithelialis mucosae (arrowhead). I. Bank vole, colon. Bacterial aggregates fill capillaries (arrow) and are present within cells, also in the lamina epithelialis mucosae (arrowhead). Horseradish peroxidase method, Papanicolaou’s hematoxylin counterstain. Bars = 20 μm (A-D, G), 50 μm (E), 10 μm (F, H, I).
Table Detection of *Francisella tularensis* in organs and excretions of experimentally infected voles

| Species and specimens | Day 1 | Day 3 | Day 4 | Day 5 |
|-----------------------|-------|-------|-------|-------|
|                       | PCR   | IH    | PCR   | IH    | PCR   | IH    | PCR   | IH    |
| *Microtus agrestis*, field vole |       |       |       |       |       |       |       |       |
| Spleen                | 0/3   | 0/3   | 3/3   | 1/2   | 1/1   | 1/1   | 5/5   | 5/5   |
| Kidney                | 0/3   | 0/3   | 0/3   | 0/3   | 1/1   | 1/1   | 5/5   | 5/5   |
| Feces                 | 0/3   | 0/3   | 0/1   |       |       |       | 5/5   |       |
| Urine                 | NA    | NA    | 1/1   |       |       |       | 2/2   |       |
| *Myodes glareolus*, bank vole |       |       |       |       |       |       |       |       |
| Spleen                | 0/3   | 0/3   | 3/3   | 3/3   | 6/6   | 6/6   |       |       |
| Kidney                | 0/3   | 0/3   | 2/3   | 2/3   | 6/6   | 6/6   |       |       |
| Feces                 | 0/3   | 0/3   | 2/3   | 2/3   | 6/6   | 6/6   |       |       |
| Urine                 | 0/3   | 0/3   | 3/3   |       |       |       |       |       |

*a*real-time PCR targeting the bacterial 23 kDa gene of *F. tularensis* (27, 28)

*b*IH, Immunohistology using a mouse monoclonal antibody against *F. tularensis* lipopolysaccharide (clone T14; IgG3)

No, number; PCR, polymerase chain reaction; IH, immunohistology; NA, not available

Denominators represent the total amount of screened animals.
Response to Reviewers

Helsinki, July 23, 2014

Response to the decision letter
PONE-D-14-20237
Experimental infection of voles with Francisella tularensis indicates their amplification role in tularemia outbreaks

Dear Prof. Dumler,

Attached is a revised version of our manuscript “Experimental infection of voles with Francisella tularensis indicates their amplification role in tularemia outbreaks”. Thank you for the opportunity to improve and resubmit our work and to the reviewers for their thorough work and helpful comments. We have revised the manuscript according to their suggestions and replied to each comment in detail. We used the track changes mode to mark the changes in the manuscript. We hope that the revised manuscript is now suitable for publication in PLOS ONE.

Sincerely,

Heidi Rossow

Journal requirements:

1. We note that you stated “data are available upon request” at submission. Could you please confirm that all data underlying the findings in your study are freely available in the manuscript, supplemental files, or in a public repository?

RESPONSE: All data underlying the findings in our study are freely available in the manuscript.

Comprehensive data concerning materials, methods and results can be found summarized in the manuscript. In addition, we have residual formalin fixed and frozen tissue samples, histological slides from each individual and detailed PCR-results which are overly numerous and detailed and not of interest for the reader and technically impossible due to space and text limitations to attach to the manuscript. Obviously, this is thy type of detailed individual data that is “normally” not included in a manuscript. However if for some reason this is requested, it will be accessible from the authors.

Re: PONE-D-14-20237, Rossow et al.
Title: “Experimental infection of voles with Francisella tularensis indicates their amplification role in tularemia outbreaks”

Responses to Reviewer:
Francisella t.h. kills voles, I would recommend that this paper be shortened to a short report or something similar.

2.2. RESPONSE: As none of the other reviewers nor the academic editor proposed shortening the paper, we interpreted that it is possible to keep the length as it is.

REVIEWER 3:

REVIEWER: 1. But if voles have “massive bacteremia” (page 14, line 323), could mosquitoes not also become infected by feeding on voles and then transmit to humans?

3.1. RESPONSE: Thank you for pointing this. There are few species of mosquitoes that, as adults, first take a blood meal from a vole and then human. In theory, if that would happen, it could be possible that they transmit tularemia directly from voles to humans. However, we did not find evidence for that. Instead, there is evidence for developing mosquitoes picking F. tularensis up from the water. This is definitely an issue that should be addressed in future studies. We now have added this point to the discussion section (lines 326-8).

REVIEWER 2: The authors could also mention a bit more explicitly the possibility that environmental contamination of water/grass may -- through ingestion -- propagate the outbreak among voles.

3.2. RESPONSE: We have now added this point in the discussion section (lines 330-1).

REVIEWER 3: Figure 4 legend – I think the last two panels may be mislabeled (second “G” should be “H” and following “B” should be “I”?)

3.3. RESPONSE: This has been corrected.