Susceptibility of selected murine and microtine species to infection by a wild strain of *Francisella tularensis* subsp. *holoarctica*

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ABSTRACT: The purpose of this study was to compare susceptibility of BALB/c mice, common voles (*Microtus arvalis*) and yellow-necked mice (*Apodemus flavicollis*) to infection by a virulent *Francisella tularensis* subsp. *holarctica* strain. Median survival in these three species following experimental infection with 320 colony forming units of *F. tularensis* (both intraperitoneally and subcutaneously) amounted to 4.5, 7 and 4 days, respectively. Survival curves of BALB/c and yellow-necked mice were very similar and were significantly different from that of common voles. LD_{50} was 0.5 and 37.9 colony forming units in BALB/c mice and common voles, respectively. The bacterial burden in the spleen, liver, lung, kidney and blood of common voles started to develop later post exposure and amounted to lower levels (except in kidneys) than in BALB/c mice. The results demonstrate that yellow-necked mice are even more susceptible to infection by *F. tularensis* than BALB/c mice and that the common vole is a small mammalian host with a susceptibility which is two-orders-of-magnitude lower.

Keywords: tularaemia; survival time; minimum infectious dose; LD_{50}; bacterial burden

Tularemia, a zoonotic disease caused by *Francisella tularensis,* has recently become important both due to its emergence in areas with no previous known risk, re-emergence in long recognised nosoareas (Pikula et al., 2004a,b) and its biothreat potential (Petersen and Schriefer, 2005). Current and emerging assays for *F. tularensis* have been thoroughly reviewed (Pohanka et al., 2008). Although at present four subspecies of *F. tularensis* have been described, the majority of human and animal infections are caused by *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* (Cerny, 2001; Farlow et al., 2005; Petersen and Schriefer, 2005; Stanek, 2005; Staples et al., 2006) acquired from numerous sources depending on the ecology of tularemia in endemic areas (Pikula et al., 2003, 2004c). Basically, there are terrestrial and aquatic disease cycles (Moerner, 1992), in which more than 150 wild mammalian and to a lesser extent avian species participate (Ellis et al., 2002). Zoonoses with a wildlife reservoir, including tularemia, represent a major public health problem (Kruse et al., 2004), and necessitate ongoing epidemiologic surveillance (Hubalek et al., 1993; Kozuch et al., 1995; Gurycova et al., 2001; Vyrostekova et al., 2002; Zhang et al., 2006). Surveys of tularemia reservoirs often report positive findings in some rodent species as well as in lagomorphs (Pohanka et al., 2007). Cases of human disease are associated with activation of endemic foci of tularemia, outbreaks in voles and hares (Tarnvik et al., 1996) and higher prevalence of *F. tularensis* in vectors such as ticks (Hubalek et al., 1996) or mosquitoes (Eliasson et al., 2002), and frequently are the result of handling tularemic hares (Cerny, 2001; Treml et al., 2007).

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Nevertheless, it is still not clear whether small mammals are the true reservoir of the bacterium in the environment (Ellis et al., 2002). In some authors' opinion, the natural reservoir of the bacterium remains mysterious and even some ubiquitous protozoa might be an important environmental reservoir for *F. tularensis* (Abd et al., 2003).

On the other hand, there are reports of chronic shedding of tularemia nephritis in rodents (Bell and Stewart, 1975), seropositive animals remaining culture positive for *F. tularensis* (Petersen et al., 2004) and chronic infection in species highly sensitive to tularemia (Dunaeva and Olsufyev, 1958; Shlygina et al., 1987; Shlygina et al., 1989).

These controversial opinions and findings demonstrate the need for further studies into the competence of various hosts as reservoirs for tularemia. Therefore, the objective of this study was to use a wild *F. tularensis* strain and compare the susceptibility of BALB/c mice, representing an important model organism for experimental infection by *F. tularensis*, with two species of wild rodents reported to harbour *F. tularensis* in European habitats, i.e., the common vole (*Microtus arvalis*) and yellow-necked mouse (*Apodemus flavicollis*). For comparative purposes we evaluated differences in survival time, minimum infectious dose and LD_{50} as well as in the development of tissue bacterial burden.

MATERIAL AND METHODS

Francisella tularensis strain subtyping

A wild strain of *F. tularensis* isolated from a European brown hare specimen from South Moravia in 2004 was used for experimental infections in this study. A liver sample from the hare was mechanically homogenized and spread onto McLeod agar supplemented with Iso VitaleX™ (Becton-Dickinson, San Jose, CA) together with bovine haemoglobin. Plates were incubated under humid aerobic conditions for at least 24 but not more than 48 hours.

First, glass slide agglutination was performed for fast identification of *F. tularensis*. For these purposes, cells were suspended in phosphate buffered saline (PBS) up to the approximate concentration of 10^9 CFU/ml and 10 µl of the suspension were injected onto glass slides together with agglutination serum (Sevapharma, Prague, Czech Republic) and the slides were checked for immunoprecipitation. An *Escherichia coli* strain (ATCC 9637) was obtained from the Czech Collection of Microorganisms (Brno, Czech Republic) and processed in a similar way as *F. tularensis* also including glass slide agglutination. A non-precipitated mixture of *Escherichia coli* and agglutination serum against *F. tularensis* served as a negative control.

Second, further *F. tularensis* subtyping was carried out by mass spectrometry. Briefly, a suspension of cells (from two cultivation plates of 5 cm diameter) in PBS was spun down at 4 000 g for 10 min and washed again in PBS. The pellet was re-suspended in 1 ml of 8M urea with the protease inhibitor Complete mini (Roche, Basel, Switzerland) and benzoase (Sigma-Aldrich, St. Louis, MO, USA). Two rounds of French press (AB Biox, Sweden) were performed to break down the cells and the material was centrifuged at 14 000 g for 10 min. 200 µl of the supernatant were diluted with 1 ml of 50mM ammonium bicarbonate buffer to adjust pH prior to tryptic digestion and 1 µg of trypsin was added and left to digest overnight at 37°C. The digested mixture was desalted on HLB cartridges (Waters, Milford, MA, USA) and the final eluate was dried and re-suspended in 50 µl of 2% acetonitrile and 0.1% (v/v) formic acid in water. 3 µl of the sample were separated on Atlantis™ C18 column, 75 µm × 150 mm (Waters), mounted on the CapLC system (Waters) interfaced through nanospray with Q-TOF Ultima™ API (Waters). 80 min gradient of acetonitrile eluted the peptides from the column and data were acquired using data dependent acquisition recording up to three channels. Data were subtracted, smoothed and centroided using the ProteinLynx script in MassLynx software (Waters) and saved as a .pkl file. Searching against the database of *Francisella* proteins (currently, genomes of 5 different strains are publicly available) was performed in Phenyx 2.1 (GeneBio, Switzerland). A minimum peptide z-score of 3 and a maximum peptide *P*-value of 1 e-1 was required for correct sequence assignment. The above procedure of *F. tularensis* subtyping has already been described elsewhere in greater detail (Hernychova et al., 2001; Hubalek et al., 2004).

Preparation of *Francisella tularensis* for experimental infection

Experimental infections were performed using a suspension of *F. tularensis* cells washed down

from cultures growing on blood agar supplemented with cystine using sterile physiological saline solution. No adjuvans was employed. After thorough mixing absorbance of the suspension at 605 nm wavelength was measured using a spectrophotometer (Unicam Helios Gamma&Delta, Spectronic Unicam, United Kingdom) in order to determine the number of bacterial cells per volume units according to Mc Farland's standard (Murray et al., 2003). The obtained number was only approximate and served as an estimate for the dilution necessary to arrive at the required dose. The exact infectious dose was then determined by plating 10-fold serial dilutions and counting colony forming units (CFU) in the suspension administered to experimental animals. Colonies were counted after 72 h incubation at 37°C.

Experimental animals

Three small mammalian species, i.e., the laboratory mouse (*Mus musculus*), the common vole (*Microtus arvalis*) and the yellow-necked mouse (*Apodemus flavicollis*), were used for the study. BALB/c mice were purchased from a commercial breeder and were included in the experiment at the age of eight weeks and a body weight of 25g. During early spring, parent common voles and yellow-necked mice were live-trapped in the wild as overwintered animals. After ensuring the good health status (healthy appearance, excellent nutritional state, freedom from tularemia based on agglutination test) and determining the sex of the captured specimens, pairs were formed and kept under laboratory conditions in boxes for rodents. The produced offspring were then included in the experiment at the age of two months and at body weights ranging from 14 to 21 g. Experimental animals were fed granules for rodents, a mixture of seeds, meadow grass and hay and were provided with drinking water *ad libitum*. Experiments were performed in compliance with laws for the protection of animals against cruelty and were approved by the Ethical Committee of the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic.

Survival time

Experiments to determine species-specific survival times included laboratory mice (*Mus musculus*), common voles (*Microtus arvalis*) and yellow-necked mice (*Apodemus flavicollis*). Twenty specimens of the same species, age and body weight were used in the study. Each experimental animal was injected with 320 *F. tularensis* CFU. Experimental animals were equally divided into two groups for intraperitoneal and subcutaneous routes of exposure. Animals were examined twice daily for developing signs of infection. Data on days (hours) elapsed from inoculation to death of experimental animals were then recorded and used to compute median survival values as well as compare survival curves.

Minimum infectious dose and LD_{50}

Laboratory mice as well as common voles were divided into seven groups of 10 specimens. The original suspension of *F. tularensis* obtained by washing from a plated culture resulted in a concentration of 1.6×10^5 CFU per 1 ml, which was then serially diluted in a 10-fold manner. Experimental animals were intraperitoneally injected with 0.1 ml of the suspension so as to obtain 1.6×10^4, 1.6×10^3, 1.6×10^2, 1.6×10^1, 1.6, 1.6×10^{-1}, and 1.6×10^{-2} CFU. After that, animals were examined twice daily for developing signs of infection. Data on days (hours) elapsed from inoculation to death, mortality rates within groups and minimum infectious dose still resulting in some mortality were then recorded.

Quantification of *Francisella tularensis* in tissues

Tissue bacterial burdens in organs of laboratory mice and common voles were studied following intraperitoneal infection with 160 CFU *pro toto*. A total of 20 specimens from each species were inoculated and killed by decapitation at 48, 72, 96, 120, and 144 h post exposure. Blood was collected by cardiac puncture prior to euthanasia. Cadavers were necropsied in order to examine gross pathological findings and organs were collected aseptically (liver, spleen, lung, and kidney). To enumerate viable bacterial cells in 1 g of individual organs, samples were cut into small pieces and then homogenized using a homogenizer. After that, three dilutions in a 10-fold manner were made using sterile physiological saline. Taking 0.1 ml of each dilution, samples were plated on and inspected for

growth of colonies after 72 h incubation at 37°C. Data on numbers of CFU in three dilutions from each organ and blood were then averaged and $\log_{10}$-transformed to obtain bacterial burdens per 1 g of tissue or 1 ml of blood.

Statistical analysis

GraphPad Prism 4 (GraphPad Software, San Diego, USA) was used for statistical analyses and graphing data. Survival curves in experimental animals were compared using the logrank test and the calculation of two-tailed *P*-values. Median survival values, i.e., the time at which half the animals had died following experimental infection, were also calculated. LD_{50} and its 95% confidence intervals were calculated using the method of moving average interpolation (Schaper et al., 1994).

RESULTS

Francisella tularensis strain subtyping

First, glass slide agglutination was used to identify the *F. tularensis* isolate. A commercial strain of *Escherichia coli* (Czech Collection of Microorganisms) suspended in the same manner as *F. tularensis* served as a negative control. The agglutination serum provided a significant precipitate when a suspension of the tested *F. tularensis* sample was assayed, while the tested *Escherichia coli* sample provided no precipitate. Even though the agglutination test confirmed that the tested sample was *F. tularensis*, we were not able to confirm the subspecies taxonomy. A proteomic study for subspecies determination was therefore performed. Mass spectrometry provided the best information on *F. tularensis* taxonomy. The following significant markers from *F. tularensis* subsp. *holarctica* were detected and recognized according to the Phenyx 2.1 database: 17 kDa major membrane protein precursor (TUL4), DNA-binding protein HU-beta, peroxiredoxin (EC 1.11.1), chaperone GroES, 30S ribosomal protein S5, sigma-54 modulation protein as well as specific sequences in the GroEL protein (K/GRNVVLDKSFGAPTITK/D; K/AVTAGMNPMDLKR/G; K/ALDGLTGENDDQNHGIALLRK/A). Based on these results, the obtained isolate could be classified as *Francisella tularensis* subsp. *holarctica*.

Survival time

Median survival values in laboratory mice, common voles and yellow-necked mice following experimental infection with 320 *F. tularensis* colony forming units (CFU) amounted to 4.5, 7 and 4, respectively. As shown in Figure 1, survival curves of laboratory BALB/c mice and yellow-necked mice were very similar, while the survival curve of the common vole was significantly different (logrank test, chi square = 12.01, df = 2, P = 0.0025). Comparing intraperitoneal and subcutaneous routes of exposure in all three small rodent species examined, only slight differences without statistical significance were found. However, one common vole specimen survived the subcutaneous route of infection without showing signs of disease for a much longer period. Bacterial burdens per 1 g of liver, spleen, lung and kidney tissue and 1 ml of blood ranged from 9 to 25 *F. tularensis* CFU in this animal killed on Day 37 post exposure.

Minimum infectious dose and LD_{50}

Data on mortality rates and median survival values in laboratory mice and common voles infected with seven serial (10-fold) dilutions are presented in Table 1. As shown, all animals in the group (both mice and voles) died when injected with 160 and higher numbers of *F. tularensis* CFU. On the other

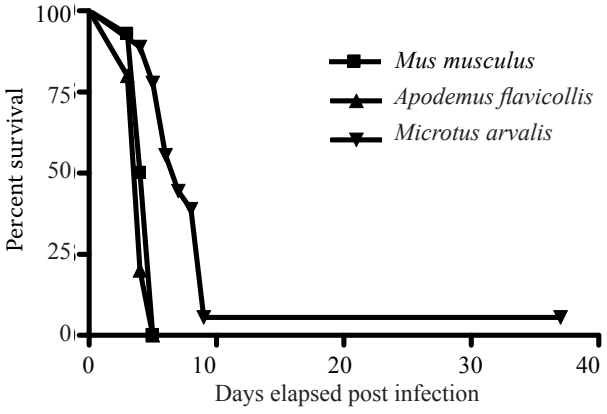

Figure 1. Survival curves of BALB/c mice (*Mus musculus*), yellow-necked mice (*Apodemus flavicollis*) and common voles (*Microtus arvalis*) following experimental infection with 320 *F. tularensis* CFU. Curves were plotted using data on both intraperitoneal and subcutaneous routes of infection

Table 1. Mortality rates and median survival values in laboratory BALB/c mice (*Mus musculus*) and common voles (*Microtus arvalis*) infected with seven serial (10-fold) dilutions of experimental *F. tularensis* suspension. CFU = colony forming units of *F. tularensis*.

Mus musculus							
Infectious dose (CFU)	1.6×10^4	1.6×10^3	1.6×10^2	1.6×10^1	1.6	1.6×10^{-1}	1.6×10^{-2}
Mortality rate (%)	100	100	100	80	60	0	0
Median survival (days)	3.4	3.8	4.8	6.0	7.6	–	–
Microtus arvalis							
Infectious dose (CFU)	1.6×10^4	1.6×10^3	1.6×10^2	1.6×10^1	1.6	1.6×10^{-1}	1.6×10^{-2}
Mortality rate (%)	100	100	100	20	0	0	0
Median survival (days)	6.2	6.8	8.4	11.0	–	–	–

hand, one and two orders of magnitude lower infectious doses (i.e., 16 and 1.6 CFU) clearly demonstrate the difference in susceptibility between BALB/c mice and common voles to *F. tularensis* infection. The dose of 16 *F. tularensis* CFU induced mortality rates of 80 and 20% in mice and

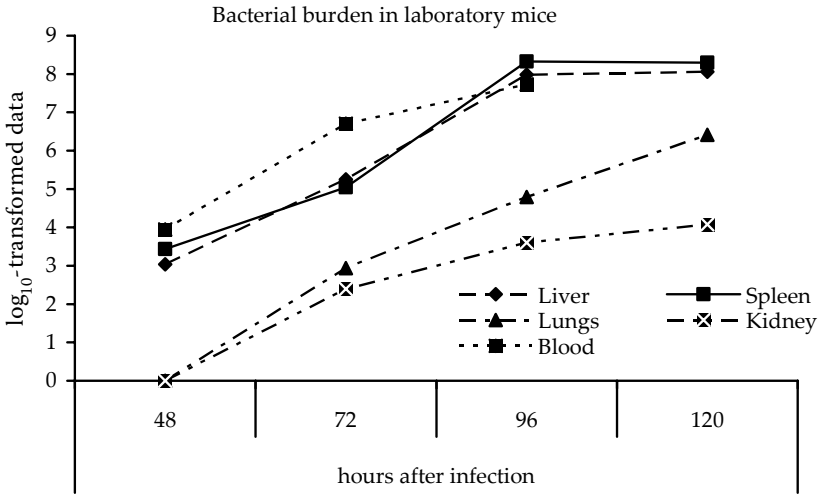

Figure 2. Tissue bacterial burdens in organs of laboratory mice (BALB/c) following intraperitoneal infection with 160 *F. tularensis* CFU *pro toto* at 48, 72, 96, and 120 h post infection. Values represent means (n = 5 at each measurement)

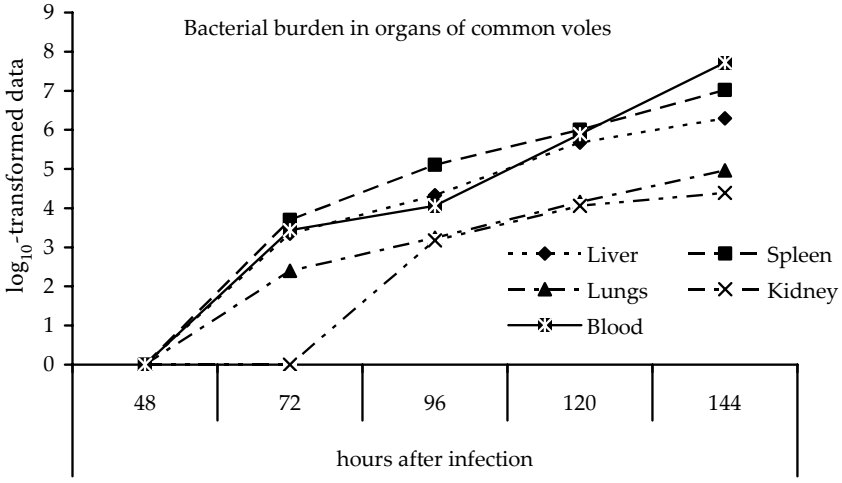

Figure 3. Tissue bacterial burdens in organs of common voles (*Microtus arvalis*) following intraperitoneal infection with 160 *F. tularensis* CFU *pro toto* at 48, 72, 96, 120, and 144 h post exposure. Values represent means (n = 4 at each measurement)

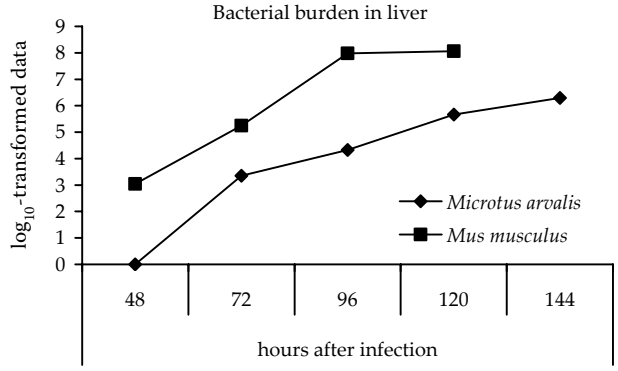

Figure 4. Comparison of tissue bacterial burden and its development in the liver of BALB/c mice (*Mus musculus*) and common voles (*Microtus arvalis*) following intraperitoneal infection with 160 *F. tularensis* CFU *pro toto* at 48, 72, 96, 120, and 144 h post exposure. Values represent means (n = 5 and 4 at each measurement in mice and voles, respectively)

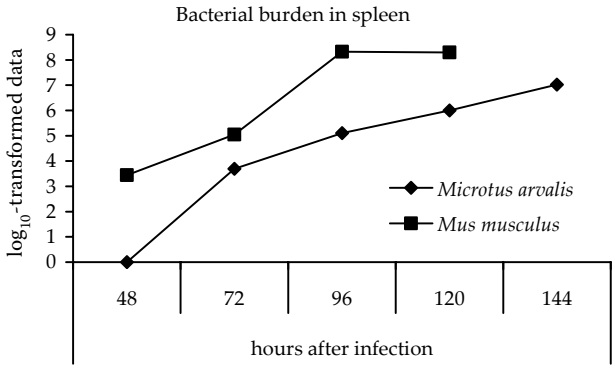

Figure 5. Comparison of tissue bacterial burden and its development in the spleen of BALB/c mice (*Mus musculus*) and common voles (*Microtus arvalis*) following intraperitoneal infection with 160 *F. tularensis* CFU *pro toto* at 48, 72, 96, 120, and 144 h post exposure. Values represent means (n = 5 and 4 at each measurement in mice and voles, respectively)

voles, respectively. Dilution of the experimental *F. tularensis* suspension down to 1.6 CFU resulted in mortality of only BALB/c mice (60%). It is also clear that the higher the infectious dose, the lower the median survival in both species studied. Depending on the infectious dose, median survival times ranged from 3.4 to 7.6 and 6.2 to 11 days in BALB/c mice and common voles, respectively. LD_{50} in the mouse and common vole was calculated to be 0.5 and 37.9 CFU, respectively.

Quantification of *Francisella tularensis* in tissues

Figures 2 and 3 present the tissue bacterial burden in the organs of laboratory mice and common voles, respectively, determined following intraperitoneal infection with 160 CFU *pro toto* at 48, 72, 96, 120, and 144 h post exposure. As shown in Figure 2, for the laboratory mouse, the highest and lowest *F. tularensis* burdens were found in

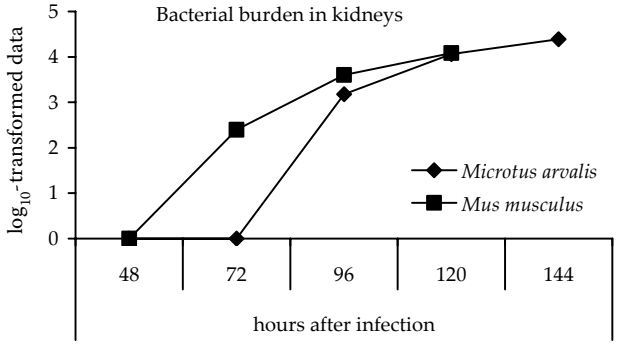

Figure 6. Comparison of tissue bacterial burden and its development in kidneys of BALB/c mice (*Mus musculus*) and common voles (*Microtus arvalis*) following intraperitoneal infection with 160 *F. tularensis* CFU *pro toto* at 48, 72, 96, 120, and 144 h post exposure. Values represent means (n = 5 and 4 at each measurement in mice and voles, respectively)

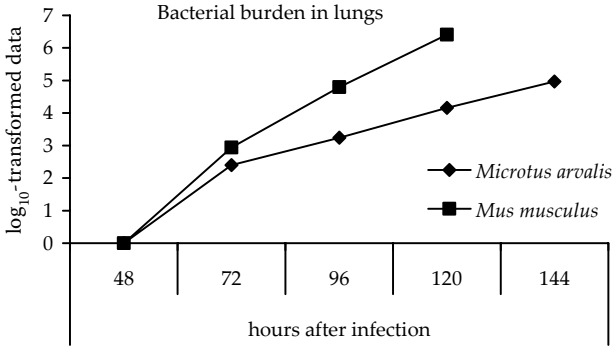

Figure 7. Comparison of tissue bacterial burden and its development in lungs of BALB/c mice (*Mus musculus*) and common voles (*Microtus arvalis*) following intraperitoneal infection with 160 *F. tularensis* CFU *pro toto* at 48, 72, 96, 120, and 144 h post exposure. Values represent means (n = 5 and 4 at each measurement in mice and voles, respectively)

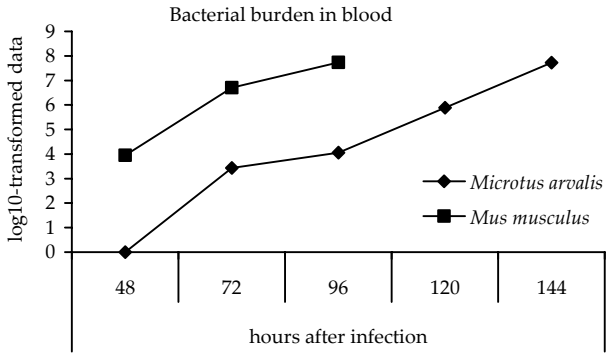

Figure 8. Comparison of tissue bacterial burden and its development in the blood of BALB/c mice (*Mus musculus*) and common voles (*Microtus arvalis*) following intraperitoneal infection with 160 *F. tularensis* CFU *pro toto* at 48, 72, 96, 120, and 144 h post exposure. Values represent means (n = 5 and 4 at each measurement in mice and voles, respectively)

the spleen and kidney, respectively. Likewise, the lowest *F. tularensis* burden in the common vole was in the kidneys. The highest bacterial burden, however, was in the blood and spleen of common voles (Figure 3). As demonstrated in Figures 4 to 8, tissue bacterial burdens in common voles started to develop later post exposure and resulted in lower levels than in laboratory mice. The only exception to this rule was that of the higher *F. tularensis* burden in the kidneys of common voles (2.45×10^4 CFU) than in laboratory mice (1.21×10^4 CFU) at 144 and 120 h post exposure, respectively, i.e., during the terminal stages of infection in both rodent species.

DISCUSSION

In this experimental study, the competence of selected murine and microtine rodent species as a reservoir for tularemia was evaluated using parameters of survival, minimum infectious dose, LD_{50}, and bacterial burden quantification. A wild strain of *F. tularensis* isolated from a European brown hare was used for this purpose. Subtyping of this strain by mass spectrometry confirmed *F. tularensis* subsp. *holarctica* (biotype B), i.e., the only subspecies isolated in European countries (Petersen and Schriefer, 2005), with the exception of one report on biotype A occurrence in Central Europe (Gurycova, 1998). Despite being as infectious as biotype A, the lower virulence of the European biotype B enables laboratory work under containment level three conditions (Tarnvik and Berglund, 2003). Nevertheless, most experimental studies during the last three decades have used not virulent clinical isolates but the live vaccine strain (LVS) of *F. tularensis* attenuated for humans (Anthony and Kongshavn, 1987; Conlan et al., 1994; Green et al., 2005). However, there are genuine concerns about using *F. tularensis* LVS as a surrogate for more virulent strains of the pathogen (Conlan et al., 2003). Many researchers think that it is time to re-examine experimental tularemia using virulent strains of *F. tularensis* and well-characterized inbred mouse strains in common usage today (Chen et al., 2003, 2004; Conlan et al., 2003). It was the objective of this study to provide data on differences in the susceptibility of *Mus musculus* (BALB/c mice), *Apodemus flavicollis* (yellow-necked mouse) and *Microtus arvalis* (common vole) to *F. tularensis* infection. While BALB/c mice represent the standard, as they are genetically defined experimental animals with much data available concerning their response to infection by *F. tularensis* (Chen et al., 2003; Conlan et al., 2003; Green et al., 2005; KuoLee et al., 2007), yellow-necked mice and common voles have been reported to harbour *F. tularensis* with as high a prevalence as 3.9% during activation of tularemic foci in Central Europe (Gurycova et al., 2001; Vyrostekova et al., 2002).

Though Dunaeva and Olsufyev (1958) consider these species highly susceptible to tularemia, the results of the present study demonstrate that there is a two-orders-of-magnitude difference in susceptibility to *F. tularensis* infection between BALB/c mice and common voles, with mice being more susceptible. While survival curves of laboratory BALB/c mice and yellow-necked mice are very much alike, it seems that the latter is even more susceptible to infection by *F. tularensis*. Similarity and dissimilarity in the responses of the above three species may be explained by intergeneric genetic relationships within the rodent family Muridae and representatives of the family Arvicolidae. The genus *Mus* has unequivocally been determined to be the sister group of the genus *Apodemus*, whereas the genetic distance to *Microtus arvalis* is much greater (Hartl et al., 1992).

The results presented here confirm the high susceptibility of BALB/c mice to infection by *F. tu-*

larensis (biotype B). They are in agreement with published data (Conlan et al., 2003) and may be used as a comparative reference for the evaluation of the susceptibility of the other two species studied. The LD_{50} score calculated as 0.5 *F. tularensis* CFU in the BALB/c mice means that approximately one CFU (administered either *i.p.* or *s.c.)* may cause infection and mortality. At this dilution of the suspension, there certainly is a possibility of injecting experimental animals with material containing not even one viable bacterial cell, and, as shown in Table 1, dilution of the experimental *F. tularensis* suspension down to 1.6 CFU resulted in mortality of only 60% of BALB/c mice. This might have been caused either by the ability of experimental animals to survive such a low-dose infection or by the absence of infectious bacteria in the inoculum.

The lowest median survival time (i.e., 4 days) following experimental infection by *F. tularensis* was found in the yellow-necked mouse. The relatively high prevalence of infection reported in live-trapped yellow-necked mice from endemic foci with increased epizootic activity (Gurycova, 2001) is rather surprising in light of this extremely high susceptibility and short survival time because, like BALB/c mice (Conlan et al., 2003), yellow-necked mice displayed overt signs such as lethargy, hunching and anorexia at least 24 h prior to death. In our opinion, the probability of obtaining a live specimen of this species from the wild positive for tularemia by culture of the spleen is very low, because there remain only three active days from infection to capture in this rodent. On the other hand, the probability of yellow-necked mice harbouring *F. tularensis* is increased by their reported higher degree of infestation by ticks as compared to other rodents (Talleklint and Jaenson, 1997; Rosa et al., 2007; Boyard et al., 2008). Unfortunately, owing to problems in producing sufficient numbers of yellow-necked mice for experiments under laboratory conditions, we were not able to obtain statistically significant data on tissue bacterial burden, minimum infectious dose, and LD_{50}. The epidemiological importance of the yellow-necked mouse as a reservoir for tularemia, however, is probably lower considering the short survival following infection and family social structure (Zgrabczynska and Pilacinska, 2002).

In contrast, common voles which live in colonies and reach population peaks every three to five years (Tkadlec and Stenseth, 2001; Lambin et al., 2006) may be considered important hosts, associated with outbreaks of tularemia (Pikula et al., 2002). Median survival times of common voles in the present study were twice longer than in BALB/c mice and yellow-necked mice and ranged from 6.2 to 11 days depending on the infectious dose. The calculated LD_{50} of 37.9 *F. tularensis* CFU in common voles is by two orders of magnitude higher than in BALB/c mice. The dose of 16 *F. tularensis* CFU induced mortality of only 20% in common voles and the surviving specimens must have been able to eliminate the infection because tissue samples taken after killing these animals 37 days post exposure were negative in culture and biological trials using mice. However, similar to the observations of other authors (Bell and Stewart, 1975; Shlygina and Olsufyev, 1982; Shlygina et al., 1987, 1989), we noticed persistent or unproductive infection in one surviving vole following experimental infection with 320 *F. tularensis* CFU administered subcutaneously. Quantification of bacteria in the tissues of this animal killed on Day 37 post exposure revealed low numbers of *F. tularensis* CFU. Nevertheless, the total numbers of bacteria in the tissues of this survivor still amounted to levels sufficient for the spread of infection by, for example, cannibalism (Bell and Stewart, 1975; Petersen et al., 2004; Petersen and Schriefer, 2005) in an overcrowded population. Generally, tissue bacterial burdens in common voles start to develop later post exposure and amount to lower levels than in laboratory mice with the only exception of the higher *F. tularensis* burden in kidneys of common voles during terminal stages of infection. This finding corresponds with reports on chronic shedding tularemia nephritis in *Microtus pennsylvanicus* (Bell and Stewart, 1975). Common voles (*Microtus arvalis*), like prairie dogs (Petersen et al., 2004), are part of the terrestrial cycle of tularemia due to their selection of grassland habitats (Delattre et al., 1996). Interestingly, the epizootics of tularemia among wild rodents have mainly been reported during autumn months (Gurycova et al., 2001; Vyrostekova et al., 2002). This seasonal variation may be due to changes in the dynamics of host and vector populations during the year, and/or in the prevalence or virulence of the pathogen or immunologic features of hosts (Dowell, 2001).

Regarding the importance of common voles in the tularemia cycle and questions concerning outcomes of infection in this host, further studies into problems such as the influence of population characteristics and cycles, overcrowding, co-exposure

to environmental stressors including organic and inorganic pollution or pathogens, are needed to enhance our knowledge of this complex zoonotic disease.

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