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Full-length manuscript

Mycobacterium microti tuberculosis in its maintenance host, the field vole (*Microtus agrestis*): characterization of the disease and possible routes of transmission

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Abstract

The field vole (*Microtus agrestis*) is a known maintenance host of *Mycobacterium microti*. Previous studies have shown that infected animals develop tuberculosis. However, the disease is also known in cats and is sporadically reported from humans and other mammalian species. We examined trapped field voles from an endemic area, using a range of diagnostic approaches. These confirmed that a combination of gross and histological examination with culture is most appropriate to identify the true prevalence of the disease, which was shown to be over 13% at times when older animals that have previously been shown to be more likely to deveop the disease, dominate the population. The thorough pathological examination of diseased animals showed that voles generally develop systemic disease with most frequent involvement of spleen and liver, followed by skin, lymph nodes and lungs. The morphology of the lesions was consistent with active disease and their distribution suggested skin wounds

or oral and/or aerogenic infection as the main portal of entry. The demonstration of mycobacteria in open skin lesions, airways and salivary gland indicated bacterial shedding from the skin and with sputum and saliva. This suggests not only the environment, but also direct contact and devouring as likely sources of infection.

Key words: field vole (*Microtus agrestis*), *Mycobacterium microti*, pathogenesis, transmission, tuberculosis

Tuberculosis in field voles (*Microtis agrestis*, Linnaeus 1761) was first reported in 1937.[43] The causative agent, initially named *Mycobacterium tuberculosis* subsp. *muris*, is now called *M. microti* and is a member of the *M. tuberculosis* complex.[41,42] Differentiation of *M. microti* from other members of the *M. tuberculosis* complex is difficult on the basis of biochemical properties, but is readily possible by genotypic methods.[7,19,40,42] Based on phylogenetic analyses, *M. microti* could now be classified as a vole adapted ecotype of the *M. tuberculosis* complex.[31]

The field vole has been identified as the most likely maintenance host of *M. microti* in Great Britain.[5,7,32] However, natural infection has also been observed in other small rodents.[7,44] Sporadic human cases have been reported throughout Europe, and a range of other mammalian species, including cats, alpacas, llamas, badgers, pigs, cattle, horses, dogs and ferrets have been diagnosed with tuberculosis due to *M. microti*; among these, cats are most frequently affected.[28,32] Molecular typing of a large number of *M. microti* strains has identified a geographically localized genotypic diversity, similar to that seen for *M. bovis* in cattle.[32]

A total of 27 human cases of *M. microti* tuberculosis have been reported, both in immunocompetent and immunosuppressed patients in Western Europe (including Great Britain, The Netherlands, Germany and France), and in the vast majority (19/27) as pulmonary tuberculosis.[28] *M. microti* has in the past also been used in anti-tuberculosis vaccine trials.[4,25]

Vole tuberculosis is also of ecological interest due to its adverse effect on the condition and possibly also the survival of field voles in endemic areas.[5] In the wild, the disease does not affect juvenile voles and has an increasing prevalence with age.[5] Skin lesions, which have been used to diagnose the disease in host individuals, only occur in advanced stages and are therefore most prevalent in older animals.[5,32,43,44] So far, however, more detailed studies on

the pathological features and the pathogenesis of vole tuberculosis are scarce and focussed mainly on the hallmark lesions in the skin, which can confirm the disease but have been shown to underestimate its actual prevalence.[5,8,25,29,31] Its role in the dynamics of natural populations cannot be evaluated with confidence until its prevalence can be determined accurately and major routes of transmission identified. Therefore, we have undertaken a thorough pathological examination of the type, distribution and composition of *M. microti* induced tuberculosis in wild field voles from which we draw conclusions on the possible natural routes of transmission to voles, and from these to other species, such as cats.

MATERIALS AND METHODS

Field Study and Animals

The study was performed on wild field voles in Kielder forest, a 600 km^2 coniferous plantation forest in Northumberland, at the border between England and Scotland, UK. Field voles in Kielder forest had previously been identified as an endemically *M. microti*-infected population, with an 8% prevalence of tuberculosis, based on the presence of characteristic skin lesions.[7,43] Trapping was performed biannually, in spring (March), before the breeding season, when only old, over-wintered animals (6-8 months old) are present, and in autumn (September), during and after the breeding season (dominance of 2-3 month old voles), between September 2001 and March 2003 (4 surveys) and between March 2005 and March 2007 (5 surveys).[5] Field voles were trapped in grassy clear-cuts within a coniferous forest. Field voles in Kielder exhibit cyclic dynamics and hence a wide range of population densities were sampled throughout the study period.[23] All animals were euthanized by exposure to isofluorane and subsequent dislocation of the cervical vertebrae. They were necropsied and thoroughly examined to identify any gross tuberculosis lesions. From the first three surveys,

gross lesions were collected for histological confirmation of the disease, and culture was performed on lymph nodes (pooled superficial cervical, axillary and brachial lymph nodes) of voles from the survey in March 2002 and lymph nodes and lung of animals from the survey in September 2002. From all animals trapped in March 2003, bacterial cultures were undertaken from lungs and lymph nodes, and organs (lungs, lymph nodes, spleens and liver) were examined histologically in addition to and regardless of the presence of any gross lesions. In addition, from animals trapped in March 2006, lungs, spleens and livers were examined histologically and cultured independent of the presence of gross lesions. From all other animals in the five surveys from March 2005 to March 2007, organs/tissues with gross tuberculosis lesions were examined by histology and bacterial culture. PCR was employed on cultures and/or gross lesions to confirm *M. microti* infection.[5,24] The mycobacterial species was confirmed by spoligotyping and IS6110 RFLP typing of positive cultures.[5,19,40]

Case Selection and Tissue Processing

For a thorough assessment of the frequency and distribution of tuberculosis lesions, all voles from the survey in March 2003 (n=327) were used. From these animals, sections of lungs, spleen, liver, kidneys, small intestine and lymph nodes (see above) as well as all gross lesions indicative of tuberculosis were examined histologically. Gross lesions were classified as "external lesions", when obvious skin lesions with crust formation were observed, whereas "internal lesions" comprised those that were only obvious after skinning (mainly in lymph nodes) and any gross lesions within internal organs. In addition, gross lesions consistent with tuberculosis from all surveys were examined histologically.

Tissues were fixed in 10% non-buffered formalin, either fresh (surveys in 2001-2003 and in March 2006) or after freezing and subsequent thawing (surveys in 2005, September 2006 and March 2007) and routinely paraffin embedded. 3-5 µm-thick sections were prepared and

stained with hematoxylin-eosin (HE), the Ziehl-Neelsen (ZN) stain for the demonstration of acid-fast bacilli (AFB) and, in selected cases, the van Kossa stain for the demonstration of calcium salt deposition (mineralization). Additional sections were used for immunohistological examinations.

Immunohistology

For the identification of inflammatory cells within the tuberculous lesions, antibodies known to cross-react in many mammalian species were tested in the spleens of field voles and applied to sections of tuberculous lesions to demonstrate T cells (rabbit anti-human CD3, Dako, Glostrup, Denmark), B cells (rat anti-human CD79a, Dako) and neutrophils as well as macrophages including epithelioid cells and multinucleate giant cells (rabbit anti-swine lysozyme, Dako). The peroxidase anti-peroxidase (PAP) and the avidin biotin peroxidase complex (ABC) method were applied according to previously published protocols.[18,21]

Consecutive sections, incubated with normal rabbit serum or a non-reacting mouse monoclonal antibody, respectively, were used as negative controls for polyclonal and monoclonal antibodies. A canine lymph node served as positive control for all antigens and the mesenteric lymph node section included from all voles served to confirm cross-reactivity with vole leukocytes.

Transmission Electron Microscopy (TEM)

Sections of grossly identified lung lesions that were subsequently confirmed as tuberculous lesions by histology on adjacent tissue sections from a vole trapped in March 2006 were routinely processed for TEM. Briefly, tissue blocks (2 mm^3) were prepared and fixed in 4% paraformaldehyde (pH 7.4) with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4°C, then washed in 0.1M sodium cacodylate buffer and secondarily fixed in 1% osmium

159 tetroxide$_{(aq)}$ for 90 min, followed by routine resin embedding and the preparation of semi-thin
160 sections (0.5µm) for the identification of areas of interest, and the ultrastructural examination
161 of subsequently prepared ultrathin sections (60 nm).
162
163
164 **Results**
165 *Comparison of Diagnostic Methods*
166 For a comparison of the sensitivity of the different methods (assessment of external and
167 internal gross lesions, histology for the identification of tuberculous lesions, mycobacterial
168 culture) for the detection/assessment of tuberculosis and mycobacterial infection, all 327
169 animals collected in March 2003 were examined (Fig. 1). Based on the presence of external
170 gross lesions (prior to dissection; all confirmed by histology), a disease prevalence of 4.3%
171 (n=14) was identified. Internal gross lesions were observed in about twice as many cases
172 (8.9%, n=27), whereas the histological examination identified tuberculous lesions in 9.8%
173 (n=32) of the voles. Four of the latter animals (12.5%) were negative in the culture. On the
174 other hand, *M. microti* was isolated from 11.9% (n=39) of animals, nine of these (23.1%) did
175 not exhibit any gross and histological alterations indicative of the disease. Altogether, 13.2%
176 (43/327) of animals were identified as infected with *M. microti*, by histopathology and/or
177 culture.
178
179 *Gross Tuberculosis Lesions*
180 **External lesions** were represented by (multi)focal, nodular skin areas with superficial
181 ulceration and crust formation, often between the shoulder blades (March 2003: n=10 (31.3%
182 of voles with histologically confirmed tuberculosis); Fig. 2a), but also in the neck midline
183 (Fig. 2a, b), on one shoulder, and, less frequently, on the head, back, abdomen, chest and

rump. Lesions often extended into the subcutaneous adipose tissue (Fig. 2b). In severe cases, large areas of the skin at any site of the body were affected (Fig. 3). Occasionally, skin lesions were inconspicuous and externally obvious only as a subcutaneous nodule (Fig. 4).

Internal lesions were represented by granulomas in the adipose tissue between the shoulder blades (consistent with the pyramid shaped so-called anterior subcutaneous fat compartment described in mice) or in the neck midline (Fig. 2b), (multiple) granulomas within enlarged lymph nodes (mainly axillary/brachial and superficial cervical lymph nodes; Fig. 5) often extending into the adjacent (adipose) tissue, enlargement of mediastinal lymph nodes and granulomas within lungs (Fig. 6). Other internal organs did not exhibit any gross changes, apart from each one case with obvious granulomas in the mesenteric lymph nodes and the kidneys as well as the adrenal glands.

Distribution of Lesions, Based on Histological Examination

In the 32 diseased animals collected in March 2003, the histological examination identified tuberculous lesions in skin/subcutis, lymph nodes, salivary glands, lungs, liver and spleen (Fig. 7). Interestingly, liver (90.6%; n=29) and spleen (83.9%; n=26) were most frequently affected, confirming that all but two animals had developed systemic disease. Among other tissues, the **skin and subcutis** were most frequently affected (71.9%; n=23), almost exclusively at the cranial part of the body and mainly between the shoulder blades or at one shoulder, less frequently at the neck, head or thorax. In all but one animal where lesions were only found in the subcutis between the shoulder blades and the axillary lymph nodes, skin involvement was seen in association with overt systemic disease (i.e. lesions in spleen, liver, lung) and in the majority of animals (19/22) also with involvement of the local lymph nodes (superficial cervical and brachial/axillary), suggesting that the skin in areas where bite wound occur most frequently might have been the site of the primary lesion. The **brachial/axillary**

lymph nodes were affected in 46.9% of the cases. In two animals, one brachial lymph node was the only affected tissue and in a third vole, the only other lesion was in the subcutis between the shoulder blades. This suggests that the disease was confined to these lymph nodes (and their tributary skin area) and possibly at an early stage at the time of death of these voles. All other animals with lesions in the brachial/axillary lymph nodes showed systemic disease, as confirmed by the presence of liver and spleen lesions. The lack of further lesion sites in four of these voles (12.5%) suggests systemic spread of bacteria from an initial lymph node lesion, whereas the presence of lesions in tributary skin areas in the remaining 8 animals (25%) again indicates infection via the skin. Similarly, lesions in the **superficial cervical lymph nodes** (observed in 37.5% of voles with confirmed tuberculosis) were in the majority (9/12) seen with skin lesions at the head, neck or shoulder. In the three animals that did not exhibit skin lesions, involvement of this lymph node might be the consequence of oral uptake of bacteria. The **lung** exhibited lesions in 65.6% of affected animals, always together with the mediastinal lymph nodes and other tissues, i.e. spleen and liver as well as skin and draining lymph nodes (13/21), skin (6/21) or superficial cervical or brachial/axillary lymph nodes (2/12).

Morphological Features and Cellular Composition of Tuberculosis Lesions

Tuberculosis lesions generally exhibited similar morphological features. They represented non-demarcated focal granulomatous infiltrates without obvious organization or evidence of peripheral fibroblast proliferation, neovascularization or lymphatic follicle formation (Figs. 8, 9). Larger infiltrates showed central or multifocal caseous necrosis (Fig. 8) that often exhibited large irregular areas of dystrophic calcification (Fig. 8), as confirmed by the von Kossa stain. The latter also occurred as numerous variably sized, often concentric, laminated mineralization bodies cell-free in the infiltrate and in the periphery of the large necrotic areas

(Figs. 8, 9). Mineralization was also seen intracellularly as small laminated pearls within scattered macrophages (Fig. 8) and appeared to develop within mitochondria, as the ultrastructural examination showed (Fig. 10a, b). Apart from macrophages and epithelioid cells which were the dominant cells in the infiltrate, variable numbers of multinucleated giant cells (MGC) and numerous neutrophils were generally observed (Fig. 9). All these cells exhibited weak to intense lysozyme expression (Figs. 11, 12), which confirmed their activation.[20] Lymphocytes were generally numerous in the infiltrates, both disseminated and as variably sized aggregates mainly in the periphery. They were in the majority T cells, whereas B cells dominated in the focal aggregates. In most lesions, AFB were numerous. They were found within macrophages, epithelioid cells and MGCs where they often appeared to aggregate within large cytoplasmic vacuoles (phagosomes), and were evident as large cell-free clumps within necrotic areas (remnants of bacilli-laden, necrotic cells; Fig. 12).

In the **skin**, lesions were represented by a non-demarcated, focal granulomatous dermatitis, often with involvement of the subcutaneous adipose tissue (cellulitis) and superficial ulceration and crust formation (Figs. 8, 13). Occasionally, AFB were found within these crusts (Fig. 13, inset), confirming bacterial shedding into the environment. In one vole with skin lesions below the left ear, the granulomatous infiltration extended into the salivary gland.

Subcutaneous lesions between the shoulder blades and at the neck often extended into the underlying skeletal muscle layers and adipose tissue (Fig. 9), but were frequently seen without involvement of the overlying skin. In some animals, the subcutaneous adipose tissue between the shoulder blades exhibited mild focal, perivascular, granulomatous infiltration, without any evidence of AFB. Occasionally, a "fistule" like formation was seen between the shoulder blades, with a focal infiltrate extending from the upper dermis to the underlying skeletal muscle/adipose tissue, where a more extensive infiltration was present. Some

granulomatous infiltrates, however, were entirely restricted to the skeletal muscle in this area and contained large areas of necrosis and mineralization and usually myriads of AFB.

In affected **lymph nodes**, a focally extensive or multifocal coalescing granulomatous inflammation with extensive necrosis and mineralization was generally seen. Mineralization pearls were also present. The cellular composition was as described for the skin lesions. Inflammatory infiltrates often extended into the adjacent (adipose) tissue and, when the superficial cervical lymph node was affected, occasionally into the salivary gland (Fig. 9), likely leading to bacterial shedding with the saliva.

The typical focal granulomatous lesions as they were generally seen in affected **lungs** were occasionally associated with a (multi)focal necrotizing bronchitis and bronchiolitis with clumps of AFB in the inflammatory exudate (Fig. 15a, b), indicating discharge of bacteria with the sputum. In addition, variably intense perivascular and peribronchial B cell dominated lymphocyte accumulations, consistent with bronchus-associated lymphatic tissue (BALT) were observed (Fig. 15a).

Tuberculous lesions in the **spleens** differed from those in other tissues. They represented small focal aggregates of macrophages and epithelioid cells with occasional neutrophils in the red pulp, immediately adjacent to T cell zones and follicles (Fig. 16). MGC were not observed and AFB were very rare. However, occasional mineralization pearls were present, both cell free and within macrophages.

Livers exhibited disseminated lesions that were often located adjacent to portal areas and were represented by small granulomatous infiltrates composed of macrophages and epithelioid cells, often together with variable numbers of MGC and moderate numbers of lymphocytes, which were in the majority T cells (Figs. 17). AFB were very rare, but intra- and extracellular mineralization pearls were almost always observed (Fig. 17).

Apart from the presence of a bilateral granulomatous nephritis and adrenalitis and a granulomatous myocarditis with the presence of abundant AFB in each one case, the other examined tissues did not exhibit any histological changes and AFB were not identified.

Discussion

The present study comprises a thorough evaluation of the distribution and morphological features of *M. microti* induced tuberculosis in field voles, the maintenance host of the bacterium.[5,7,32] In particular in Great Britain, vole tuberculosis has been of interest as a factor affecting population dynamics of field voles in endemic areas.[5,7,8,43,44] In recent years, however, its association with feline tuberculosis has been identified and there are occasional reports of *M. microti* induced tuberculosis in other species including man, with sporadic geographic clustering.[14,32] Furthermore, a mutual geographic exclusivity of *M. microti* and *M. bovis* has been shown in the UK, indicating that *M. microti* may be a determinant of *M. bovis* epidemiology. [32]

In ecological studies, the prevalence of tuberculosis in field voles has usually been assessed on the basis of gross lesions.[5,7,8,44] While this is certainly the most efficient approach to assess large animal cohorts and for longitudinal monitoring of the disease status, it does not reflect the true disease prevalence. We observed the typical external tuberculous skin lesions in less than 50% of field voles with the disease, of which more than 15% did not exhibit any gross lesions at all. It has previously been shown in studies on tuberculosis in badgers that detection rates vary depending on the examination protocol.[11] Our study confirms these results and shows that for an accurate assessment of the disease prevalence in voles, a detailed histological examination of a range of tissues, including particularly liver and spleen, is essential. However, despite the detailed histological assessment, we did not

identify tuberculosis lesions in more than a quarter of the culture-positive voles. Mycobacterial infection without associated pathological changes has previously been shown in humans and badgers.[10,16] Macrophages are the primary site of mycobacterial replication and a means of dissemination, suggesting that low level infection of tissue macrophages as a result of bacteremia is responsible for positive culture results.[12,30,33] It remains to be clarified, whether infected voles will invariably develop the disease, can remain latently infected, or can clear the infection. On the other hand, we also identified a proportion of culture-negative animals with histologically confirmed tuberculosis lesions. This indicates that also culture has its limitations for the confirmation of *M. microti* tuberculosis and further confirms that a combined diagnostic approach is currently still required to identify the true prevalence of mycobacterial disease and/or infection in voles.

The distribution pattern of tuberculosis lesions in affected animals in our study confirms that vole tuberculosis is generally a systemic disease. Only in less than 10% of affected animals did we not find evidence of systemic disease (voles with lesions only in the brachial/axillary lymph node with or without involvement of a tributary area, i.e. the subcutis between the shoulder blades), which indicates that animals can generally not confine the bacteria locally.

Vole tuberculosis frequently involves the skin, and we found lesions in the subcutis and/or exophytic processes with ulceration and crusts/scab formation in more than 70% of affected animals.[7,8,44] These lesions did not represent a local process, but were in all but one case associated with systemic disease. In ulcerated lesions, we occasionally found mycobacteria in the superficial crusts, indicating that the skin lesions can be a source of infection for other animals, through release of bacteria into the environment, but also via direct contact.

Subcutaneous (and skin) lesions were almost exclusively observed in the cranial part of the body and were frequently seen between the shoulder blades and in the neck midline.

Thorough examination of the dorsal neck between atlas and thoracic spinal cord of disease-free voles did not identify lymphatic tissue or specific (scent) glands in this location. Instead, the known lymph nodes draining the affected skin areas, i.e. brachial/axillary and superficial cervical lymph nodes, were the most frequently involved. It therefore appears likely that the disease develops from a cutaneous infection, through skin lesions, such as bite wounds from fights or scratches that become infected from the contaminated environment. Since overt skin lesions are generally only seen in older voles, it seems also possible that they evolve at some stage from small inflammatory foci in the adipose tissue, which we also observed in some animals.[32] Alternatively, some skin lesions might be the consequence of bacterial spreading from the adjacent lymph nodes or bacteremia.[25,44] A more recent study provided evidence of the latter, since North American prairie voles (*Microtus ochrogaster*) developed "crusted caseous skin lesions" in particular between the shoulder blades, after experimental intraperitoneal infection with the vole strain ATCC 19422 at a high dose (Manabe, personal communication).[25] It remains to be elucidated whether the blood or lymphatic vessels supplying the mixed adipose tissue depots that voles exhibit at the dorsal neck and shoulder have some specific features that might “catch” infected monocytes.

Oral and/or aerogenic uptake of bacilli would be an alternative way of infection, and indeed, the presence of lesions in the superficial cervical lymph nodes without any associated skin lesions in two cases would be consistent with this. Similarly, Wells observed the development of tuberculous lesions in the “glands in the neck” in a proportion of voles housed together with an animal that had developed generalised lesions after subcutaneous infection with *M. microti*. He considered an oral infection from the shared feed as the likely source and showed in the same study that drinking of *M. microti* infected water induces lesions not only in this location, but also in lungs and mesenteric lymph nodes, thereby suggesting a combined oral and aerogenic infection.[44] We observed extension of the

358 inflammatory process from the superficial cervical lymph nodes into the adjacent tissue
359 including the salivary glands in two animals, which provides evidence of bacterial shedding
360 with the saliva.

361 We found tuberculous lesions in liver and spleen in more than 90% and 80% of the
362 affected animals, respectively. This indicates that the bacteria spread readily through the
363 lymphatic fluid and/or blood.[13] They would then be taken up by specific tissue macrophages,
364 Kupffer cells in the liver and red pulp macrophages in the spleen, leading to the development
365 of the observed disseminated small granulomas. Similar lesions have been reported in spleen
366 and liver of laboratory mice after intravenous infection with *M. tuberculosis* and *M. bovis*,
367 and in the spleen of *Microtus ochrogaster* voles that had been infected intraperitoneally with
368 *M. microti*.[1,9,25]

369 We never found tuberculous lesions in the lungs without systemic disease, i.e.
370 involvement of spleen and/or liver as well as skin and/or non-tributary lymph nodes. This
371 suggests that lung lesions are more often a consequence of bacteremia than of aerogenic
372 infection in naturally infected voles.[13] Pulmonary lesions in the voles represented
373 granulomas, but lacked all relevant features of the classical tuberculoma that dominates in
374 non-progressive pulmonary tuberculosis of human patients, such as the organized peripheral
375 arrangement of follicle-like B cell aggregates and the high vascularization of the tissue, as
376 well as a fibrotic capsule. Instead, they were similar to the disorganized granulomas of active
377 cavitary pulmonary tuberculosis in humans, from which bacilli are released into the
378 sputum.[33,37-39] In the voles, we also found bacilli within cell debris in the lumen of airways
379 and, occasionally, a necrotizing bronchitis, a feature also observed in laboratory mice after
380 intravenous *M. bovis* infection.[9] These findings confirm that diseased voles generally do not
381 develop latency, cannot contain bacteria within lesions, and can shed bacteria with the
382 sputum.

Interestingly, none of the recent studies, including ours, on both naturally and experimentally (intraperitoneally) infected voles, provided any evidence of involvement of the gastrointestinal tract. In contrast to Wells' early studies, who describe frequent involvement of the mesenteric lymph nodes, none reported lesions in the intestine and associated lymph nodes, or evidence of bacterial shedding with the feces.[7,25,44] There was also no evidence of bacterial shedding with the urine, neither by culture nor by histology in our study which only observed lesions in the kidneys in one out of more than 4000 examined animals.[7]

In the voles, the larger tuberculous lesions are granulomas with a core of caseous necrosis containing cell-free bacilli, surrounded by a variable mixture of partially bacilli-laden macrophages, epithelioid cells and MGC.[29] Caseous necrosis in tuberculous granulomas has been interpreted in different ways. Some authors believe that it is correlated with the strength of the hypersensitivity reaction mounted by an infected animal.[13] This would suggest that field voles develop a strong delayed type hypersensitivity (DTH), but weak cell mediated immunity (CMI), different from badgers with tuberculosis and laboratory mice infected with *M. bovis* and *M. tuberculosis*, where granulomas exhibit far less extensive necrosis and are believed to reflect a weak DTH reaction and good CMI through activated macrophages.[13,17] However, other studies indicated that the necrotic areas are simply the result of hypoxia and macrophage and neutrophil death with release of neutrophil enzymes.[36] We observed marked dystrophic mineralization within necrotic areas. A study on experimental tuberculosis in guinea pigs suggests that this is related to the presence of activated macrophages, since mineralization was seen in association with the accumulation of extracellular ferric iron as a consequence of transferrin receptor, H ferritin and lactoferrin expression on activated macrophages and neutrophils.[2] Mycobacteria, like all living organisms, require iron as a cofactor for proteins and have been shown to scavenge it from the host within phagosomes,

but might also be able to make use of free iron. However, extracellular iron can also initiate extracellular calcification and is toxic for both cells and mycobacteria by catalyzing the generation of reactive oxygen intermediates and can thereby contribute to necrosis and the reduced bacterial viability observed in granulomas.[2,33] Interestingly, *M. bovis* appears not to induce significant necrosis and mineralization within lesions in field voles, which suggests that the reaction mounted in response to the bacteria is influenced by the bacterium rather than the host.[2,29] However, we also observed marked intracellular mineralization of macrophages within granulomas, mainly at the border to necrotic areas. Some of these cells also contained mycobacteria, but the ultrastructural examination did not identify bacilli as the mineralisation core. Instead, it revealed that the mineralization started in the mitochondria, a phenomenon that is initiated by the influx of calcium into mitochondria of dying cells. In tuberculous granulomas, this is likely a consequence of hypoxia, which could also be mediated by vascular leakage.[2,35,36]

The dominance of macrophages and neutrophils and the limited lymphocyte contribution, i.e. the lack of lymphocyte accumulations in the periphery of tuberculous infiltrates in our study indicates a dominance and/or persistence of an innate immune response. Neutrophils in the lesions could contribute to a reduction in bacterial load. They have been shown to rapidly phagocytose *M. microti* and form phagolysosomes, different from macrophages, in which the bacilli inhibit phagosome-lysosome fusion by inhibition of lysosomal movements.[12,30] However, they are likely to contribute also to the tissue damage due to their degranulation with death.[36] T cells, which would be the hallmark of the DTH, are present but do not lead to the formation of an organized granuloma which walls off the necrotic core in humans and other animal model species of tuberculosis.[30,36,37-39] It is possible that in vole tuberculosis the proinflammatory cytokine response is only limited, which would inhibit tuberculoma formation and persistent infection, but allow constant progression of the disease, a scenario

433 that has also been proposed for some strains of *M. tuberculosis*.[33] In field voles, granulomas
434 contain ample MCG, cells that generally mediate physical containment of the bacilli,
435 inhibiting cell to cell spread by forming a peripheral ring of nuclei around the bacilli that
436 aggregate centrally in the cytoplasm.[6] The latter has been induced *in vitro* by IFN-γ in
437 combination with IL-3 or GM-CSF, cytokines that could be released by macrophages and
438 infiltrating T cells.[26]

439 While a range of species including humans have been shown to be susceptible to the
440 disease, tuberculosis due to *M. microti* is particularly relevant in cats in Great Britain, where
441 it is considered a spillover from tuberculosis in small mammals, such as the field vole, in the
442 wild.[32] In cats, the disease mainly affects the skin and associated lymph nodes of the head or,
443 though less frequently, the limbs, tail, neck and torso, but generalized disease involving lung,
444 liver, spleen and mesenteric lymph nodes is also seen. Affected cats mainly live in extra-
445 urban areas and the case distribution confirms that the agent is endemic in certain areas which
446 at the same time show a low *M. bovis* tuberculosis incidence in cattle, badgers and cats,
447 suggesting that both bacteria induce protective immunity against the other.[15,32] While most
448 lesions in cats appear to be a consequence of hunting, i.e. wounds inflicted by the prey
449 animal, cases without skin lesions would indicate oral or aerogenic infection. Considering the
450 results of the present study, infection of cats from the environment contaminated with
451 bacteria from sputum, saliva or skin crusts of diseased voles would also be a possibility. Wild
452 mammalian carnivores such as foxes (*Vulpes vulpes*) and weasels (*Mustela nivalis*) that rely
453 on field voles as their main prey are also likely to have substantial exposure to *M. microti*
454 both from ingestion and their environment.[3,27] Further studies would be needed to investigate
455 the potential implications for their health status.

456 In humans, the disease mainly manifests as pulmonary tuberculosis. Since the first case in
457 1998, a total of 27 cases have been reported; both immunosuppressed and immunocompetent

458 patients were affected.[28] One report from Scotland identified some regional clustering of
459 human (four patients) and animal (two cats, each one badger, ferret and llama) cases,
460 although the genotyping showed a mixture between vole and llama type.[14] Epidemiologic
461 investigation of the reported human cases have failed to link them with infected cats or wild
462 rodents. However, we have shown that diseased voles can shed the bacilli into the
463 environment. At least in endemic areas, this could offer an explanation for sporadic human
464 infections. At present, studies on the geographic distribution of *M. microti* in wild rodents in
465 Europe are limited to Great Britain.[5,7,8] They were initiated due to the interest of our group in
466 the population dynamics of wild rodents suffering from endemic pathogens generally.[43,44]
467 Field voles (*Microtus agrestis*) are widely distributed throughout Europe including Russia to
468 south-east Siberia, with the exception of Ireland, Iceland and southernmost Europe, and with
469 fluctuation in the population density in 3-4 year cycles
470 (http://www.iucnredlist.org/details/13426/0). However, the presence of the disease might not
471 be recognized easily in endemic areas with low disease prevalence despite the frequent
472 external lesions; also, Wells assumed that it was very unusual to find dead voles in the field
473 because they were rapidly devoured by other voles.[43] Nonetheless, since geographic
474 clustering has been shown, a systematic approach could clarify whether the field vole is the
475 maintenance host for *M. microti* also in other regions in Europe or whether other common
476 small rodents have to be considered.[32]
477
478
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627

628 **Figure Legends:**

629

630 **Fig. 1**. Prevalence of tuberculosis/*M. microti* infection in one survey (survey 4; n=327)
631 assessed by different detection methods (external and internal gross lesions, histologically
632 confirmed tuberculosis lesions, mycobacterial culture). "Infection total" represents the results
633 of histology and culture.

634

635 **Fig. 2**. Gross lesions of *M. microti* tuberculosis in a field vole. a) Skin scab in dorsal midline,
636 between shoulder blades (arrow). b) After partial skinning of the animal in a), the
637 inflammatory process underlying the external skin lesion is obvious as an area of hyperaemia
638 in the adipose tissue cushion between the shoulder blades (arrow). A further granulomatous
639 lesion is present in the adipose tissue in the neck midline (arrowhead).

640

641 **Fig. 3**. *M. microti* tuberculosis in a field vole, represented by severe, multifocal skin lesions
642 with superficial ulceration and crust formation (arrows).

643

644 **Fig. 4**. *M. microti* tuberculosis in a field vole. Foreleg with extensive multifocal, coalescing
645 granulomatous cellulitis (arrow), involving the axillary lymph node.

646

647 **Fig. 5**. *M. microti* tuberculosis in a field vole. Foreleg with multifocal, granulomatous
648 lymphadenitis (brachial lymph node; arrow).

649

650 **Fig. 6**. *M. microti* tuberculosis in a field vole. Lung, left main lobe. Multifocal granulomatous
651 pneumonia, represented by well-demarcated, elevated whitish nodules with central
652 mineralisation (arrows).
653
654 **Fig. 7**. Organ distribution of histologically confirmed tuberculosis lesions in affected animals
655 (n=32; Survey 4). "Skin" refers to external lesions and those involving and/or restricted to the
656 subcutis. Brachial and axillary are counted together, since they could not be discerned when
657 extensive granulomatous lesions that extended into the surrounding adipose tissue were
658 present. Mediastinal lymph nodes are not mentioned separately, since they were involved
659 when lesions were observed in the lungs.
660
661 **Fig. 8**. *M. microti* tuberculous lesion in a field vole. Histogical features of tuberculous skin
662 lesion. Granulomatous infiltrate with extensive focal caseous necrosis (highlighted by stars).
663 Multifocal mineralisation is seen, within the necrotic area (M), between infiltrating
664 inflammatory cells (arrows) and intracellularly, within individual macrophages (arrowhead;
665 see also inset). HE stain.
666
667 **Fig. 9**. *M. microti* tuberculous lesion in a field vole. Subcutaneous adipose tissue (A) between
668 shoulder blades. Non-demarcated granulomatous infiltrate dominated by macrophages, with a
669 large proportion of neutrophils (arrows). Scattered foci of mineralization are present
670 (arrowheads). HE stain.
671
672 **Fig. 10**. *M. microti* tuberculous lesion in a field vole. Epithelioid cell in granulomatous
673 infiltrate. a) The cell exhibits several mineralisation foci (consistent with mitochondria) in the
674 cytoplasm (arrows). Arrowheads: intact mitochondria. b) Cytoplasm. Beside intact

675 mitochondria (arrowheads), there are three enlarged and mineralised mitochondria, in one of
676 which remnants of the normal mitochondrial structures are still evident (arrow).
677 Ultramicrographs.
678
679 **Fig. 11**. *M. microti* tuberculous lesion in a field vole. Epithelioid macrophages (arrowheads)
680 and multinucleated giant cells (arrows) exhibit strong lysozyme expression. Peroxidase anti-
681 peroxidase method, Papanicolaou's haematoxylin counterstain.
682
683 **Fig. 12**. *M. microti* tuberculous lesion in a field vole. Macrophages and acid fast bacilli.
684 Epithelioid macrophage (arrowhead) and multinucleated giant cell (arrow) with large
685 aggregate of acid fast bacilli within phagosome. ZN stain.Upper inset: Lysozyme expression
686 in epithelioid macrophages and in particular in multinucleated giant cells. Peroxidase anti-
687 peroxidase method, Papanicolaou's haematoxylin counterstain. Lower inset: Epithelioid
688 macrophages are laden with intracytoplasmic bacilli that are located within phagosomes
689 (arrows). HE stain.
690
691 **Fig. 13**. Field vole with *M. microti* tuberculosis. Skin lesion with granulomatous dermatitis
692 and superficial crust formation. HE stain. Inset: Epidermal serocellular crust with aggregate
693 of acid fast bacilli on the surface (arrowhead). ZN stain.
694
695 **Fig. 14**. Field vole with *M. microti* tuberculosis. Salivary gland. The majority of the glandular
696 tissue is effaced by the extensive granulomatous infiltrate. Glandular acini are only seen in
697 the periphery (arrows); D: secretory duct. HE stain.
698

699 **Fig. 15**. Field vole with *M. microti* tuberculous lesions in the lung. a) Non-demarcated
700 granulomatous infiltrate (highlighted with stars) with a few perivascular lymphocyte
701 infiltrates in the periphery (arrows). HE stain. b) Within the granulomatous infiltrate, bronchi
702 exhibit focal loss of epithelial cells (highlighted by star) and mild neutrophil and macrophage
703 infiltration (arrowhead). The lumen of the bronchi is filled with exudate containing acid fast
704 bacilli both cell free and within macrophages. ZN stain.
705
706 **Fig 16**. Field vole with *M. microti* tuberculosis. Spleen with multifocal granulomatous
707 infiltrates (highlighted by asterisks) in the red pulp, close to T cell zones (T). Scattered foci of
708 mineralization are seen (arrowheads); inset: higher magnification of focal mineralisation. HE
709 stain.
710
711 **Fig. 17**. Field vole with *M. microti* tuberculosis. Liver with small focal granulomatous
712 infiltrates (arrows) composed of lysozyme positive macrophages (upper inset) and T cells
713 (CD3+; lower inset). Small mineralisation pearls (arrowheads) are present. HE stain and
714 Peroxidase anti-peroxidase method, Papanicolaou's haematoxylin counterstain.