Long-term survival of tuberculosis complex mycobacteria in soil

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INTRODUCTION

Tuberculosis is a deadly infectious disease that is caused by one of nine closely related mycobacterial species, which form the Mycobacterium tuberculosis complex (MTC) (Alexander et al., 2010; van Ingen et al., 2012). Tuberculosis plagues several mammalian species, including humans, non-human primates and other mammal species, as well as psittacine birds (Ghodbane & Drancourt, 2013). Every MTC species has a limited range of natural hosts, but there is no specific barrier and any MTC species can potentially infect any susceptible host species (Djelouadji et al., 2011). Humans are infected worldwide by sympatric M. tuberculosis sensu stricto families (Gagneux et al., 2006), whereas patients who are exposed in Africa may also be infected by Mycobacterium africanum (de Jong et al., 2010) and Mycobacterium canettii (Koeck et al., 2011; Van Soolingen et al., 1997). Zoonotic tuberculosis can be due to Mycobacterium caprae, Mycobacterium microti (Panteix et al., 2010) or the bovine tuberculosis agent Mycobacterium bovis (Thoen & LoBue, 2007). Conversely, captive mammals that are in close contact with humans, such as non-human primates and Asian elephants, can be infected by M. tuberculosis (Ghodbane & Drancourt, 2013).

The circulation of the MTC species between hosts is not fully understood. M. tuberculosis is an airborne organism transmitted from one individual with pulmonary tuberculosis to a new individual through direct contact. The ingestion of M. bovis-contaminated animal stuffs is another route of zoonotic tuberculosis (Thoen & LoBue, 2007). This route of transmission has also been discovered in mammals that were diagnosed with bovine tuberculosis (Palmer et al., 2012). In these herbivores, it was postulated that infected soil could be a source of contamination. Accordingly, there is evidence for the persistence of M. bovis in soil. M. bovis mycobacteria have been recovered from soil specimens after the experimental inoculation of soils (Duffield & Young, 1985; Fine et al., 2011), as well as from soils naturally infected by the excreta of various mammal species (Young et al., 2005). Whether survival in soil occurs for the other MTC species is unknown and has not been well studied.

Here, we investigated the possibility that representatives of three MTC species that are encountered in human

While there is evidence for the persistence of Mycobacterium bovis in soil, there are no reports for the other Mycobacterium tuberculosis complex (MTC) mycobacteria. Here, soil was inoculated with $10^8$ c.f.u. g$^{-1}$ M. tuberculosis, M. bovis and M. canettii and subcultured monthly for 12 months. The pathogenicity of mycobacterial colonies, identified by using matrix-assisted laser desorption/ionization time of flight mass spectrometry, was assessed in a mouse model. Moreover, mice were fed with food that contained 16.7% M. tuberculosis-contaminated soil. The three tested MTC species survived in soil for 12 months with a final inoculum of $2 \times 10^3$ c.f.u. g$^{-1}$ for M. tuberculosis, $150$ c.f.u. g$^{-1}$ for M. bovis and $2 \times 10^4$ c.f.u. g$^{-1}$ for M. canettii. In an experiment that included negative controls, all (5/5) mice inoculated with such M. tuberculosis and M. canettii developed 0.03–0.3 granulomas mm$^{-2}$ in their lungs and spleen and grew mycobacteria; five mice that were inoculated with M. bovis from soil did not develop granulomas but grew mycobacteria. Furthermore, 0.2–0.4 granulomas mm$^{-2}$ were observed in the lungs and spleen of 3/5 mice fed with M. tuberculosis-contaminated soil in the presence of two negative control mice. M. tuberculosis grew in the stomach, intestine, spleen and lung in 5/5 challenged mice, whereas the negative controls remained M. tuberculosis-free ($P=0.008$, Fisher exact test). This study provides clear evidence that MTC mycobacteria survive in soil, and that M. tuberculosis remains virulent while in the soil, outside its hosts, for extended periods of time.

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Abbreviations: MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; MTC, Mycobacterium tuberculosis complex.
pulmonary tuberculosis, namely, *M. tuberculosis*, *M. canetti* and *M. bovis*, could survive in soil and that such contaminated soil could be a source for experimental infection.

**METHODS**

**Ethics statement.** Protocols used for mouse experiments were approved by Marseille Medical School Animal Ethics Committee.

**Soil inoculation and sampling.** All manipulations involving MTC organisms were performed in a Biosafety Level 3 laboratory (BSL3). A clinical isolate of *M. tuberculosis*, *M. canetti* CIP140010059⁷ and *M. bovis* CIP671203 was cultured on Middlebrook7H10 agar supplemented with oleic–albumin–glucose–catalase (OADC) (Becton Dickinson) and sodium pyruvate for culturing *M. bovis* at 37 °C in a 5% CO₂-enriched atmosphere. Natural soil was collected in the Marseille area. No specific permissions were required for these activities; field studies did not involve endangered or protected species. The soil sample was analysed for granulometry, mineral and biological composition (LCA Laboratories). The results were repeated and validated according to the accreditation of the laboratory (Table 1). Soil was then steam-sterilized and further humidified by adding 2 ml sterile distilled water per 50 g sterilized soil. Sterility was assessed by inoculating 5 g humidified soil onto 7H10 agar incubated at 37 °C under a 5% CO₂ atmosphere for 4 weeks. Sterilized soil was used as negative control in all experiments. Sterilized soil was then thoroughly mixed with each of the MTC organisms studied and suspended in sterile PBS to achieve a homogeneous inoculum of 10⁶ c.f.u. (g soil)⁻¹, and 50 g of this preparation was placed in an opaque sterile plastic container that was incubated at room temperature for 12 months. Room temperature was monitored weekly and sterile distilled water (1 ml) was gently added twice per month to the container. Before each sampling, soil was mixed with a sterilized fork to ensure the homogeneity of the sample. An averaged 250 mg aliquot of inoculated soil was sampled 24 h post-inoculation and then every month for 12 months. Aliquots of 250 mg were suspended in 250 ml PBS and were serially diluted (from 10¹ to 10⁸) and immediately plated on OADC-enriched Middlebrook 7H10 agar supplemented with sodium pyruvate for *M. bovis* in parallel with negative controls of non-inoculated soil. Plates were incubated at 37 °C for 4 weeks and colonies were counted. Colonies were identified as MTC organisms after Ziehl–Neelsen staining and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) identification; colonies were collected and prepared using the previously described protocol and then applied to a MALDI-TOF-MS 96 Microflex target (Bruker Daltonic) (El Kheîchine et al. 2011). Further, an aliquot of soil inoculated for 12 months with *M. tuberculosis* was filtered at 5 µm to remove large particles, and mycobacteria were observed by optical microscopy after Ziehl–Neelsen staining and by electron microscopy.

**Inoculation of mice.** BALB/c female mice weighing 17–18 g (Charles River) were kept under the BSL3 cabinet for 5 days before inoculation. This experiment aimed to determine whether mycobacteria isolated from soil 12 months post-contamination were still virulent; thus, MTC mycobacteria grown from soil inoculated for 12 months were used. The mice were separated into four groups of five each. Three groups were infected intraperitoneally with 100 µl of a 1 × 10⁶ c.f.u. ml⁻¹ suspension of *M. tuberculosis*, *M. bovis* or *M. canetti* that had been cultured from soil inoculated for 12 months; one negative control group was inoculated with 100 µl sterile PBS. The weight, clinical examination and survival of mice were followed weekly for 90 days. All mice (all survivors) were killed by exsanguination at day 90 post-infection. The lungs, spleen and liver were removed. One portion of each organ was homogenized in Middlebrook 7H9 broth to determine the mycobacterial load after serial dilutions and plating on Middlebrook 7H10 at 37 °C under a 5% CO₂ atmosphere for 10 days. The remaining portion of each organ was fixed with 10% formalin for histopathological analyses.

**Feeding of mice with infected soil.** In order to determine whether soil contaminated for 12 months with *M. tuberculosis* could be a natural source of infection, a group of five BALB/c female mice weighing 17–18 g were fed for 60 days with food contaminated with *M. tuberculosis*-inoculated soil (20 g soil contaminated for 12 months was added to 100 g food). A negative control group that consisted of five mice was fed with food mixed with mycobacteria-free soil in the same proportion. Weight, clinical examination and survival of mice were followed weekly during 60 days of feeding. The mice (all survivors) were killed by exsanguination after 60 days of feeding. The lungs, spleen, stomach and large intestine were removed and examined for histopathology and cultured for *M. tuberculosis*, as described above.

**Histopathology.** The lungs, spleen and liver of mice were perfused with 10% formalin dissolved in PBS, immersed for 24 h in the same fixative and paraffin-embedded. Transverse sections, 5 µm, taken via the hilus, were stained with haematoxylin and eosin. Histopathology was performed blind, as the pathologist (H. L.) examined coded slides that did not reveal whether specimens had been collected from controls or from challenged animals. Granuloma was defined as an area of nodular inflammatory lesions that comprised lymphocytes and macrophages.

**Statistical analyses.** All statistical analyses in this study were performed using the Fisher exact test with a significance level of *P* = 0.05.

**RESULTS**

**Axenic culture of MTC organisms from contaminated soil**

During the 12-month observation period, the room temperature varied from 18 °C to 24 °C in the BSL3 laboratory where inoculated soil samples were kept. Whereas negative controls remained sterile, soil seeded with *M. tuberculosis* reproducibly grew *M. tuberculosis* at every checkpoint with a yield of 2 × 10⁵ c.f.u. g⁻¹ at month 12 post-inoculation; the soil seeded with *M. bovis*...
reproducibly grew *M. bovis* at every checkpoint, with a yield of 150 c.f.u. g\(^{-1}\) at month 12 post-inoculation. Lastly, soil seeded with *M. canettii* reproducibly grew *M. canettii* at every checkpoint with a yield of 2\(^\times\)10\(^4\) c.f.u. g\(^{-1}\) at month 12 post-inoculation (Fig. 1). Colony morphology did not vary with time and remained characteristic of each species, with the expected morphology of mycobacteria after Ziehl–Neelsen staining. Also, Ziehl–Neelsen staining confirmed the presence of mycobacteria in the soil sample (Fig. 2a) and electron microscopy imaging showed normal mycobacteria morphology (Fig. 2b).

**Mouse model**

In this experiment, at 90 days p.i. with mycobacteria cultured from the seeded soil specimen, we observed no significant difference (\(P>0.05\)) in the weight of mycobacteria-inoculated and negative control mice. Gross organ examination at 90 days post-infection revealed macroscopic lesions (Fig. 3) but no significant difference (\(P>0.05\)) in the weight of spleen, liver and lungs between mycobacteria-inoculated and negative control mice. However, the lungs of mice inoculated with *M. tuberculosis* or *M. canettii* exhibited means of 0.033 nodules mm\(^{-2}\) and 0.320 nodules mm\(^{-2}\), respectively. For the spleen, mice challenged with *M. tuberculosis* and *M. canettii* showed means of 0.0332 nodules mm\(^{-2}\) and 0.157 nodules mm\(^{-2}\), respectively. For the liver, only mice challenged with *M. canettii* showed nodules, with a mean of 0.087 nodules mm\(^{-2}\). For mice challenged with *M. bovis*, all tissues were free of nodules. Likewise, tissues collected from negative controls remained free of nodules. At 90 days post-infection the organs collected from negative control mice remained sterile whereas the mycobacterial load in the liver was 2\(^\times\)10\(^7\)\(\pm\)0.5\(\times\)10\(^3\) c.f.u. ml\(^{-1}\) for all mycobacteria; in the spleen it was of 8.4\(\times\)10\(^4\)\(\pm\)4.45\(\times\)10\(^4\) c.f.u. ml\(^{-1}\) for *M. tuberculosis*, 4.96\(\times\)10\(^4\)\(\pm\)1.21\(\times\)10\(^4\) c.f.u. ml\(^{-1}\) for *M. bovis* and 10\(^5\) c.f.u. ml\(^{-1}\)\(\pm\)4.08\(\times\)10\(^4\) for *M. canettii*; in the lungs it was of 3.29\(\times\)10\(^5\)\(\pm\)3.14\(\times\)10\(^5\) c.f.u. ml\(^{-1}\) for *M. tuberculosis*, 2.43\(\times\)10\(^5\)\(\pm\)1.38\(\times\)10\(^5\) c.f.u. ml\(^{-1}\) for *M. bovis* and 6.56\(\times\)10\(^4\)\(\pm\)1.76\(\times\)10\(^4\) c.f.u. ml\(^{-1}\) for *M. canettii*.

**Feeding of mice**

All mice used in this study survived experimental infection. Sixty days after feeding with *M. tuberculosis*-inoculated soil, there was no statistically significant difference between negative control and challenged mice in regard to weight and macroscopic findings in the lungs, spleen, stomach and large intestine. Tissues collected from the negative control mice were free of granulomas. Furthermore, no granulomas were observed in the stomach and the intestine of five challenged mice, 0.38 granulomas mm\(^{-2}\) lung were observed in one mouse, and 0.2 and 0.285 granulomas mm\(^{-2}\) spleen in two other challenged mice (Fig. 4). The organs collected from the negative control mice remained sterile, in contrast to the challenged animals, which all grew *M. tuberculosis*, with bacterial loads of 120\(\pm\)100 c.f.u. ml\(^{-1}\).
in the lung, 0 in the spleen, 1760 ± 1160 c.f.u. ml⁻¹ in the stomach and 140 ± 100 c.f.u. ml⁻¹ in the intestine (Fig. 4).

**DISCUSSION**

Here, we observed that three MTC species can survive in soil for 12 months. This observation was verified by the fact that the negative control plates, which were incubated in parallel with inoculated plates, remained sterile throughout the 12-month duration of the experiment, indicating that the growth of MTC mycobacteria reported herein did not merely result from contamination of the soil prior to or after experimental inoculation. Moreover, all of the colonies were conclusively identified using MALDI-TOF-MS as being the same *Mycobacterium* species as that used for the inoculation (El Khechine et al., 2011). In particular, non-tuberculosis mycobacteria that are known as common soil inhabitants (Salah et al., 2009) were not identified in either control or inoculated plates.

**Fig. 3.** Number of granulomas by organ (a) and bacterial load in each organ (b) in mice inoculated with mycobacteria seeded in soil for 12 months. Error bars indicate ± SD/SE.

**Fig. 4.** Histological and culture results for mice challenged by feeding: (a) histology of the spleen; (b) histology of the lung; (c) *M. tuberculosis* load in each organ; (d) mean number of granulomas in organs. Error bars in (c) indicate ± SD/SE. Magnification 10X. The arrows in (a) and (b) indicate granulomas.
These observations are entirely novel for M. canettii. This particular species, the agent of human tuberculosis in the Horn of Africa, is thought to have an environmental, yet unknown, reservoir (Koeck et al., 2011). Data herein reported indicate that M. canettii can significantly achieve more sustained survival in soil than M. tuberculosis and M. bovis. These data suggest it would be interesting to investigate the presence of M. canettii in soil samples collected in M. canettii-endemic regions.

A unique Russian report indicated that three different strains of M. tuberculosis, including the H37Rv strain studied herein, could be cultured for no longer than 3 months after being seeded in natural turf–podzol sandy soil (Kozlov & Rotov, 1977). Several reports have indicated that M. bovis is able to survive in soil (Walter et al., 2012). One previous study, using PCR-based detection, found mpb64 and mpb70 MTC-specific sequences in soil sampled from an Irish farm that had a history of bovine tuberculosis, and no incoming infected bovines and badgers after culling, for as long as 15 months after possible contamination. The sequences exhibited >99% identity to reference M. bovis sequences (Young et al., 2006). Nevertheless, experimental inoculation of other farm soil with the M. bovis BCG Pasteur strain revealed a rapid decrease in both living cells and DNA content, and the influence of both temperature and humidity in such decay processes (Young et al., 2005).

Likewise, PCR-based detection of the mpb70 gene was positive in 47% of the soil samples collected in badger setts (tunnels) from 60 UK farms in six bovine tuberculosis-endemic regions; furthermore, 16S rRNA detection was indicative of viable organisms (Courtenay et al., 2006). Refined real-time PCR detecting the DR4 region confirmed that all badger setts tested on a UK farm in 2006 were positive for M. bovis (Sweeney et al., 2007).

More convincing evidence was provided by culture-based observations. M. bovis organisms were previously isolated from a water sample, but not from soil samples, collected from a naturally infected badger yard (Little et al., 1982). Moreover, M. bovis organisms could be isolated 4 weeks after artificial contamination of the soil (Duffield & Young, 1985), for 6 weeks from exposed naturally infected buffalo tissues and for 4 weeks from spiked buffalo faeces; however, M. bovis could be isolated for only 5 days from buried tissues (Tanner & Michel, 1999).

In these previous studies, contamination by rapidly growing soil inhabitants may have hampered the further recovery of viable M. bovis cells from infected soil samples (Young et al., 2005). Refined immune-trapping of M. bovis was crucial to avoiding contamination and helped to grow M. bovis mycobacteria within 4 weeks from the soil of 7/7 badger setts (Sweeney et al., 2007). It was recently shown that M. bovis survived as long as 88 days in soil under natural weather conditions (Fine et al., 2011).

We further observed that contaminated soil was a source of disseminated M. tuberculosis infection in mice. Indeed, we observed granulomas in the lungs and spleen of challenged animals that were not observed in negative control animals. Moreover, M. tuberculosis grew from such lesions, confirming that they were due to tuberculosis. These data indicate that soil-borne M. tuberculosis mycobacteria retained their pathogenicity. M. tuberculosis in soil could be the source of some cases of cutaneous tuberculosis in underdeveloped countries (Frankel et al., 2009).

The experimental conditions here reported are far from the natural ones, and numerous factors such as variation in sunshine exposure (Duffield & Young, 1985), temperature and humidity, competition with other soil inhabitants and protection by protists such as encysted amoebae (Mba Medie et al., 2011) may interfere in one way or another with the survival of MTC mycobacteria in the soil. Therefore, the data herein reported could prompt further field studies to confirm the survival of MTC in soil and contaminated soil as a source for natural infection of mammals.

REFERENCES


infection suggest the existence of an environmental reservoir. *Clin Microbiol Infect* 17, 1013–1019.


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