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Rapid dissemination of *Mycobacterium bovis* from cattle dung to soil by the earthworm *Lumbricus terrestris*

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ABSTRACT

Indirect transmission of *Mycobacterium bovis*, the causative agent of bovine tuberculosis (bTB), between wildlife and livestock is thought to occur by inhalation or ingestion of environmental substrates contaminated through animal shedding. The role of the soil fauna, such as earthworms, in the circulation of *M. bovis* from contaminated animal feces is of interest in the epidemiology of bTB. The objective of this study was to assess the impact of earthworm activity on *M. bovis* transfer from animal dung to castings and the surrounding soil. For this purpose, microcosms of soil containing the anecic eathworms *Lumbricus terrestris* were prepared and covered with cattle feces spiked with the *M. bovis* BCG strain Pasteur to carry out two separate experiments. The dissemination, the gut carriage and the excretion of *M. bovis* were all monitored using a specific qPCR-based assay. Our results showed that the earthworm *L. terrestris* was able to rapidly disseminate *M. bovis* from the contaminated cattle feces to the surrounding soil through casting egestion. Moreover, contaminated earthworms were shown to shed the bacteria for 4 days when transferred to a *M. bovis*-free soil. This study highlights for the first time the possible role of earthworms in the dissemination and the persistence of *M. bovis* in soils within bTB endemic areas.

1. Introduction

Mycobacterium bovis is a pathogenic mycobacteria that can infect and cause bovine tuberculosis (bTB) in cattle and in a wide range of domestic or wild mammals, as well as in humans (Biet et al., 2005). Tuberculosis caused by *M. bovis* is a primarily respiratory disease, but it may affect various organs in animals (Morris et al., 1994). Although cattle are considered as the principal host of *M. bovis*, wild fauna such as badgers (*Meles meles*), wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*) have also been identified as potential wildlife reservoirs (Palmer, 2013). Transmission of infection between cattle mainly occurs by inhalation of contaminated aerosols during close contacts with infected animals (Morris et al., 1994; Phillips et al., 2003). An inoculum of less than ten bacilli is enough to cause respiratory disease in cattle (Dean et al., 2005). Moreover, indirect transmission by inhalation of environmental bioaerosols or ingestion of infected soil and water could also be a potential route of transmission (Palmer et al., 2004; Vicente et al., 2007).

Infected animals (badgers, wild boar and deers) were shown to shed *M. bovis* (through different routes depending on the species) *via* oro-nasal mucus, sputum, urine, feces and wound discharges (Corner et al., 2012; Palmer et al., 2004; Barasona et al., 2015) that may contaminate environment, especially pastures shared by cattle and wild animals. Thus, investigation of soil as a potential reservoir of *M. bovis* is an important step towards understanding the epidemiology of the bacteria.

Among soil fauna, earthworms constitute the largest animal biomass in most terrestrial ecosystems, especially in grasslands (Lavelle and Spain, 2001). Earthworms play the role of engineers in soil processes, impacting on soil functions and ecosystem services (Blouin et al., 2013), affecting soil structure, water movement, organic matter decomposition, nutrients releasing, plant growth and pollution remediation. Earthworms are usually classified into three ecological types according to their feeding habits: (i) the epigeic species are restricted to the surface organic layers where

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they feed, (ii) the endogeic species live in the subsurface where they make lateral tunnels as they ingest soil and organic matter and (iii) the anecic species feed preferentially on surface litter but live in burrows in the soil (Lee, 1985). Earthworms ingest soil particles mixed with organic matter that are mechanically and chemically digested by both gut fluid and microbiota (Curry and Schmidt, 2007) and release castings (undigested soil egested on or under the soil surface). Earthworm gut microbiota act as a selective filter for soil microorganisms, shape microbial soil communities and either up- or down-regulate microbial populations (Byzov et al., 2007; Egert et al., 2004; Gómez-Brandón et al., 2011). It has been shown that earthworms may have beneficial microbial activities especially in the transport of bacterial inocula through soil (Thorpe et al., 1996), but earthworms may also act as pests by promoting the dissemination of plant pathogens such as *Phytophtora cactorum* and *Fusarium reticulatum* (Montecchio et al., 2015). More interestingly, a few studies have revealed that human and animal pathogenic bacteria, viruses and parasites, especially *Escherichia coli* O157:H7, foot-and-mouth disease virus and *Cestoda* tapeworms, may be carried and spread by earthworms (Edwards and Bohlen, 1996; Prysor Williams et al., 2006).

Although animal dung is a very attractive food for earthworms (Lowe and Butt, 2005), few studies have evaluated the role of earthworms in the survival and the dissemination of potentially pathogenic mycobacteria from animal dung or manure to surrounding soil (Fischer et al., 2003). In addition, since earthworms represent an important part of the diet of various species, including badgers and wild boar (Granval and Muys, 1995; Lee, 1985), the consumption of contaminated earthworms (gut carriage) might be a source of contamination for wild fauna.

The current study set out to assess the impact of earthworm activity on *M. bovis* transfer from animal dung to their released castings and the surrounding soil. We also investigated *M. bovis* gut carriage and casting contamination following ingestion of infected feces.

2. Material and methods

2.1. M. bovis *strain preparation*

M. bovis BCG strain Pasteur 1173P2 was grown aerobically at 37 °C in Middlebrook 7H9 broth supplemented with 10% Middlebrook ADC Growth Supplement (Sigma-Aldrich, France). The *M. bovis* inoculum was prepared as follows: a 10 mL stationary phase culture was pelleted and once the supernatant was removed, the cell aggregates were disrupted by vortexing with glass beads for 15 s. The pellet was suspended in 1 mL of sterile water and serial 10-fold dilutions of the suspension were spiked on modified Middlebrook 7H11 plates Middlebrook 7H11 supplemented with 10% heat inactivated bovine serum (Dominique Dutscher, France) and 10% Middlebrook OADC Growth Supplement (Sigma-Aldrich, France) for enumeration. The plates were always incubated at 37 °C for 2 months. A 10-fold dilution of the prepared *M. bovis* suspension was also directly quantified with the RD4 qPCR assay described below.

2.2. Soil, cattle feces and earthworm characteristics

A *M. bovis*-free pasture (that had never been grazed by bTB infected cattle) located in Burgundy (Middle East of France) was selected to sample the **s**oil used in this study. This latter is a clayey loamy soil with a pH of 7.75. Organic carbon and nitrogen content was 3.61 and 3.8 g kg^{-1}, respectively. The soil was sieved to 4 mm and stored at 4 °C until its use.

Fresh cattle feces were collected from bTB-free Charolais cattle in a bTB-free area of Burgundy and immediately used.

The adult anecic earthworms (*L. terrestris)* were provided by Lombri'Carraz (France) and kept at 4 °C in large buckets containing soil and organic matter until they were used in the study. Soil, earthworms and cattle feces used in this work were previously proved to be negative for the presence of *M. bovis* using the qPCR-based assay.

2.3. DNA extraction and purification

DNA from all the samples collected was extracted as follows: 8 mL of lysis buffer (100 mM Tris pH 8.0, 100 mM EDTA pH 8.0, 100 mM NaCl and 2% (w/v) SDS), 4 g of silica beads (100 μm diameter), 5 g of ceramic beads (1.4 mm diameter) and 8 glass beads (4 mm diameter) were added to 2 g of sample in a 15 mL sterile vial—whereas, 1 mL of lysis buffer, 0.5 g of silica beads, 0.625 g of ceramic beads, 1 glass beads were added to 250 mg-samples. Samples were disrupted for 3 × 30 s at 4 m/s in a FastPrep®-24 Instrument (MP Biomedicals Europe, Illkirch, France), incubated for 30 min at 70 °C and centrifuged for 5 min at 7000 × *g* at room temperature. Supernatants were incubated on ice for 10 min with 1/10 volume of 3 M potassium acetate pH 5.5 and then centrifuged for 5 min at 14,000 × *g*. One volume of ice-cold isopropanol was added to the supernatant for DNA precipitation overnight at −20 °C. DNA was collected by centrifugation (30 min at 14,000 × *g*), and DNA pellets were washed with ice-cold ethanol (70%) and dissolved in 100 μL of water. Crude DNA extracts (100 μL) were loaded on Microbiospin™ columns (Biorad, Marnes-La-Coquette, France) filled with 100 mg of polyvinyl polypyrrolidone (PVPP, Sigma Chemical Co., France). After centrifugation (4 min, 1000 × *g*, 4 °C), eluted DNA was further purified using the Geneclean Turbo Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Purified DNA concentrations were estimated by spectrophotometry (λ = 260 nm) using a NanoDrop® 2000 spectrophotometer (Thermo Scientific, Illkirch, France).

2.4. M. bovis *quantitative PCR (qPCR)*

M. bovis-specific qPCR was set up targeting the RD4 flanking region using previously described forward RD4 flanking primer (5′-TGTGAATTCATACAAGCCGTAGTCG-3′) and probe (5′-**6**-FAM-AGCG-CAACACTCTTGGAGTGGCCTAC-3′ TAMRA) (Sweeney et al., 2007). In order to improve qPCR efficiency, the reverse RD4 flanking primer based assay was modified (5′-ATCTTAGCTGGTCAATAGC-CATTTTT-3′) to reduce the size of the qPCR product to 116 bp.

qPCR reactions were carried out in triplicate with a 25 μL reaction mix containing 12.5 μL of ABsolute™ QPCR ROX Mix (Thermo Scientific, France), 1 μL (final concentration 25 ng μL^{-1}) of T4 bacteriophage gene 32 product (MP Biomedicals Europe, France), 1 μL of each primer (final concentration 400 nmol L^{-1}), 0.5 μL of each probe (final concentration 200 nmol L^{-1}), 4 μL of ultrapure water and 5 μL of pure or diluted (10- or 20-fold) DNA. Absolute quantification was achieved using standard DNA dilutions. A recombinant plasmid containing one copy of the RD4 fragment of *M. bovis* BCG strain Pasteur 1173P2 cloned in the pCR®II-TOPO® vector (Invitrogen, France) was used as a standard. A calibration curve was obtained by amplification of serial dilutions of the plasmid ranging from 1 to 10^6 copies per PCR reaction. All DNA samples extracted from the soil, castings and feces samples were screened with the RD4-based qPCR assay and results were expressed as the number of *M. bovis* gene copies per g of soil, castings or feces. We previously assessed that when Cycle threshold (Ct) was above 39, detection was possible but unreliable (unpublished results). The word "traces" was then chosen in all further experiments in order to indicate that *M. bovis* targets are present in very low quantities (below 250 CFU g^{-1} of soil). The