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Anatomical distribution of *Mycobacterium bovis* genotypes in experimentally infected white-tailed deer

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ABSTRACT

Mycobacterium bovis (*M. bovis*) causes tuberculosis in white-tailed deer (WTD). Natural infection of WTD with *M. bovis* is most closely mimicked by instilling inoculum into palatine tonsillar crypts. One hundred fifty days after intratonsillar inoculation, *M. bovis* was cultured from 30 tissues originating from 14 deer. Whole-genome sequencing (WGS) was performed on the original inoculum, single colonies subcultured from the original inoculum, and *M. bovis* isolated from each culture positive tissue. Single nucleotide polymorphisms (SNP) were identified by comparing the derived sequences to the reference strain AF2122/97. Results indicate that the majority of the SNPs that were identified were homogeneous between the inoculum and the isolates from the tissues. The majority of individual tissues had different WGS genotypes from each other, suggesting that dissemination of *M. bovis* beyond the initial site of infection may require few mycobacteria representing a bottleneck.

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1. Introduction

Mycobacterium bovis (*M. bovis*) is the primary causative agent of tuberculosis in animals and has a broad natural host range. *M. bovis* has been isolated from a large number of free-ranging mammalian species including rodents, carnivores, and ungulates, including deer (Hardstaff et al., 2014; Miller and Sweeney, 2013). In the United States, free-ranging white-tailed deer (WTD) serve as a reservoir of *M. bovis* that periodically spills over into domestic cattle (O'Brien et al., 2011). The mechanism of transmission between deer and cattle is believed to be indirect oral transmission since there is little evidence of direct aerosol transmission between WTD and cattle through nose-to-nose contact (Witmer et al., 2010). Experimentally, oral inoculation through feed or instillation in the tonsil more closely mimics the natural distribution of tuberculous lesions than does infection by aerosol (Palmer et al., 1999, 2002, 2003), the most common route of transmission of tuberculosis among humans and cattle.

The most common site for gross lesion development in naturally infected WTD is the medial retropharyngeal lymph node, followed by the lung and lung associated lymph nodes (i.e. tracheobronchial and mediastinal). Less commonly, gross lesions may be seen in cranial, mesenteric and hepatic lymph nodes, liver, and parietal pleura. *M. bovis* may also be cultured from tissues without grossly visible lesions (O'Brien et al., 2001; Palmer et al., 2000).

Dissemination of mycobacteria from the initial site of infection plays an important role in establishing long-term infection. Experimental deposition of *M. bovis* in palatine tonsillar crypts rarely results in lesion development within the tonsil (O'Brien et al., 2001; Palmer et al., 2000). However, the medial retropharyngeal lymph node, which receives efferent lymphatics from the palatine tonsil, routinely develops lesions from which *M. bovis* can be isolated. Lymphatic linkage between the tonsil and medial retropharyngeal lymph node suggests that mycobacteria may be transported from one location to another via hematopoietically derived cells. Observations of human tuberculosis cases from the mid to late 1800s suggest that the earliest tubercle involvement was associated with the lymphatics (reviewed by Behr and Waters, 2014). More recently, Basaraba et al. report that the pulmonary lymphatics are the primary site of *Mycobacterium tuberculosis* infection in an aerosol infection model of guinea pigs (Basaraba et al., 2006). Utilizing the rabbit model, Abadie et al. demonstrated that

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BCG was transported to the regional lymph nodes via neutrophils rather than macrophages or dendritic cells (Abadie et al., 2005). Involvement of the lymph nodes and associated tissues plays a principle role in tuberculosis development (Behr and Waters, 2014).

Dissemination of *M. bovis* in WTD has not been studied to date. To begin to understand mycobacterial dissemination, in vivo, *M. bovis* was cultured from tissues of experimentally infected WTD, and analyzed using whole-genome sequencing.

2. Materials and methods

2.1. Inoculum preparation

M. bovis strain 95–1315 was isolated from a Michigan WTD and represents one of the predominate endemic strains in the United States. 95–1315 was grown in Middlebrook's 7H9 media supplemented with 10% oleic acid–albumin–dextrose complex (Difco, Detroit, MI) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, MO) at 37 °C. Mid log-phase growth bacilli were pelleted by centrifugation at 750 × *g*, washed twice with phosphate buffered saline (PBS) (0.01 M, pH 7.2), and diluted to the appropriate cell density. Inoculum was stored in 1-ml aliquots at −80 °C until used. Twenty-four hours after freezing, a single 1-ml aliquot was removed and bacilli were enumerated by counting colonies of serial dilutions grown on Middlebrook's 7H11 medium (Becton Dickinson, Cockeysville, MD). At the time of inoculation, aliquots of enumerated stock were diluted to the appropriate concentration. Plate counts were repeated the day of inoculation to retrospectively confirm inoculum dosage. DNA was isolated from 1 ml of the inoculum and sequenced as described below.

2.2. Animals

Thirty-three WTD (~1 year-old) were obtained from a tuberculosis-free captive breeding herd at the National Animal Disease Center in Ames, Iowa, USA. Deer were randomly assigned to one of two groups; orally vaccinated *M. bovis* BCG Danish ($n = 17$); and non-vaccinated ($n = 16$).

Deer were vaccinated as previously described (Nol et al., 2008). Briefly, with the aid of a swine mouth speculum, a 1.0 ml preparation of 1×10^8 colony forming units (CFU) BCG in PBS was administered to the posterior oropharynx using a 3-ml syringe and a 10 French 25-cm sterile urinary catheter (Monoject, St. Louis, MO, US). All deer were experimentally infected 111 days after vaccination as described previously (Palmer et al., 1999). Briefly, deer were anesthetized by IM injection of a combination of xylazine (2 mg/kg) (Mobay Corporation, Shawnee, KS) and ketamine (6 mg/kg) (Fort Dodge Laboratories, Fort Dodge, IA). A laryngoscope was used to aid visualization of tonsillar crypts. Approximately 150 CFU of virulent *M. bovis* strain 95–1315 (low passage) placed into each palatine tonsillar crypt for a total dose of 300 CFU per deer. Strain 95–1315, used for challenge, was originally isolated from a WTD in MI. After infection the effects of xylazine were reversed by IM injection of tolazoline (4 mg/kg) (Lloyd Laboratories, Shenandoah, IA). Deer were housed separately, by vaccination status, inside a biosafety level 3 building. The Institutional Animal Care and Use Committee approved all procedures prior to implementation.

2.3. Mycobacterial culture from tissues

At necropsy, the following tissues were collected; mandibular, parotid, medial retropharyngeal, tracheobronchial, mediastinal, mesenteric, hepatic and superficial cervical lymph nodes, palatine and pharyngeal tonsils, liver and lung. A portion of each tissue was removed for histological examination and the remaining tissue was submitted to the National Veterinary Services Laboratories for mycobacterial culture. Culture was performed as previously described (Robbe-Austerman et al., 2013). Bacteria for whole genome sequencing were selected by pooling all colonies from the surface of the solid media.

2.4. Inoculum preparation for sequencing

Initially, DNA was isolated from a 1 ml aliquot using a phenol-chloroform method (Higgins et al., 2011). A sample of *M. bovis* from the same lot as the original inoculum was serially diluted in 7H9 broth and plated on 7H11 agar to retrieve individual colonies. Eighteen individual colonies were picked from two different plates and inoculated into 5 ml 7H9 media. When cultures became turbid, an additional 20 ml of 7H9 was added. Cultures were grown for an additional 2 weeks. DNA was isolated using a phenol-chloroform method (Higgins et al., 2011).

2.5. Whole-genome sequencing and single nucleotide polymorphism (SNP) analysis

DNA was isolated using MasterPure Kit (Epicentre, Madison WI) from *M. bovis* cultures free of contamination by following the manufacturer's directions or by a phenol-chloroform method as described (Higgins et al., 2011). Sequencing libraries were prepared as directed by the manufacture using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA). Libraries were normalized using (Agencourt AMPure XP, Beckman Coulter, Brea, CA) according to the manufacturer's directions. Sequencing was performed on a MiSeq using the MiSeq Reagent Kit version 2 (Illumina). The run was performed according to the manufacturer's recommendations to produce 2 × 250 paired-end reads. The targeted genome coverage was between 100X and 150X. Samples with coverage less than 50X were sequenced again.

Sequences from the MiSeq were aligned to the *M. bovis* reference genome AF2122/97 (NCBI Accession number NC_02945) using Burrows-Wheeler Aligner 0.7 (BWA) (Li and Durbin, 2009) with default settings. The resulting SAM file was converted to a sorted and indexed BAM file using Samtools 0.1.18 (Li et al., 2009). Duplicates were identified and removed using the MarkDuplicates program from picard tools 1.81 [http://picard.sourceforge.net]. Genome Analysis Tool Kit (GATK) (McKenna et al., 2010) was used to recalibrate base quality scores, indel realignment, and SNP discovery using standard hard filtering parameters or variant quality score recalibration according to GATK Best Practices recommendations (DePristo et al., 2011; Van der Auwera et al., 2013). Two additional filters were applied to remove SNPs in the respective variant call file (VCF). First, SNPs that had qual scores <150 and AC = 1 were removed. Secondly, SNPs that fell into repeat regions (PPE, PE_PGRES gene families, transposable elements), or that were in loci with poor read density were removed (similar to Bryant et al., 2013; Ford et al., 2011; Kato-Maeda et al., 2013). All remaining SNPs from each sample were compiled and aligned against the reference utilizing the genome position of the reference. The alignment and SNP analysis Pipeline is available online (https://github.com/stuber/SNP_analysis). Alignments were visualized using the Integrated Genomics Viewer (Robinson et al., 2011; Thorvaldsdóttir et al., 2013).

2.6. Effect of SNPs on protein function

The predicted effect of a mutation on protein function was assessed using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) for each SNP contained within a known gene using default parameters (Adzhubei et al., 2010). PolyPhen-2 utilizes an iterative greedy algorithm to automatically analyze SNPs using 8 sequence