1 **Anatomical distribution of *Mycobacterium bovis***
2 **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.

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., 2003; 2002; 1999), 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 1800's 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 *M. tuberculosis* infection in an aerosol infection model of guinea pigs (Basaraba et al., 2006). Utilizing the rabbit model, Abadie et al. demonstrated that 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.

Materials and Methods

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 $750 \times g$, 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.

Animals

Thirty-three WTD (~1 yr-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) and a laryngoscope 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.

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.

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).

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 x 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).

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 and 3 structural based predictive features to classify the likelihood of a SNP being benign, possibly damaging, or probably damaging. The algorithm also calculates Naïve Bayes posterior probability that a mutation is damaging.

Statistical Analysis

Fisher's exact test was used to analyze the proportional differences between the non-vaccinated and vaccinated animals using Prism Software (version 6.0c).

Results

Mycobacterium bovis was isolated from 14/33 deer (Palmer et al., 2014). Nine of the infected deer were from the non-vaccinated group with the remaining 5 from the vaccinated group. *M. bovis* was isolated from the following tissues in more than one animal; medial retropharyngeal lymph node (n=11), tonsil (n=6), tracheobronchial lymph node (n=4), and lung (n=3). From a single deer, *M. bovis* was isolated from the mediastinal, mesenteric, parotid, mandibular, hepatic and superficial cervical lymph nodes (Table 1).

M. bovis cultures derived from tissues were subjected to DNA isolation and whole genome sequencing. The original inoculum was sequenced utilizing two methods of DNA preparation. First, a 1 ml aliquot was subjected to DNA isolation and sequenced as described in Materials and Methods. Secondly, to test homogeneity of the inoculum, 18 individual colonies were selected from the culture plates used for titration then sequenced

as described. Single nucleotide polymorphisms (SNPs) were identified by comparing the derived sequence to the reference, AF2122/97 (NCBI Accession No. NC_002945). Isolates had an average of 514 SNPs ($SD \pm 11$) relative to the reference. Low quality SNPs were removed from the analysis using a very conservative approach to reduce the risk of falsely identifying SNPs. The majority of the remaining SNPs were uninformative and were removed from further analysis because they were homogeneous between all strains. The remaining 11 SNPs were informative and separated the isolates into 11 genotypes (Table 2 and Supplemental Table 1). SNPs specific to BCG were not detected in any of the isolates sequenced. Difference between the non-vaccinated and vaccinated group were not significant, likely due to the small number of isolates from the vaccinated group; therefore, the groups are treated as one for the remainder of the paper.

Sequencing of individual colonies from the inoculum revealed the presence of at least 4 genotypes: 1, 2, 4, and 5 (Table 2 and Supplemental Table 1). The majority of the colonies sequenced from the inoculum were genotype 4 (14 of 18). Genotype 1 was found in 2 of 18 colonies. The remaining 2 colonies, A2 and A6, were genotype 2 and 5, respectively. Sequencing of the inoculum rather than individual colonies resulted a genotype that resembled the genotype 1 and the reference (Table 2). This result appeared to be in contrast to the high proportion of genotype 4 among the individual colonies. The inoculum sequence file for the inoculum was examined visually for evidence of a mixed genotype. Genotype 1 and 4 only differ by one SNP located at nucleotide position 1664895. Visual examination of the alignment file revealed that 64% of the reads aligning to this region contained a Cytosine at this position (genotype 4) with the remaining reads containing a Thymine (Table 3). This percentage was similar to that

208 reported for the proportion of genotype 4 to genotype 1 among the sequenced colonies.

209 The discrepancy between the colonies and the inoculum is due to the conservative
210 algorithm used to call SNPs. These data also confirm that mixed infections can be
211 detected. This methodology was used to determine if tissues were infected with more
212 than one genotype.

213 All sequence alignment files were examined for evidence of mixed genotypes present in
214 the tissues (Table 3). Six tissues and one colony contained evidence of multiple
215 genotypes. The culture from the tonsil of Animal 1103 (Table 3) contained two
216 genotypes. The most prominent genotype was 8 with a T and C (65%) at nucleotide
217 positions 1664895 and 3674323, respectively. The remaining 35% was genotype 4 with a
218 C and A at the same respective nucleotide positions. The remaining samples had variation
219 at a single location (Table 3). Colony A9 contained two genotypes 1 and 4, consistent
220 with the inoculum both in content and ratio.

221 Deer were inoculated with a mixture of genotypes. Infection using a mixture of genotypes
222 allowed the examination of mycobacterial dissemination within the host. Eighty percent
223 of tissues were infected with a single genotype (24 of 30 tissues)(Table 4). The most
224 commonly isolated genotypes from these tissues were 4 (25%), 1 (21%), and 8 (16%).
225 Genotypes 1 and 4 were the primary genotypes found in the inoculum; however,
226 genotype 8 was only identified in tissues. Multiple genotypes were isolated from 20% of
227 the tissues. Genotype 8 was the most commonly isolated in this group (5/6 tissues). In
228 contrast to tissues with more than one isolate, genotype 8 was cultured from only 4/24
229 tissues where only one genotype was cultured. Genotypes 1 and 4 were isolated from 4/6
230 and 2/6 tissues, respectively.

Vaccination did not appear to have a significant impact on distribution of genotypes in the infected WTD. There were no significant ($P>0.05$) differences in genotype distribution or frequency between the vaccinated and non-vaccinated groups.

SNPs may alter the protein function when located within a gene and affect the amino acid at that location. They may also affect gene expression when located in a non-coding region with promoter activity. The potential impact of the SNPs on gene composition was investigated.

Two of the SNPs were located between genes (3325411 and 3674323). SNP 3325411 was located 2 nucleotides upstream from *gatB* gene (genotype 2), while 3674323 was located 16 nucleotides upstream from *sugI* (genotype 8)(Table 5). Two additional SNPs, 1896416 and 2458135, were predicted to be synonymous, therefore, unlikely to have an impact on protein function (genotype 9 and 11, respectively). The remaining SNPs occurred within genes and resulted in amino acid substitution or premature nascent protein termination.

Two SNPs were associated with Genotype 3. SNP 3674354, a C to T substitution, resulted in the creation of a termination codon that truncated *sugI* to only 6 amino acids. *SugI* is a putative integral membrane protein that is believed to transport sugar across the cellular membrane. The second SNP 466472, an A to T transversion, results in a lysine to methionine mutation that occurred in the RNA polymerase subunit beta and is probably damaging (score: 0.988, see Table 5). Genotype 3 was only found in the tonsil of animal 1152.

SNP 1664895, a T to C transition that resulted in a valine to alanine substitution that was

probably damaging (score: 0.866) to *ripA*, a peptidoglycan endopeptidase (Table 5). This SNP is the defining SNP for genotype 4 and is also found in genotypes 5, 6 and 7 (Table 2). Genotype 4 was widely distributed within infected animals; having been isolated from tonsil, medial retropharyngeal, hepatic, and prescapular lymph nodes (Table 4).

Genotype 5 contained the same SNP found in genotype 4 plus SNP 1366125 (Table 2). This SNP was a G>T transversion that resulted in a glutamic acid to aspartic acid substitution in *sigE* that was likely benign (score: 0.006, Table 5). Genotype 5 was not isolated from infected animals, but was identified in the original inoculum.

The nucleotide transitions associated with genotypes 6 and 7 resulted in amino acid substitutions (A196V and N274D respectively) were predicted to be benign (score: 0.007 and 0.0006 respectively) and occurred in *embR*, a transcriptional regulator, and a predicted methyltransferase (Table 5). These genotypes also contained the SNP that characterized genotype 4. These genotypes were only isolated from the medial retropharyngeal lymph node.

A G>A transition at 1953936 created genotype 10. This nucleotide transition resulted in a methionine to isoleucine substitution that was predicted to be likely benign (score: 0.207) in an uncharacterized protein (Table 5). Genotype 10 also contained the characteristic SNP found in genotype 8 and was isolated from the lung, tracheobronchial lymph node, and mesenteric lymph node from the same animal.

Discussion

The details of natural *M. bovis* transmission and infection of WTD are relatively

unknown. Experimentally, transmission has been shown to occur through both direct and indirect contact (Palmer et al., 2010; 2001). The number of mycobacteria transmitted naturally, and required for successful infection, is not known, but is hypothesized to be relatively small, possibly as low as a single CFU (Dean et al., 2005). Here we describe infection with multiple genotypes and subsequent tissue distribution.

The original inoculum was shown to contain at least 4 genotypes (1, 2, 4, 5 see Table 2 and Supplemental Table 1), but likely contained all 11. The most widely distributed genotypes were 4, 1, and 8. Genotype 1 and 4 were represented in the colonies sequenced from the original inoculum; however, genotype 8 was not. The fact that genotype 8 was isolated from different animals and tissues, and the fact the SNP is found in genotypes 9, 10 and 11 makes it unlikely that a mutation that generated the 3674323A>C SNP occurred after infection. These data suggest that genotype 8 was present in the original inoculum, but at a frequency below the level of detection.

In 80% of affected tissues only a single genotype was recovered suggesting that an individual tissue may be colonized with a single mycobacterium or CFU. This observation is consistent with a recent study where molecularly identifiable strains of *M. tuberculosis* were bronchoscopically instilled into the segmented bronchus of the right caudal lung lobe of cynomolgus macaques. Individual granulomas were cultured (Lin et al., 2014), and of the granulomas that contained cultivable *M. tuberculosis*, 79% contained a single genotype, suggesting that each lesion was established by a single bacterium.

Transmission of *M. bovis* from the tonsil to other anatomical sites may represent a

bottleneck to establishment of infection in other tissues. Several factors may contribute to single genotypes establishing infection. First, replication may take place in the tonsil prior to dissemination. Genotypes that are more fit for in vivo replication or survival are more available for dissemination. This possibility does not seem likely since not all genotypes from an animal were cultured from the tonsil, nor was *M. bovis* always cultured from the tonsil. Second, the dissemination event may be relatively rare, thus the chance for more than one genotype being transported to secondary tissues would be unlikely to occur resulting in a single genotype per tissue. A third possibility is that the genotypes have a tissue specificity conferred by genetic variation. The fourth possibility is that the SNPs result in changes to the bacterial proteome that alter the ability to be disseminated (Supplemental Table 2). This may be especially true if a transport mechanism, such as attachment to neutrophils, as demonstrated in the rabbit model, is active (Abadie et al., 2005). The last two possibilities were investigated by in silico examination of the potential consequences of the mutation. There were no obvious advantage/disadvantage to the mycobacteria from the SNP. Genotype 8 was a potential candidate for a SNP that provided an advantage because genotype 8 was not present in the colonies sequenced in the original inoculum, but was present in 30% of all tissues that were culture positive. The SNP that defines genotype 8 is also present in genotypes 9, 10 and 11 which, like genotype 8, were not identified in the inoculum but were isolated from tissues of infected animals. The defining SNP in genotype 8 is located between two genes, *DacC* and *SugI*. The SNP may not directly produce a mutant protein, but may influence gene expression. The direct impact cannot be determined from the current studies, additional work will be required to quantitate any advantage conferred by the

SNP and to determine the mechanism involved.

The role of genetic variation of *M. bovis* has been the primary focus of this paper; however, the genetics of the WTD may also play a role in *M. bovis* dissemination and pathology. Mackintosh et al. in an elegant genetic experiment, reported that susceptibility and resistance to *M. bovis* in red deer (*Cervus elaphus*) is heritable (Mackintosh et al., 2000). They also show that there is a differential immune response between the susceptible and the resistant deer. These data suggest that some of the variation reported here might be due to differences in the host genetics and immunological response rather than the genetic differences within the pool of *M. bovis* genotypes. Additional experimentation would be required to differentiate the role of host variation versus the mycobacterial variation and is beyond the scope of this paper.

Infection of WTD with *M. bovis* through intratonsillar instillation in the tonsillar crypt results in an infection that mimics natural infection. Dissemination of *M. bovis* from the initial site appears to be a relatively rare event since tissues infected subsequently usually only contained a single genotype. The proportion of genotypes in secondary tissues were not proportional to the original inoculum suggesting that some selective pressure limits spread whether the selection is due to the genetic variation of the mycobacteria or the deer it is not clear from the current studies.

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Table 1

Table 1: Isolation of *M. bovis* from tissues of infected deer.

Animal ID	Tissue		Lymph node ¹							
	Tonsil	Lung	Parotid	Medist	M. Retro	Trach	Hepatic	Mesen	Mandib	Prescap
Non Vaccinated										
260					+	+				
261		+				+				
1102	+				+					
1103	+	+	+	+			+		+	+
1113		+			+	+		+		
1150					+					
1155	+				+					
1160						+				
1170					+					
Vaccinated										
1141					+					
1151	+				+					
1152	+				+					
1161					+					
1167	+				+					

¹ Abbreviations used in this table Mediastinal (Medist), Medial Retropharyngeal (M. Retro), Tracheobronchial (Trach), Mesenteric (Mesen), Mandibular (Mandib), Superficial Cervical (Prescap) lymph nodes.

Table 2

Table 2: Genotypes of *M. bovis* isolated from tissues of infected deer and colonies grown from the original inoculum

Animal ID	Origin of Isolate	Nucleotide Position of SNP ¹										Genotype	
		3325411	3674354	766472	1664895	1366125	1414908	820643	3674323	1896416	1953936		2458135
Reference		C	C	A	T	G	C	T	A	C	G	G	0
95-1315	Inoculum	C	C	A	T	G	C	T	A	C	G	G	NA ²
1103	Tonsil	C	C	A	T	G	C	T	A	C	G	G	1
1103	Parotid	C	C	A	T	G	C	T	A	C	G	G	1
1103	Medist	C	C	A	T	G	C	T	A	C	G	G	1
1103	Lung	C	C	A	T	G	C	T	A	C	G	G	1
261	Lung	C	C	A	T	G	C	T	A	C	G	G	1
1150	Med Retro	C	C	A	T	G	C	T	A	C	G	G	1
1151	Med Retro	C	C	A	T	G	C	T	A	C	G	G	1
1167	Tonsil	C	C	A	T	G	C	T	A	C	G	G	1
1170	Med Retro	C	C	A	T	G	C	T	A	C	G	G	1
1102	Med Retro	C	C	A	T	G	C	T	A	C	G	G	1
Inoculum	Colony A7	C	C	A	T	G	C	T	A	C	G	G	1
Inoculum	Colony A6	G	C	A	T	G	C	T	A	C	G	G	2
1152	Tonsil	C	T	T	T	G	C	T	A	C	G	G	3
Inoculum	Colony A1	C	C	A	C	G	C	T	A	C	G	G	4
260	Trach	C	C	A	C	G	C	T	A	C	G	G	4
1151	Tonsil	C	C	A	C	G	C	T	A	C	G	G	4
1113	Med Retro	C	C	A	C	G	C	T	A	C	G	G	4
261	Trach	C	C	A	C	G	C	T	A	C	G	G	4
1103	Prescap	C	C	A	C	G	C	T	A	C	G	G	4
1103	Hepatic	C	C	A	C	G	C	T	A	C	G	G	4
Inoculum	Colony A2	C	C	A	C	T	C	T	A	C	G	G	5
1141	Med Retro	C	C	A	C	G	T	T	A	C	G	G	6
1161	Med Retro	C	C	A	C	G	C	C	A	C	G	G	7
1152	Med Retro	C	C	A	T	G	C	T	C	C	G	G	8
1167	Med Retro	C	C	A	T	G	C	T	C	C	G	G	8
260	Med Retro	C	C	A	T	G	C	T	C	C	G	G	8
1160	Trach	C	C	A	T	G	C	T	C	C	G	G	8
1155	Med Retro	C	C	A	T	G	C	T	C	C	G	G	8
1103	Mandib	C	C	A	T	G	C	T	C	T	G	G	9
1102	Tonsil	C	C	A	T	G	C	T	C	T	G	G	9
1113	Trach	C	C	A	T	G	C	T	C	C	A	G	10
1113	Lung	C	C	A	T	G	C	T	C	C	A	G	10
1113	Mesen	C	C	A	T	G	C	T	C	C	A	G	10
1155	Tonsil	C	C	A	T	G	C	T	C	C	G	A	11

¹ Abbreviations used in this table Mediastinal (Medist), Medial Retropharyngeal (M. Retro), Tracheobronchial (Trach), Mesenteric (Mesen), Mandibular (Mandib), Superficial Cervical (Prescap) lymph nodes.

² Result of directly sequencing inoculum

Table 3: The presence of multiple genotypes were identified by examining each SNP position in each strain sequenced¹.

Animal ID	Tissue ²	Nucleotide Position of SNP		
		1664895	3674323	1896416
Reference		T	A	C
95-1315	Inoculum	T/C64 ³	A	C
1103	Tonsil	T/C33	C/A35	C
1103	Medist	T	A/C46	C
1151	Med Retro	C/T36	A	C
1167	Tonsil	T	C/A41	C
1102	Med Retro	T	A/C38	C
Inoculum	Colony A9	T/C57	A	C
1152	Med Retro	T	C	T/C33

¹ SNP positions without variability or where the variability was less than 25% were excluded from table.

² Abbreviations used in this table Mediastinal (Medist), Medial Retropharyngeal (Med Retro).

³ First nucleotide represents the nucleotide reported. The second represents the alternative with the percentage.

Table 4

Table 4: Genotype of *M. bovis* isolated from tissues of infected deer.

Animal ID	Tissue		Lymph node ¹							
	Tonsil	Lung	Parotid	Medist	M. Retro	Trach	Hepatic	Mesen	Mandib	Prescap
Non Vaccinated										
260					8	4				
261		1				4				
1102	9				1,8					
1103	8,4	1	1	1,8			4		9	4
1113		10			4	10		10		
1150					1					
1155	11				8					
1160						8				
1170					1					
Vaccinated										
1141					6					
1151	4				1,4					
1152	3				9,8					
1161					7					
1167	8,1				8					

¹ Abbreviations used in this table Mediastinal (Medist), Medial Retropharyngeal (M. Retro), Tracheobronchial (Trach), Mesenteric (Mesen), Mandibular (Mandib), Superficial Cervical (Prescap) lymph nodes.

Table 5

Table 5: Effect of SNPs on Protein Composition and Function

Position	Genotype	Change	Gene	Ammino Acid	Function	Effect
3325411	2	C>G	intergenic			
3674354	3	C>T	Mb3364, <i>sugI</i>	Q6ter	probable sugar transport	truncated protein
766472	3	A>T	Mb0687, <i>rpoC</i>	K445M	RNA polymerase subunit beta	probably damaging (score: 0.988) ¹
1664895	4, 5, 6, 7	T>C	Mb1513, <i>ripa</i>	V387A	peptidoglycan endopeptidase	possibly damaging (score: 0.866) ²
1366125	5	G>T	Mb1253, <i>sigE</i>	E157D	RNA polymerase factor	likely benign (score: 0.006) ³
1414908	6	C>T	Mb1298c, <i>embR</i>	A196V	transcriptional regulation	likely benign (score: 0.007) ⁴
820643	7	T>C	Mb0747c	N274D	methyltransferase	likely benign (score: 0.0006) ⁵
3674323	8, 9, 10, 11	A>C	intergenic			
1896416	9	C>T	Mb1711c	synonomous		
1953936	10	G>A	Mb1771	M98I	uncharacterize protein	likely benign (score: 0.207) ⁶
2458135	11	G>A	Mb2236, <i>pepB</i>	synonomous	aminopeptidase	

¹ Predicted with PolyPhen-2 with sensitivity 0.53 and specificity 0.95
² Predicted with PolyPhen-2 with sensitivity 0.83 and specificity 0.93. Located near the nucleophile (C383) of the active site.
³ Predicted with PolyPhen-2 with sensitivity 0.97 and specificity 0.75
⁴ Predicted with PolyPhen-2 with sensitivity 0.96 and specificity 0.75
⁵ Predicted with PolyPhen-2 with sensitivity 0.97 and specificity 0.75
⁶ Predicted with PolyPhen-2 with sensitivity 0.92 and specificity 0.88