1 Toll-like Receptor (TLR) Diversity Influences Mycobacterial Growth in African Buffalo

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19 Abstract

20 Understanding the role of wildlife in the maintenance or spread of emerging infectious diseases is a growing
21 priority across the world. Bovine tuberculosis (BTB) is a chronic, infectious disease caused by
22 *Mycobacterium bovis* (*M. bovis*). BTB is widespread within game reserves in southern Africa, and within
23 these ecosystems the primary wildlife host of this disease is the African buffalo. We used a modified
24 bacterial killing assay for mycobacteria to investigate the effect of Toll-like receptor (TLR) genetic diversity
25 and demographic parameters on the ability of African buffalo to restrict mycobacterial growth. Eosinophil
26 count, time delay, bovine PPD response and avian PPD response were negatively correlated with
27 mycobacterial growth. TLR6 diversity and the interaction of age group and sex were positively correlated
28 with mycobacterial growth. Our results suggest that that both demographic and individual immune
29 parameters influence the ability to control mycobacterial infection in African buffalo. TLR6 diversity is
30 particularly interesting as this locus has also shown associations with BTB in cattle, suggesting that further
31 research into the effects, selection and role of TLR6 variants in bovine tuberculosis will be productive.

32 **Key-words:** bacterial killing; bovine tuberculosis; buffalo; genetic; immunity; toll-like receptor

33

33 **Introduction**

34 Understanding the role of wildlife in the maintenance or spread of emerging infectious diseases is a growing
35 priority across the world. Interface zones that bring people, livestock and wildlife into contact are increasing
36 in size as the human population grows, and both urban areas and agricultural practices have expanded into
37 areas previously inhabited by wildlife (Miller et al., 2013; Siembieda et al., 2011). The One Health concept
38 was developed in recognition of the fact that the health of humans, animals and the environment is
39 inextricably linked, and as such, the way to approach infectious disease management and research is through
40 cross-disciplinary collaboration (Miller and Olea-Popelka, 2013; “One Health Initiative,” n.d.; van Helden et
41 al., 2013). Wildlife disease hosts form an important part of this strategy, as maintaining an infectious disease
42 within an ecosystem can have major impacts on people, livestock and other wildlife species.

43 Bovine tuberculosis (BTB) is a chronic, infectious disease caused by *Mycobacterium bovis* (*M. bovis*). BTB
44 is prevalent in many developing countries in cattle and wildlife, and widespread within certain game reserves
45 in South Africa. The primary BTB maintenance host within the South African savannah ecosystem is the
46 African buffalo (*Syncerus caffer*), with prevalence levels/rates reaching up to 54% in some herds (le Roex et
47 al., 2015). However, African buffalo are not the only wildlife species affected by this disease as the host
48 range of *M. bovis* is broad: upwards of 20 additional wildlife species have been diagnosed with BTB in
49 South Africa alone. These include lion, leopard, baboon, rhino, and kudu (Michel et al., 2006). While some
50 countries have had great success eradicating or drastically reducing BTB using test and slaughter schemes,
51 these efforts are severely compromised in countries where a wildlife reservoir exists (Buddle et al., 2011). As
52 such, it is imperative that we try to understand the demographic and immune factors that may influence the
53 ability of buffalo to fight BTB infection.

54 Studies of genetic diversity most often focus on neutral variation, such as microsatellite loci, to answer
55 population-level biological questions (Kirk and Freeland, 2011). In studies specifically relating to disease
56 and adaptive fitness, however, adaptive or functional variation may be more informative. Thus far,
57 functional diversity studies have predominantly utilised the major histocompatibility complex (MHC) as the
58 immune marker of choice, due to its high variability and ability to discriminate between populations and
59 individuals (Kirk and Freeland, 2011). However, gene duplications, high diversity between species and the
60 presence of MHC pseudogenes make designing studies in non-model species challenging (Babik, 2010).
61 More recently, attention has turned to Toll-like receptor (TLR) genes as a proxy for immune diversity, as
62 they are more highly conserved between species but still display sufficient variation to compare individuals
63 and populations (Kirk and Freeland, 2011).

64 TLRs are transmembrane proteins that belong to a family of pattern recognition receptors. These proteins are
65 capable of recognising particular molecular patterns associated with invading pathogens, including bacteria,
66 viruses and fungi (Akira et al., 2001; Iwasaki and Medzhitov, 2010). Mammalian TLRs expressed on
67 antigen-presenting cells initiate the innate immune response following the detection of these pathogen-
68 associated molecular patterns (PAMPs) by initiating signalling pathways that stimulate host defences (Akira,

2003; Iwasaki and Medzhitov, 2010). Evidence suggests that variation within the pathogen binding regions of TLRs can translate to functional variation in pathogen recognition (Schröder and Schumann, 2005), and therefore these markers could be informative in studies of adaptive immune potential. Gene polymorphisms in TLRs have been shown to be associated with mycobacterial susceptibility in humans (Kleinnijenhuis et al., 2011; Salie et al., 2015) and cattle (Fisher et al., 2011; Song et al., 2014; Sun et al., 2012), making this gene family a particularly good proxy for immune diversity for this study.

In the growing field of ecological immunology, bacterial killing assays (BKAs) are frequently used as a measure of the capacity of whole blood to kill bacteria *ex vivo*. These assays can be used to quantify innate immune response variation between individuals and species, as well as model the effects of life history and/or environmental factors on the immune variation. Typically utilising bacteria such as *E. coli* and *S. aureus*, BKA's provide a more functionally relevant measure of innate host immune function than assessing isolated immune components (Demas et al., 2011; Matson et al., 2006).

In this study, we developed a modified bacterial killing assay for mycobacteria, and used this assay to investigate the effect of TLR diversity and demographic parameters on the ability of African buffalo to restrict mycobacterial growth.

Methods

Study Population

African buffalo blood samples (n = 43) originated in the Kruger National Park, South Africa. The Kruger National Park covers approximately 19 000 km², and supports upward of 40 000 buffalo (pers comm, Dr Markus Hofmeyr, SANParks). The buffalo herd sampled for this study has been isolated within a sub-section of the park for more than 10 years, and the buffalo were immobilized incidentally to this research. Sex composition was 14 males and 29 females. Both sexes were represented in the 'sub-adult' and 'adult' age classes, while the 'senior' category was represented only by females. Sex composition within age classes can be visualised in supplementary Figure A1, available online. At sampling, various demographic measurements were taken by trained researchers as described in Beechler et al. (2012), and blood samples were taken within 30 minutes of immobilization by Veterinary Wildlife Services staff. The additional demographic measurements can be seen in Table 1. Heparinised whole bloods obtained from the sampling events were gently rotated at ambient temperature during transport to the laboratory, and used for the mycobacterial growth inhibition assays within 6 hours.

Modified Bacterial Killing Assay Using BACTEC MGIT System

A modified version of a traditional bacterial killing assay was used to quantify the ability of individuals to kill or restrict growth of mycobacteria, as a surrogate measure of innate immunity. The detection of mycobacteria using liquid culture systems has recently become popular as a fast, reliable and sensitive

103 alternative to traditional plating methods. The BACTEC MGIT (mycobacterial growth inhibition tube)
104 system has been successfully used to detect mycobacteria in animal tissues, milk, and sputum samples,
105 among others (Abendaño et al., 2012; Diacon et al., 2014). The BACTEC MGIT system utilises fluorescence
106 to measure the metabolic activity of viable bacteria, and results in a quantitative measure of the number of
107 bacteria within a sample, using a time to positivity (TTP) principle. In essence, the time taken for a sample to
108 register positive mycobacterial growth can be converted to a number of bacterial colony forming units
109 (CFUs) present at the outset (Diacon et al., 2014).

110 *Bacterial Preparation and Standard Curve*

111 Frozen *M. bovis* Bacille Calmette-Guerin (BCG) stock (Danish strain 1331) was serially diluted in Difco™
112 7H9 broth (Difco Laboratories, Detroit, USA) and plated in triplicate onto Difco™ 7H11 Mycobacteria agar
113 (Becton, Dickinson and Company, Sparks, USA) enriched with oleic acid-albumin-dextrose-catalase
114 (OADC) (Becton, Dickinson and Company, Sparks, USA). Plates were incubated at 37°C and colonies were
115 counted after 2 weeks to confirm CFU count. Serial dilutions of BCG were prepared in triplicate and used to
116 generate a growth curve on the Bactec 960 MGIT system and corresponding Bactec MicroMGIT instrument.
117 The predicted linear relationship between TTP and the log₁₀CFU was used to calculate CFU_(initial) and CFU₍₉₆
118 hours) in the mycobacterial growth inhibition assay.

119 *Mycobacterial Growth Inhibition Assay using Whole Blood*

120 Mycobacterial Growth Inhibition assays were performed using a protocol modified from that of Wallis et al.
121 (2001). BCG was diluted in RPMI such that the desired number of CFUs was suspended in 300µl RPMI per
122 inoculum. Heparinised whole blood (300 µl) was combined with an inoculum of BCG, in triplicate.
123 Inoculated blood samples were placed on a rotator at 37°C for 96 hours. Control MGIT tubes were prepared
124 in triplicate by inoculating with the identical inoculum of BCG, and were incubated in a 37°C incubator.
125 500µl PANTA antibiotic mixture (Becton, Dickinson and Company, Sparks, USA) in OADC was added to
126 every 4ml MGIT tube (controls and blood cultures) prior to inoculation. After 96 hours of incubation, blood
127 cultures were centrifuged at 12,000rpm in an ultrafuge for 5 min to pellet the cells, and the supernatant
128 removed. 1ml of dH₂O was added to each sample and vortexed three times, at 5 min intervals, to lyse the
129 cells. Samples were centrifuged at 12,000rpm for 10 min and the supernatant removed. The pellet was
130 resuspended in 400µl 7H9 (with 10% OADC) and added to the corresponding MGIT tube. Samples were
131 placed in a 37°C incubator.

132 *Toll-like receptor (TLR) genetic diversity*

133 Primers for six TLR loci - TLR1, TLR2, TLR4, TLR5, TLR6 and TLR9 - involved in bacterial pathogen
134 recognition (Akira and Takeda, 2004) were designed from *Bos taurus* (UMD3.1) TLR sequences in Ensembl
135 release 83 (Cunningham et al., 2015), using Primer3web v4.0 (Rozen and Skaletsky, 2000). Primer
136 sequences are given in supplementary table A1, available online. Primers were designed to cover
137 approximately 1000bp of the extracellular domain of each TLR gene, in order to encompass sequence

variation that could potentially affect pathogen recognition, as described by (Alcaide and Edwards, 2011). PCR reactions were performed in a total volume of 25µl, with 1U Supertherm Gold Taq polymerase, 1X buffer, 2mM dNTPs, 200nM each primer and 1µl DNA. Thermocycling conditions were as follows: 95°C for 7 min, 35 cycles of 94°C for 30 sec, locus-specific annealing temperature (55-58°C) for 35 sec and 72°C for 40 sec, followed by a final extension step of 5 min at 72°C. PCR products were visualised on agarose and sequenced by the Central Analytical Facility (CAF) at Stellenbosch University. Sequences were aligned and edited in BioEdit (Hall, 1999). SNPs were confirmed by visual inspection of chromatograms. Diversity statistics and proportion of heterozygous sites per TLR per individual were calculated in R v 3.2 (R Development Core Team, 2013) and included as variables in the mycobacterial growth model. Departure from a neutral model of evolution was investigated on TLRs remaining in the final model using Tajima's D in the package 'pegas' (Paradis, 2010) in R v3.2.

Comparison of immune diversity between founder and source populations

An additional sample set of 30 buffalo, born prior to 1990 and from the same region of KNP as the study population, were sequenced at the TLR loci as described above. This source population represent the genetic variation up to five generations prior to the current study population. The samples were taken at approximately the same time as the study population was isolated from the region, providing a direct comparison of before and after the founder event. In addition to basic diversity measures, diploid genotypes were resolved into individual haplotypes using PHASE v2.1 (Stephens et al., 2001; Stephens and Scheet, 2005). Calculations were carried out using the default parameters. Haplotype statistics were calculated for each population, and Kruskal-Wallis chi-squared tests were used to test for statistically significant differences between populations in R v3.2.

Factors influencing mycobacterial growth

Statistical analyses were performed in R v3.2. Linear regression was used to determine the relationship between the TTP and log₁₀CFU BCG inoculum size, and generate an equation for predicting bacterial number from TTP values. Mean TTP values were used to calculate the number of bacteria present after 96 hours, and thus a mycobacterial growth rate for each buffalo individual, with growth rate (GR) = CFU_(96 hours)/CFU_(initial). Values exceeding biological plausibility (with respect to doubling time of *M. bovis* BCG) were attributed to contamination and were removed from further analyses. To assess which variables affected the mycobacterial growth in whole blood (represented by GR) in this study, the elastic net regularization technique (Zou and Hastie, 2005) was implemented in the package 'glmnet' (Friedman et al., 2010) in R v3.2. Elastic net performs automatic variable selection and continuous shrinkage while also allowing for the selection of groups of correlated variables, in a method that combines lasso (Tibshirani, 1996) and ridge (Hoerl and Kennard, 1970) regression penalties (Friedman et al., 2010; Zou and Hastie, 2005). Penalised regression techniques are often used for genome-wide association (Waldmann et al., 2013) and risk prediction studies (Pavlou et al., 2015), and can provide improved variable selection and model fit (Ambler et al., 2012). Ten-fold cross-validation was performed to determine the optimal value of the regularization

parameter lambda (λ) at penalty parameter alpha (α) values of 0.25, 0.5 and 0.75. The alpha value determines how much weight is given to lasso ($\alpha=1$) or ridge ($\alpha=0$) regression. The variables included in the full model were age group, sex, body condition, monocyte, lymphocyte, eosinophil, neutrophil and basophil counts, avian PPD response, bovine PPD response, TLR diversity (for each TLR), batch and time delay. Two way interaction effects between age group, sex and body condition, i.e. age group:sex, age group:body condition and sex:body condition, as well as pregnancy:sex and lactation:sex were also included. Details on the measurement of the variables can be seen in Table 1.

181

182 **Results**

183 *Comparison of growth rates between individuals*

184 Regression analysis showed a linear relationship between TTP and the $\log_{10}$ CFU in each inoculum,
185 represented by the equation $y = -18.52\ln(x) + 352.29$ (Fig. A2, available online). This equation was used to
186 calculate CFU_(initial) and CFU_(96 hours) in the mycobacterial growth inhibition assay. BCG growth rates per
187 individual were determined using the mycobacterial growth inhibition assay protocol as described above.
188 Triplicate cultures established from a single blood sample per individual were used to calculate the mean
189 growth rate per individual. The variance in growth rate across individuals was 50.78, and the mean growth
190 rate was 10.89 (CFU_(96 hours)/CFU_(initial)).

191 *TLR genetic diversity*

192 Five of the six TLR loci were successfully amplified and sequenced. TLR9 primers consistently generated
193 two fragments, suggesting there may be a duplication at this locus, and TLR9 was therefore excluded from
194 downstream analyses. The number of polymorphic sites per locus ranged from 3-11, and the number of
195 haplotypes ranged from 4-9. TLR final sequence lengths and diversity statistics per population, including
196 number of heterozygous sites, mean proportion of heterozygous sites, number of private SNPs and number of
197 haplotypes, can be seen in Table 2. Number of haplotypes per population were derived from PHASE best
198 reconstruction haplotype estimates. No significant differences were seen in the mean proportion of
199 heterozygous sites between populations for any of the individual TLR genes (Kruskal-Wallis tests, $p =$
200 $0.2324 - 0.9622$). Nor were any significant differences found between populations in the number of
201 heterozygous sites, mean proportion of heterozygous sites, number of private SNPs or number of haplotypes
202 across all TLR genes combined (Kruskal-Wallis tests, $p = 0.2615 - 0.406$).

203 *Predictors of mycobacterial growth*

204 Environmental and demographic parameters were assessed for their effect on predicting mycobacterial
205 growth. Initial CFU counts of BCG inocula were incorporated into growth rate calculations and therefore
206 controlled for in the analyses. Elastic net regularization was performed to enable the best selection of
207 explanatory variables in the mycobacterial growth model. The optimal value of lambda for each α penalty

was selected by determining the minimum mean square error (minMSE) along the lambda path. At lower values of α , larger ridge regression weight favours the exploration of variables with medium or small regression coefficients and allows the inclusion of correlated variables. This is appropriate for the purposes of this study, which is to explore the variation seen in the data rather than design a predictive model with the least number of predictor variables. The model that best explained the variation in mycobacterial growth rate included the following effects: eosinophil count, time delay, bovine PPD response, avian PPD response, TLR6 diversity and the interaction of age group:sex. Regression coefficients for these variables at all values of α can be seen in Table 3. Eosinophil count, time delay, bovine PPD response (Fig. 1) and avian PPD response were negatively correlated with mycobacterial growth. TLR6 diversity (Fig. 2) and age group in males (Fig. 3) were positively correlated with mycobacterial growth. Age group, sex, body condition, batch, basophils, neutrophils, lymphocytes, monocytes, TLR1, TLR2, TLR4 and TLR5 diversity, age group:body condition and body condition:sex were eliminated from the final model. No statistically significant deviation from a neutral model of evolution was seen for TLR6 (Tajima's $D = 0.79$, $p = 0.429$).

221

222 Discussion

223 *Factors influencing mycobacterial growth*

Eosinophil count, time delay, bovine PPD response, avian PPD response, TLR6 diversity and the interaction of age:sex were selected as the best explanatory variables in the model of mycobacterial growth in the study population of African buffalo. Eosinophil count, time delay, bovine PPD response and avian PPD response showed negative correlations with mycobacterial growth rate, while TLR6 diversity and the interaction between age and sex showed positive correlations.

An increase in time delay (measured in hours) from blood collection to infection may result in increased cell death in the whole blood sample prior to inoculation with BCG. As such, fewer cells would be available for intracellular infection by BCG and thus growth rate may be lower. Bovine and avian PPD responses measure the strength of the interferon gamma ($IFN\gamma$) response to stimulation with particular mycobacterial antigens. Released by CD4 and CD8 cells after infection, $IFN\gamma$ is a potent immune mediator, activating the immune system in response to mycobacterial antigens (Travar et al., 2016). Animals with a strong $IFN\gamma$ release following PPD exposure were better able to restrict mycobacterial growth in the whole blood assay, supporting the role of this gene in the innate immune response to mycobacteria. Furthermore, the inclusion of the bovine and avian PPD responses in the model shows that these values represent a meaningful signal of immune reactivity even under the threshold of test positivity, as all animals tested below 0.385 (Michel et al., 2011). The upstream processes that result in variation in the $IFN\gamma$ response may therefore be good candidates for further research. Eosinophils play an important role in the innate immune defence; they are activated by mycobacteria, are recruited in large numbers to sites of mycobacterial infection and secrete granules and secondary proteins that have potent anti-bacterial capabilities (Lasco et al., 2004; Pulido et al., 2013).

243 Although the effect size is the smallest seen in the model, the negative correlation between eosinophil count
244 and mycobacterial growth rate supports the role of eosinophils in mycobacterial defence.

245 TLR6 diversity was positively correlated with mycobacterial growth rate. Individuals with high TLR6
246 diversity (a higher proportion of heterozygous sites) were less capable of restricting mycobacterial growth
247 after infection. In mammals, TLR6 forms heterodimers with TLR2 that interact with mycobacterial cell wall
248 features. This recognition triggers a signalling cascade that leads to the activation of transcription factors and
249 the production of pro-inflammatory cytokines such as IL1 β and TNF (Bulut et al., 2001; Kleinnijenhuis et
250 al., 2011). The activation of these heterodimers influences host protection, immune evasion and immune
251 regulation, indicating that their role is multi-faceted and may differ between different stages of infection
252 (Gopalakrishnan and Salgame, 2016). Early stages of infection utilise bacterial containment strategies, with
253 the accumulation of blood-derived macrophages at the site of infection to engulf and contain multiplying
254 bacteria. After some weeks, the activation of antigen-specific T lymphocytes results in the macrophage-
255 mediated destruction of intracellular bacteria (Kleinnijenhuis et al., 2011). As such, it may be that the TLR6
256 diversity acts to improve recognition and macrophage activation at the expense of bacterial growth in the
257 initial stage of infection.

258 Alternatively, TLR6 in African buffalo may be under selection in response to the endemic bacterial species
259 present in the natural environment. Selection for a particular immune trait or profile can affect other
260 components of the immune system, which may reduce immune competency to fight other infections. For
261 example, selecting for stronger tolerance or resistance to extracellular bacteria may reduce the ability of the
262 individual to fight intracellular bacterial infections (Allen et al., 2010; Ardia et al., 2011). African buffalo are
263 host to a wide range of bacterial infections which may have led to selection pressure at immune loci in the
264 past. These include, but are not limited to, brucellosis (Gradwell et al., 1977), chlamydia (Pospischil et al.,
265 2012) and anaplasmosis (Sisson et al., 2017). The study population has been isolated from BTB thus far, and
266 were founded from a buffalo population prior to long-term BTB exposure. Thus while selection for
267 resistance to endemic bacterial species may be occurring at TLR6, there has not yet been the opportunity for
268 selection for BTB resistance in these animals. However, as the role of TLR6 in endemic diseases in African
269 buffalo has not been elucidated, we are unable to expand on this hypothesis. While Tajima's D did not detect
270 any departures from neutral evolution over this section of TLR6 in African buffalo in our study, the length of
271 DNA sequence analysed may not have been sufficient to detect selective pressure. Specific TLR6 variants
272 have been associated with BTB in Holstein cattle (Song et al., 2014), suggesting that it may be the presence
273 of particular haplotypes or amino acids that increase susceptibility to mycobacterial growth rather than
274 diversity. Unfortunately, we were unable to use haplotypes in the analysis due to sample size limitations.
275 Interestingly, there were 10 non-synonymous vs 1 synonymous substitution sites in the TLR6 sequence
276 analysed (data not shown). An excess of non-synonymous to synonymous substitutions may indicate
277 diversifying selection, which occurs when diversity is selectively maintained in a population. Diversifying
278 selection can include processes such as positive selection for particular alleles within a locus, and this is one
279 of the crucial drivers of the evolution of immune genes in animals (Potts and Slev, 1995), plants (Allen et al.,

280 2004) and pathogens (Guttman et al., 2006). It is also possible that there are additional molecular
281 mechanisms involved with the copy number of particular recognition sites, but in the absence of more
282 information associating allelic variants to recognition site changes, this cannot be explored further.

283 Age shows a positive correlation with mycobacterial growth rate in males. We hypothesize that this may be
284 due to the immunosuppressive action of testosterone on the immune system. Androgenic suppression of the
285 immune response has been shown in a number of species, including salmon (Slater and Schreck, 1993),
286 chimpanzees (Muehlenbein and Watts, 2010) and badgers (Tomlinson et al., 2013). The immuno-
287 competence handicap hypothesis postulates that there is a trade-off between sexually-selected traits
288 (mediated by testosterone) and the immune system, most likely due to the unequal allocation of limited
289 resources (Roberts et al., 2004). As an anabolic steroid, testosterone increases metabolic rate, fat metabolism
290 and muscle function, and moderates the physiological and neurological changes associated with aggression
291 and dominance behaviour (Muehlenbein and Watts, 2010). As age increases, sexual maturity and
292 competition for breeding dominance would result in higher testosterone levels in buffalo bulls. This is
293 therefore a plausible explanation for the presence of this interaction effect on mycobacterial growth rate in
294 our model.

295 *TLR immune diversity*

296 In this study population of African buffalo, we found a total of 38 SNPs and 31 haplotypes across five TLR
297 loci, with an average of one SNP per 120bp across all TLR loci sequenced. This is comparable to TLR
298 diversity in the cow; (Fisher et al., 2011) determined the average polymorphism density in *Bos taurus* cattle
299 to be one SNP per 158bp across all TLR loci amplified. No statistically significant differences were found in
300 any of the immune diversity parameters between the study population and the source population. This
301 suggests that despite a low level of genetic drift, the isolated study population was founded with a sufficient
302 number of individuals to retain the genetic diversity seen at these loci. The small differences seen may also
303 be a function of the sample sizes; if more individuals from the source population were sampled, the few
304 alleles that appear private to the study population may in fact be represented in the source population. For the
305 purposes of this study, the TLR genetic diversity in the study population is essentially representative of that
306 found in the free-ranging source population prior to any BTB selection.

307 In conclusion, our results suggest that both demographic and individual immune parameters influence the
308 ability to control mycobacterial infection in African buffalo. Toll-like receptor diversity, age in males, and
309 immune response markers all affected the growth of mycobacteria in this study population. TLR6 diversity is
310 particularly interesting as this locus has also shown associations with BTB in cattle, suggesting that further
311 research into the effects, selection and role of TLR6 variants in bovine tuberculosis will be productive.

312

313

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 486

492 Table 1. Demographic and genetic variables included in the mycobacterial growth model

Parameter	Assessment	Measurement
Age	Incisor wear	Age class: sub-adult (1-4), adult (5-14), senior (>14)
Sex	Visual examination	Male, Female
Pregnancy status	Internal examination	Yes, No*
Lactation status	Physical assessment	Yes, No*
Body condition	Physical assessment	Scale of 1 (very poor condition) to 5 (excellent condition)*
White cell count	Automated cell counter	$\times 10^3$ cells per millilitre*
Monocytes	Blood smear	$\times 10^3$ cells per millilitre*
Neutrophils	Blood smear	$\times 10^3$ cells per millilitre*
Lymphocytes	Blood smear	$\times 10^3$ cells per millilitre*
Eosinophils	Blood smear	$\times 10^3$ cells per millilitre*
Avian PPD response	Interferon gamma release assay	OD value*
Bovine PPD response	Interferon gamma release assay	OD value*
TLR diversity	Proportion of heterozygous sites	0-1
Batch	Sample groups	Experimental batch 1-6
Time Delay	Time	Hours between blood draw and inoculation

493 * details of test measurements in Beechler et al. (2012)

494

494 Table 2. Immune diversity statistics for study and source populations

Locus	Pop	Length (bp)	N	Het sites	Mean Prop Het	Private SNPs	Haplotypes
TLR1	Study	901	43	7	0.08	2	5
	Source	901	25	6	0.13	1	6
TLR2	Study	884	43	4	0.22	1	5
	Source	884	29	3	0.24	0	4
TLR4	Study	930	43	5	0.12	0	4
	Source	930	27	7	0.11	2	5
TLR5	Study	871	43	11	0.27	2	9
	Source	871	28	10	0.26	1	8
TLR6	Study	928	43	11	0.24	3	8
	Source	928	26	11	0.27	3	8

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497 Table 3. Regression coefficients of explanatory variables of mycobacterial growth in African buffalo at
 498 different penalty weights of α .

Variable	Coeff ($\alpha = 0.25$)	Coeff ($\alpha = 0.5$)	Coeff ($\alpha = 0.75$)
(Intercept)	3.0131	3.0086	3.0073
Eosinophils	-0.0173	-0.0064	n/a
Time Delay	-0.1114	-0.1128	-0.1129
ODbovC	-0.5727	-0.5598	-0.5567
ODavC	-0.2931	-0.2018	-0.1284
TLR6 prop	0.2880	0.2449	0.2090
AgeGroupAdult:SexM	0.4910	0.5050	0.5053

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500 **Figure Legends**

501 **Fig. 1** Correlation between *M. bovis* BCG growth rate and bovine PPD response

502 **Fig. 2** Correlation between TLR6 diversity and growth rate of *M. bovis* BCG in African buffalo

503 **Fig. 3** Growth rate of *M. bovis* BCG in whole blood of African buffalo as a function of the interaction
504 between age group and sex

505

Growth Rate

Age Group