1 **Domestic pigs experimentally infected with *Mycobacterium bovis* and *Mycobacterium***

2 ***tuberculosis* exhibit different disease outcomes**

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Abstract

Domestic pigs share many similarities with humans in their pulmonary anatomy, physiology, and immunology. Accordingly, pigs have been shown to be valuable models to study human tuberculosis (TB). Here we examined the outcome of disease in domestic pigs challenged via two different routes with either the human-adapted TB bacillus *Mycobacterium tuberculosis* or the zoonotic bovine TB bacillus *M. bovis* in head-to-head comparisons. We found that pigs challenged intravenously with *M. bovis* AF2122/97 exhibited severe morbidity and rapid onset of mortality, accompanied by higher tissue bacterial burden and necrosis compared to pigs challenged similarly with *M. tb* Erdman. Concordantly, pigs challenged with aerosolized *M. bovis* AF2122/97 exhibited reduced weight gain and more severe pathology than pigs challenged similarly with *M. tb* Erdman. Moreover, pigs aerosol-challenged with *M. bovis* AF2122/97 exhibited a spectrum of granulomatous lesions ranging from small well-contained granulomas to caseous-necrotic lesions mimicking active TB disease in humans. In contrast, pigs aerosol-challenged with *M. tb* Erdman exhibited arrested granuloma development. Irrespective of challenge dose and pathological outcome however, peripheral IFN-γ responses were similar in both *M. bovis* AF2122/97 and *M. tb* Erdman challenged pigs. This study demonstrates domestic pigs can support infections with *M. bovis* and *M. tb* and develop pathology similar to what is observed in humans. And although *M. bovis* AF2122/97 appears to be more virulent than *M. tb* Erdman, both strains can be used to model TB in domestic pigs, depending on whether one wishes to recapitulate either acute and active TB or latent TB infections.

Introduction

Tuberculosis (TB) is a pulmonary infectious disease that affects a wide range of mammals, and it is one of the leading causes of death in humans. In fact, 1.4 million people globally are estimated to have died in 2019 alone due to TB [1]. TB is caused by members of the *Mycobacterium tuberculosis* complex (MTBC). Most cases of human TB are caused by *Mycobacterium tuberculosis* (*M. tb*) although *M. bovis,* which is another member of the MTBC that affects livestock and wildlife, can cause zoonotic TB [1]. TB transmission occurs mainly through bacteria-containing aerosol particles and primarily affects the lungs. Extra-pulmonary TB like meningeal and miliary TB on the other hand, occurs mostly in children and immune-compromised people respectively [2,3]. TB manifests as two clinical forms - latent infection which is asymptomatic and non-transmissible, and an active disease which is symptomatic as well as transmissible. However, it is now believed that the two forms are the extremes of a dynamic pathological continuum [4].

Much of our current understanding of TB pathogenesis is based on studies of the disease in animal models that include but are not limited to mice, hamsters, guinea pigs, rabbits and non-human primates (NHPs) [5–7]. Except for NHPs however, none of the other models recapitulate the full spectrum of pathology and clinical signs of human TB [6,8]. For example, *M. tb*-infected mice which are the most frequently used model, tend to produce pseudo-granulomas and do not recapitulate different aspects of human TB pathology [9]. Guinea pigs are hyper-susceptible to *M. tb,* develop disseminated multi-organ infections and rapidly succumb to the disease [10]. Rabbits, unlike mice and guinea pigs, develop cavitary lung lesions upon infection with virulent MTBC strains [11]. However, they do not mimic the dynamic pathological changes seen in humans and the clinical signs are not obvious which limits their usefulness as a model [6,12].

68   Non-human primates (NHPs) remain the best models for human TB due to their close

69   phylogenetic relationship with humans [13,14], and their inherent susceptibility to infection with

70   MTBC [15,16]. Indeed, a relatively low dose (10 - 100 CFUs) of *M. tb* is sufficient to establish

71   infection in NHPs and induce a wide spectrum of clinical outcomes ranging from no disease to a

72   rapidly progressing fulminant disease to chronic infection [14,17]. As in humans, TB granulomas

73   in NHPs are well-organized, consisting of a central core of infected and non-infected macrophages

74   surrounded by a layer of lymphocytes and multi-nucleated giant cells enclosed by a fibrous capsule

75   [17,18]. Likewise, the formation of caseous necrotizing granulomas seen in humans with active

76   TB also occurs in NHPs [19]. Despite these features, the NHP model has its drawbacks. First, it

77   can be challenging to procure NHPs and facilities capable of maintaining large NHP colonies are

78   limited [20]. Second, NHPs require a lot of space, which can be challenging in biocontainment

79   level-3 labs. Finally, NHP use in research is becoming less socially acceptable due to their well-

80   developed emotional and social behaviors [21].

81   Due to its similarities to humans with respect to anatomy and physiology [22], the domestic

82   pig (*Sus scrofa domesticus*) has become an increasingly attractive model for studying human

83   diseases like pertussis, influenza, genetic disorders like cystic fibrosis, and xenotransplantation

84   [22–24]. Moreover, the immune system of a pig is similar to that of a human by more than 80 %

85   of the studied parameters compared to a mere 10 % similarity between a human and a mouse [25].

86   For instance, out of 419 clusters of differentiation (CD) molecules found on the cell surface of

87   humans leukocytes, 359 orthologs have been described in pigs [26]. Likewise, both share

88   similarities in T-cells subtypes and Toll-like receptors which are critical for host-pathogen

89   interactions and immune responses [27]. Both human and porcine lungs are divided into different

90   lobes, which are further demarcated into multiple lobules by connective tissue septa, a feature

91   shown to be important for granuloma encapsulation [28,29]. The larger size of pigs enables

92   repeated sampling, and the availability of porcine specific reagents has also increased. Since pigs

93   are a livestock species and reared commercially in large numbers, there is a steady supply for

94   medical research.

95   Wild boars (*Sus scrofa*) infected naturally with *M. bovis* show similar pathology to humans

96   infected with *M. tb* [30,31]. These include a range of well-contained, non-necrotizing granuloma

97   to caseous-liquifying and calcified lesions [31]. Bolin *et. al.* were the first to show experimental

98   disseminated *M. bovis* infection in mixed-breed domestic pigs [32]. However, the route of

99   challenge in their study was either intravenous, intra-tracheal, or tonsillar deposition, none of

100   which mimic the natural route of MTBC infection [32]. Gil *et.al* on the other hand challenged

101   minipigs with *M. tb* and showed the pig's pulmonary lobular structure helps establish fibrosis and

102   encapsulation to contain the infection [29]. Likewise, Ramos *et.al* used minipigs to study TB

103   infection and natural transmission in adolescents and infants [33]. Although they challenged

104   minipigs with infectious aerosols of the hypervirulent Beijing strain of *M. tb,* they did not compare

105   *M. bovis* and *M. tb* [33].

106   In this study, we infected domestic pigs (*Sus scrofa domesticus*) with *M. tb* Erdman and *M.*

107   *bovis* AF2122/97 strains. Although *M. tb* and *M. bovis* have been compared in cattle and goats

108   [34,35], we wanted to examine differences in outcomes upon infection with these two MTBC

109   members in pigs. In the first trial, pigs were challenged intravenously with *M. tb* and *M. bovis*. In

110   the second trial, pigs were challenged either with *M. bovis* or *M. tb* at two different doses via the

111   aerosol route. We evaluated clinical signs, size and extent of lesion development and

112   histopathology. In both trials, *M. bovis* induced more severe clinical symptoms and pathology and

113   compared to *M. tb*.

114 **Results and Discussions**

115 **1. Intravenous (IV) challenge**

116 **1.1. Clinical Signs**

117   Pigs challenged intravenously (IV) with *M. bovis* AF2122/97 exhibited signs of elevated

118 respiration and reduced activity by as early as 7 days post-challenge (pc). All six pigs in the *M.*

119 *bovis* group exhibited abdominal breathing, stooped posture, inappetence and reduced mobility by

120 day 9 pc (Supplementary file 1). One out of six *M. bovis* challenged pigs died on day 11 pc, while

121 two more pigs reached their humane endpoint and had to be euthanized on day 14 pc. By day 16,

122 the remaining *M. bovis* challenged pigs also reached their humane endpoints and were euthanized

123 (Figure 1). In stark contrast, *M. tb* challenged pigs appeared healthy, ate, and behaved normally,

124 and showed no signs of respiratory distress (Supplementary file 2). Five out of six pigs of this

125 treatment group went on to survive the duration of the trial (Figure 1). There was an earlier onset

126 of fever in pigs challenged with *M. bovis* with all six pigs in the group exhibiting increasing body

127 temperatures that started 3 days pc before peaking around 9 to 10 days pc (Figure 2). In contrast,

128 *M. tb* infected pigs exhibited delayed onset of fever with increases in body temperatures that started

129 6 to 7 days pc before peaking at about 14 days pc (Figure 2). The body weights of pigs were also

130 measured on the day of the challenge and then at multiple time points pc for the duration of the

131 trial. Although most *M. bovis* challenged pigs survived till 16 days pc, there was a clear indication

132 these pigs were losing more weight compared to the *M. tb* challenged group. Indeed, *M. bovis*

133 infected pigs that were still alive weighed 1.5 kg less on average than *M. tb* infected pigs after two

134 weeks despite starting out at similar weights. Although *M. tb* infected pigs did not lose weight for

135 the entire 35 days of the trial, they did not achieve the type of weight gain expected in uninfected

136 pigs over the same period (Figure 3).

137   That *M. bovis* IV challenged pigs exhibited rapid onset of morbidity and mortality in this

138   study is consistent with the findings of Bolin *et. al* where pigs IV challenged with 10^8 CFU of a

139   clinical isolate of *M. bovis* developed severe respiratory distress, fever, exertion and had to be

140   euthanized by day 22 pc [32]. However, Bolin *et. al*. also observed that pigs challenged with a

141   similar dose of *M. bovis* by the intratracheal route exhibited mild symptoms while pigs challenged

142   by tonsillar deposition did not show any signs of infection [32]. This led them to conclude that

143   route of challenge is an important determining factor in the development of overt clinical signs

144   [32]. Our results also highlight MTBC strain is an important determinant of TB disease severity.

145   Additionally, Bolin *et. al.* suggested that the more severe disease seen in their IV challenged pigs

146   was due to the involvement of the central nervous system (CNS) as granulomatous meningitis was

147   found in 3 pigs and *M. bovis* was recovered from the brain tissues and cerebrospinal fluid (CSF)

148   of 4 additional pigs without meningitis [32]. Although we did not sample and analyze the meninges

149   or CSF in our IV challenge trial, Bolin's findings could explain the higher morbidity and mortality

150   seen with our *M. bovis* challenged pigs. Indeed, studies in other animal TB models have shown

151   high dose bacterial IV challenge can lead to CNS involvement [36,37].

152   **1.2.Pathology**

153   Detailed necropsy of pigs from both challenge groups revealed the development of

154   edematous, non-collapsing lungs which is indicative of severe inflammation (Figure 4A and B).

155   However, none of the pigs in either challenge groups presented at the gross level with

156   granulomatous lesions on the pleural or visceral surfaces of the lungs (Figure 4A-D). Despite the

157   absence of granulomatous lesions, hemorrhaging on the surface of both the lungs and liver were

158   observed mostly in *M. bovis* challenged pigs (Figure 5A). Moreover, this feature was sometimes

159   accompanied by the presence of fluid in the thoracic and abdominal cavities (Figure 5B).

160   Typical histopathological lesions included loose aggregation of inflammatory cells

161 including macrophages, lymphocytes, and neutrophils (Figure 6A-E). Necrosis with loss of

162 cellular architecture was a common finding in both *M. bovis* and *M. tb* infected animals. Vasculitis

163 and occluding inflammatory cellular aggregates in the arteriole and terminal bronchioles within

164 the lungs were observed (Figure 6A and B). Bronchioles of both *M. tb* and *M. bovis* infected pigs

165 were inflamed with an accumulation of inflammatory cells such as neutrophils, epithelioid

166 macrophages, and multinucleated giant cells (Figure 6C). These occluding lesions are consistent

167 with observations made in previous studies of *M. bovis* infected pigs and cattle [32,38]. Although

168 areas of necrosis were observed in lung tissues of both *M. tb* and *M. bovis* infected pigs, the extent

169 and frequency of such lesions appeared higher in *M. bovis* infected pigs (Figure 6D). Large areas

170 of necrosis were found in the lymph nodes of *M. bovis* infected pigs while *M. tb* infected pigs

171 tended to display what appears to be small nascent granulomas (Figure 6E). The lesions were

172 microscopic granulomas in early stages not yet surrounded by fibrous capsules. The short duration

173 of the trial most likely did not allow for the development of larger more organized spectrum of

174 granulomas normally seen in naturally infected pigs and humans with active TB. Classification of

175 the granuloma into different stages was not possible in the IV challenge trial because most

176 observed lesions were early inflammatory aggregates without much distinction.

177

178   **1.3. Bacterial burden**

179   Bacteria were recovered from the caudal, mid, and cranial lung lobes, the tracheobronchial

180 lymph nodes, and spleens of both groups of IV infected pigs. Based on the bacterial colony-

181 forming unit (CFU) counts, the bacterial burden was much higher in pigs challenged with *M. bovis*

182 compared to the pigs challenged with *M. tb* (Figure 7). *M. bovis* challenged pigs had on average

183   10-fold higher bacterial burden in all tissues compared to *M. tb* infected pigs despite being sampled

184   earlier. The difference was also observed in Ziehl-Neelsen (ZN) stained tissue sections from lungs

185   and lymph nodes of the infected pigs (Figure 6F). However, as the route of the challenge was IV,

186   it was difficult to determine whether the presence of bacteria in the spleen and tonsils was due to

187   primary bacterial inoculation or secondary dissemination from the lung. Nevertheless, the lower

188   bacterial burden in *M. tb* challenged pigs suggests *M. tb* but not *M. bovis* replication may be better

189   controlled by pigs.

190   Despite being IV challenged with similar doses, the greater morbidity and higher rate of

191   mortality accompanied by marked differences in lack of weight gain, onset of fever, pathology,

192   and bacterial burden in *M. bovis* challenged pigs suggests *M. bovis* is more virulent in the pig

193   model than the human adapted *M. tb*. However, neither *M. tb* nor *M. bovis* IV challenged pigs

194   develop granulomatous lesions due to the short duration of the trial. Also an IV challenge is unlike

195   what occurs during aerosol infection where the disease establishes first as a localized pulmonary

196   infection followed by dissemination to other organs months or years later [39–41].

197

198   **2. Aerosol challenge**

199   **2.1. Clinical Signs**

200   Pigs challenged with *M. bovis* and *M. tb* by the aerosol route did not develop overt clinical

201   signs such as cough, fever, and respiratory distress even after 9 weeks post infection. This is

202   consistent with published findings where aerosol infection of minipigs with 10^4 CFU of *M. tb*

203   failed to generate coughing and fever even after 36 weeks pc [33]. However, significant differences

204   with respect to weight gained by *M. bovis* versus *M. tb* challenged pigs were observed 9 weeks

205   post infection (Figure 8). Pigs challenged with high dose *M. tb* (*M. tb*-HD) registered average

206   weight gains of 44 kg compared to 36 kg in pigs challenged similarly with high dose *M. bovis* (*M.*

207   *bovis*-HD). However, pigs challenged with low dose of *M. bovis* and *M. tb* (*M. bovis*-LD and *M.*

208   *tb*-LD) had weight gains of 45 kg and 47 kg respectively (Figure 8). It should be noted that in

209   studies of aerogenic TB in NHPs it takes more than 24 weeks to determine whether an animal has

210   developed latent infection or active disease, based on the presence or absence of clinical signs such

211   as cough and weight loss, AFB in the broncho-alveolar and gastric aspirates, and elevated ESR in

212   the latter [8]. As such, the lack of observable clinical signs in aerosol challenged pigs could be due

213   to the shorter duration of our trial.

214

215   **2.2. Pathology**

216   Most pigs aerosol challenged with *M. bovis*-HD and *M. tb*-HD exhibited visible pulmonary

217   granulomatous lesions at necropsy 9 weeks post infection. These were uniformly distributed in all

218   the lobes (Figures 9A-D). Entire lungs from pigs of both challenge groups, cut into several slices

219   along their cross-sections further revealed multiple granulomas in the interior surface (Figure 9B

220   and D). Lesions in the lungs varied from very small pin-point hemorrhages to large, calcified

221   nodules with sizes ranging from 2 mm to more than 5 cm in diameter. Notably, cut sections of

222   lungs from *M. bovis*-HD infected pigs presented with large caseating granulomas (Figure 9B). The

223   texture of some of these were hard and gritty due to fibrosis and calcification. Moreover, these

224   mature granulomas were often located close to or encircling the conducting airways (Figure 9B).

225   In several pigs, bronchus stricture was apparent with their walls being affected by caseation

226   indicating possible shedding of bacteria (Figure 9B). In contrast, lungs of pigs infected with *M. tb*

227   showed considerably fewer granulomas (Figure 9C). They were also smaller in size and compact

228   (Figure 9D).

229   Scoring revealed significantly higher lung lesion scores in *M. bovis*-HD infected pigs than

230   both *M. bovis*-LD and *M. tb*-LD infected pigs (Figure 10). Meanwhile, *M. bovis*-HD infected pigs

231   had qualitatively higher lung lesion scores than *M. tb*-HD infected animals, but this was not

232   statistically significant (Figure 10). Likewise, a qualitatively higher but non-significant lung lesion

233   score was observed between *M. bovis*-LD and *M. tb*-LD pigs. Multiple granulomas were observed

234   in the mediastinal lymph nodes (LNs), tracheobronchial LNs and tonsils of all the pigs, irrespective

235   of the challenge strain and dose (Figure 11A-D). Although the granulomas in the LNs were not

236   scored, *M. bovis*-HD infected pigs appeared to have larger and more distinct lesions (Figure 11A).

237   The lesions appeared as large, pale indurations on the serous surface which revealed white caseous

238   masses when cut open (Figure 11A-D).

239   Microscopically, pigs aerosol challenged with either *M. tb* or *M. bovis* showed

240   granulomatous lesions in at least one organ (Figure 12A-F). These include lesions with thickening

241   of lung parenchyma (Figure 12A), multiple satellite granulomas (Figure 12B), collapsed

242   bronchioles (Figure 12C), and with accumulations of macrophages, lymphocytes, and neutrophils

243   (Figure 12D), and organized granulomatous lesions surrounded by thick fibrous capsule (Figure

244   12D). Advanced stage lesions with fibrosis encapsulating granulomas (Figure 12E) as well as with

245   necrotic cores (Figure 12F) were also observed. We also observed foamy macrophages, epithelioid

246   macrophages and multinucleated giant cells that comprise the core of granulomas (Figure 13). A

247   large number of neutrophilic infiltrations was observed in advanced stage granulomas indicating

248   the start of liquefactive necrosis (Figure 13). ZN staining showed varying numbers of acid-fast

249   bacilli (AFB), which did not appear to be associated with CFU burden in our analysis.

250   Interestingly, Santos *et. al.* reported that AFB cannot be reliably associated with the presence or

251   absence of infection in wild pigs [42]. Moreover, there were differences in the types of granulomas

252   observed even within the same pig, a feature commonly associated with active TB in cynomolgus

253   macaques [43]. Due to the differences in the number and nature of granuloma in different challenge

254   groups in this study, a classification scheme was developed.

255   Histologically, lung granulomas can be classified into different stages [31,44,45]. Stage-1

256   granulomas are characterized by the loose aggregation of inflammatory cells such as epithelioid

257   macrophages, lymphocytes, neutrophils, and very few multinucleated giant cells (MNGCs)

258   without fibrous capsule [31]. Stage-2 granulomas are characterized by fibrous encapsulation of an

259   organized lesion composed of the central core of neutrophils, foamy macrophages and abundant

260   epithelioid macrophages, and a peripheral rim of T and B lymphocytes [31]. Stage-3 granulomas

261   have much thicker and complete fibrous encapsulation and while the cellular constituents are

262   similar to stage-2, there is more neutrophilic infiltration towards the core of the lesion which ensues

263   necrosis [31,44,45]. Smaller granulomas may begin to fuse to form a multifocal granuloma at this

264   stage. Stage-4 granuloma is marked by extensive caseous necrosis and liquefaction such that there

265   is an amorphous to acellular zone in the center of the granuloma. This is when the granuloma loses

266   its integrity and the animal is believed to be transmissive [46]. Stage-4 lesions may be surrounded

267   by few to numerous satellite granulomas in stage-1 or stage-2 [31].

268   In our study, at least one lung H&E-stained sample from all the animals in each group of

269   the aerosol challenge trial was studied and the granulomas were categorized into one of the four

270   stages described above (Figure 13). For analysis, stages-1 and 2 lesions were considered as early

271   and intermediate granulomas respectively whereas stages-3 and 4 were considered as the advanced

272   stage granulomas. It was interesting to note that 9 (81%) out of 11 lung samples from *M. bovis-*

273   HD infected pigs had advanced granuloma suggesting that these animals are manifesting an active

274   form of TB (Table 2). In contrast, only 3 (50%) out of 6 lung samples had an advanced lesion in

275   *M. tb*-HD infected pigs (Table 2). Similarly, only 1 out of 11 tissues had stage-4 granuloma in *M.*

276   *bovis*-LD challenged pigs while no stage-4 granulomas were found in *M. tb*-LD infected pigs

277   (Table 2).

278   In this study, we have provided a detailed characterization and classification of

279   histopathological lesion in pigs which is an advancement over the existing body of knowledge

280   available from previous works on minipigs [29,33]. Although the number of tissue samples

281   available from the aerosol challenge trial for H&E staining were few, there is clear indication that

282   infection with *M. bovis*-HD results in the production of more advanced stage granulomas than

283   infection with *M. tb*-HD. Significantly, this demonstrates pigs can develop caseous granulomas

284   with central necrosis – a hallmark of active human TB [47]. A comparative study done in cattle

285   reported that *M. bovis* challenged calves had lesions ranging from stage 1 to 4 while the calves

286   challenged with two different strains of *M. tb* had arrested granuloma development with lesions

287   mostly at stages 1 and 2 [34]. The differences in pathological outcomes observed in our study is

288   consistent with the cattle study, which attributed the difference in pathology to attenuation of *M.*

289   *tb* in the animal host [34,48]. This finding suggests that *M. tb* could be used to generate latent

290   infection model while *M. bovis* could be used to develop an active disease model in pigs, although

291   the dose might have to be adjusted to a fewer CFU per animal to mimic latent infection in humans.

292

293   **2.3. Bacterial burden**

294   Bacteria were only recovered from the lungs (pooled data of cranial, mid, and caudal lobes

295   tissues) of 2 out of 6 pigs for both *M. bovis*-LD and *M. tb*-LD challenged groups (Figure 14).

296   Similar bacterial burden was seen for lymph nodes (pooled data of tracheobronchial, mediastinal,

297   and retropharyngeal lymph node tissues) of *M. bovis*-LD and *M. tb*-LD challenged groups (Figure

298   14). Bacteria was not recovered from the spleens of either *M. tb*-LD or *M. bovis*-LD challenged

299   pigs (Figure 14). With the HD challenge groups, bacteria were recovered from the lungs (pooled

300   data of cranial, mid, and caudal lobes tissues) of *M. tb*-HD challenged pigs and *M. bovis*-HD

301   challenged pigs. Notably, lungs samples of *M. bovis*-HD challenged pigs had higher median

302   burdens. Similarly, there was a higher bacterial burden in the lymph nodes (pooled data of

303   tracheobronchial, mediastinal, and retropharyngeal lymph node tissues) of *M. bovis*-HD

304   challenged pigs compared to *M. tb*-HD challenged pigs (Figure 14). Viable bacteria were

305   recovered from the spleens of two pigs challenged with *M. bovis*-HD only, suggesting post-

306   primary dissemination likely through hematogenous route. A similar finding was reported in goats

307   where dissemination of infection outside the thorax was seen only in *M. bovis* infected goats [35].

308   Overall, pigs aerosol challenged with *M. bovis* were found to have a relatively higher

309   pulmonary burden than *M. tb*, especially in the high dose challenge groups. This suggests that pigs

310   are able to contain infection with *M. tb* better than infection with *M. bovis*. Indeed, mycobacteria

311   were recovered from the lungs of only 3 *M. tb* challenged pigs. This was despite the similar

312   challenge dose and duration of infection for *M. tb* and *M. bovis*. The differences in tissue bacterial

313   burden also correlates positively with the difference in the lung lesion scores. Our study also

314   demonstrates heterogeneity in bacterial burden and lesion severity which has previously been

315   reported in the minipig model as well as in cattle [33,49]. Furthermore, the differences in pathology

316   and bacterial burden are likely associated with the differences in overall weight gains exhibited by

317   *M. bovis*-HD and *M. tb* -HD infected pigs.

318

319   **2.4. Peripheral T-cell response after challenge with aerosolized *M. bovis* and *M. tb***

320   TB disease progression in aerosol challenged pigs was done using the Interferon-gamma

321   release assay (IGRA), a sensitive and specific test for diagnosing TB in pigs [50]. The assay

322   quantifies the production of Interferon gamma (IFN-γ) in response to mycobacterial antigens by

323   infected animals. A steady increase in IFN-γ concentrations over time was observed in all four

324   treatment groups irrespective of the doses and strains used (Figure 15). The immune conversion

325   of IGRA from negative to positive typically occurs within 4-6 weeks post infection in humans

326   [51]. In this study, most pigs showed detectable IFN-γ levels 4 weeks after challenge which

327   correlates with the beginning of the adaptive immune response and activation of effector T-cells,

328   which are the main producers of IFN-γ. The concentration reached a maximum value at 9 weeks

329   post-challenge suggesting the highest T-cell immune activity at that period. Similar kinetics of T-

330   cell immune response is documented in minipigs [29], wild pigs [52], goats [53] and cattle [34].

331   Moreover, infection of pigs with *M. tb* and *M. bovis* at both high and low doses induced similar

332   peripheral immune responses. This is consistent with findings from a study comparing IFN-γ

333   responses to bPPD in cattle that were also infected with *M. bovis* and *M. tb* for 16 weeks but

334   displayed no differences in IFN-γ responses [54].

335

336   **Conclusion**

337   In this study, we have determined that *M. bovis* AF2122/97 is more virulent in pigs compared to

338   *M. tb* Erdman regardless of route of infection. Our extensive analysis of the histopathological

339   lesions found in the tissues of both *M. bovis* and *M. tb* infected pigs revealed domestic pigs mimic

340   the pathological features of a human TB. Specifically, pigs aerosol-challenged with high doses of

341   *M. bovis* developed wide spectrum of granulomatous lesions including caseation and necrosis,

342   much like in humans with active TB, while pigs challenged with low dose *M. tb* exhibit arrested

343   granuloma development much like in humans with latent TB. These findings support the notion

344   that the domestic pig is a useful animal model to study active and latent TB.

345   **Materials and Methods**

346   **1. Preparation of challenge material**

347   Frozen glycerol stock of *Mycobacterium tuberculosis* (Erdman) and *M. bovis* (AF2122/97) were

348   thawed and plated on Middlebrook 7H11 agar plates. A single colony was used to inoculate

349   Middlebrook 7H9 liquid medium, supplemented with 10% Albumin Dextrose Catalase (ADC) and

350   0.05% Tween-80. *M. bovis* culture media was additionally supplemented with 0.5% sodium

351   pyruvate. Bacterial cultures were incubated at 37^0C until mid-log phase growth was achieved. A

352   subculture was prepared with a starting OD_{600} of 0.1 twenty-four hours before the animal

353   challenge. On the day of challenge, bacterial culture was spun at 300g for 3 minutes and

354   supernatant, which contains single-cell suspension, was harvested for enumeration by OD. An OD

355   of 1 corresponds to 3 x 10^8 CFU/mL for *M. tb* and 1 x 10^8 CFU/mL for *M. bovis.* Necessary

356   volumes of each culture were diluted in PBS to make the required challenge inoculum.

357

358   **2. Study Design and Challenge**

359   • Intravenous challenge

360   Twelve 4-weeks old, domestic pigs were sourced from Prairie Swine Center and brought

361   to Agriculture Biosafety Level-3 (Ag-BSL-3) facility at VIDO. Animals were tagged with unique

362   numbers and randomly divided into two groups (Group A and Group B) with 6 pigs in each group

363   (Table 1). The pigs were allowed to acclimatize in the containment facility for 7 days before the

364   challenge. Group A was challenged with intravenous injection of 3×10^8 CFU of *M. bovis* per pig

365   while group B was challenged with intravenous injection of 3×10^8 CFU of *M. tb* per pig. Six pigs

366   from each challenge group were housed in 14 feet x 5.5 feet pens. They were kept on a raised

367   platform and were under continuous surveillance through a camera and in person. Although both

368   the challenged groups were housed in the same containment room, they were separated by a 2.5

369   feet high physical barrier enclosing each pen. The animal containment rooms had a net negative

370   pressure with the air exchange maintained at 10 cycles per hour allowing for whole air volume

371   replacement multiple times in an hour. The exhaust mechanism thereby greatly minimizes the

372   chances of cross-infection. The room was provided with 12-12 hours of light and dark cycles. The

373   animals were monitored for changes in clinical signs such as respiratory distress, fever, anorexia,

374   weight loss, and other morbidities. Daily weight and temperature measurements were taken for 10

375   consecutive days.   Food and water were provided ad-libitum for the entire period of the

376   experiment.

377   • Aerosol challenge

378   24 three-week-old domestic pigs were brought to the containment facility at VIDO and

379   allowed to acclimatize for a week. These animals were tagged and randomly divided into four

380   groups containing 6 animals each. Two challenge doses were prepared for each strain as mentioned

381   above and the inoculum was aerosolized through a Collison nebulizer (CH Technologies, USA),

382   connected to a tank of compressed oxygen. Aerosol generated was passed through a tube into a

383   mask that fitted snugly to the pig's snout. Each pig received 2 mL of the challenge inoculum. Post-

384   challenge, the animals were housed similarly as described for the IV challenge.

385

386   **3. Gross pathology and Histopathology**

387   Pigs that died during the experiment or those euthanized were subjected to a detailed gross and

388   histopathological examination. During necropsy, the presence of tuberculosis-associated lesions

389   was monitored in the lungs, liver, spleen, lymph nodes and other visceral organs in the thoracic

390   and cervical regions. For histopathological evaluation, a 2x2x2 cm^3 section of tissue was sampled

391   and secured within a tissue cassette, immersed into 10% buffered formalin for fixation inside the

392   BSL-3 containment. These samples were later taken to Prairie Diagnostic Services, Saskatoon for

393   Hematoxylin-Eosin (H&E) and Ziehl-Neelsen (ZN) staining.

394   • Lung lesion scoring

395   Using visual and palpation skills, the chief veterinarian at VIDO scored each lung lobe

396   based on the scoring scheme as developed by Palmer *et. al.* in cattle with minor modifications [38]

397   (Table 1). The total lung lesion score for each animal was obtained by adding the lesion score of

398   individual lobes. Both sizes of the granuloma and number were considered while scoring the

399   lesions.

400   **Table: 1 Lung lesion scoring scheme for cattle** (developed by Drs. Carly Kanipe & Mitch

401   Palmer, USDA)

Lesion score	Observation
0	No visible lesion
1	**1.** Less than 10 lesions **2.** All lesions less than 10 mm in diameter
2	**1.** 6-10 lesions **2.** Rare (≤ 2) lesions between 10-20 mm in diameter
3	**1.** 11-20 lesions **2.** Occasional (3-5) lesions between 10-20 mm in diameter
4	**1.** More than 20 lesions **2.** Frequent (≥5) lesions between 10-20 mm in diameter
5	**1.** Countless & coalescing lesions or **2.** More than half of the lesion is>10 mm or

	3. Any lesions > 20 mm in diameter	

402

- Staging of granulomas

Tissue sections were categorized into different stages based on their cellular composition, architecture, degree of necrosis and extent of fibrosis [31,44,45] For each H&E stained tissue sample, 10 field-of-views were chosen randomly within a slide and each field was categorized into one of the four stages. The sample would then be classified into one of the four stages if the occurrence of one particular lesion was in at least 5 field-of-views (50% cutoff limit). Stage I indicates early-stage loose aggregation of cells due to localized immune response whereas stage IV indicates advanced stage granuloma with caseation and liquefactive necrosis. This stage is considered to represent transmissible pathology. Only lung tissue sections were examined for classification.

4. Evaluation of tissue bacterial burden

Approximately 1 g of tissue was obtained from at least three different sections of the lungs and lymph nodes using a sterile biopsy punch. The samples were stored in 3 mL of PBSA + tween-80 (0.05%) + ampicillin (80µg/ml) + cycloheximide (100µg/ml) and frozen. Later, the tissue samples Frozen samples were homogenized, and different dilutions were plated in Middlebrook 7H11 selective media. Tissue homogenizer was washed thoroughly to prevent carry over of bacteria from one sample to the other. Once grown, the colonies were enumerated using automated colony counter (Scan Interscience).

422   **5. Interferon-gamma release assay (IGRA) protocol**

423   The progression of the disease was monitored by a blood-based interferon-gamma release assay.

424   Blood samples from each pig were collected a day before the challenge and every two weeks post

425   challenge. 10 ml blood per animal was collected in lithium heparin tubes, placed in a rocker to

426   allow proper mixing with an anticoagulant. A 24-well tissue culture (Costar, Corning Incorporated,

427   USA) plate was set up with 100 µL of mycobacterial antigen per well. The antigens used were

428   bovine PPD (300 IU/mL), EsxA (5 µg/mL) and PBS control. Mycobacterial culture filtrate

429   contains soluble antigens secreted by the bacteria in the culture. 1.5 ml blood from each animal

430   was transferred to individual wells and mixed by gentle rotation of the plates. The plate was

431   covered with a sterile breathable membrane and incubate at 37^0C for 24 hours. The next day, the

432   breathable membrane cover was removed, and the plate was spun at 800 g for 15 mins at room

433   temperature. Plasma was harvested from the wells without disturbing the cell pellet and transferred

434   into Spin-X centrifuge tube filter (Corning Incorporated, USA) and spun at 15000 g for 20 mins

435   at room temperature. The filtrate obtained was transferred to a fresh screw-capped tube and stored

436   at -80^0C until used.

437

438   **6. Statistical Analysis**

439   The rate of survival between IV challenged *M. bovis* and *M. tb* was compared by Log-rank test.

440   Comparison of weight gained by pigs in different groups in the aerosol challenge experiment was

441   done by 2-independent sample T-test. Likewise, comparison of lung lesion scores was done by

442   Kruskal-Wallis test with subsequent multiple pairwise comparisons using Mann-Whitney U test.

443

444

Acknowledgements

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615   Immune responses in cattle inoculated with Mycobacterium bovis, Mycobacterium

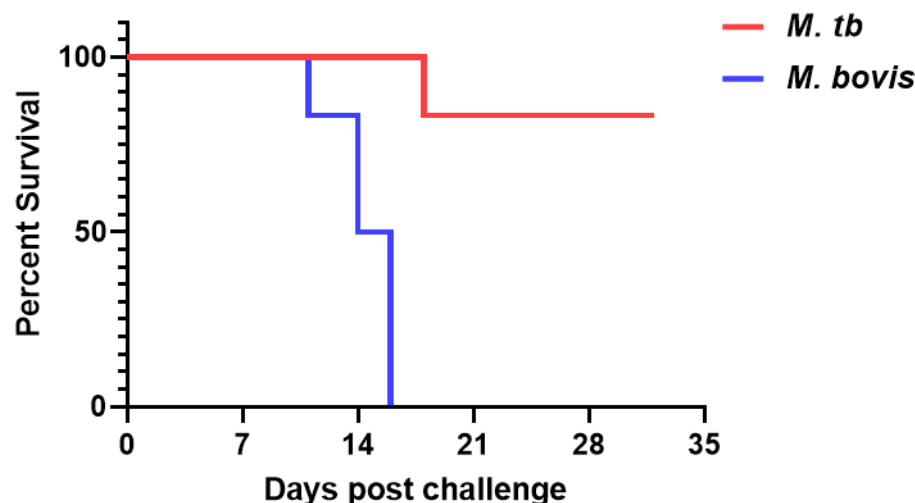616   tuberculosis, or Mycobacterium kansasii. Clin Vaccine Immunol 2010;17:247–52.

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622   **Figures and Table**

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624

625   **Figure 1. Kaplan-Meier curve of survival rate of pigs challenged intravenously with *M. tb***

626   **and *M. bovis*.** Pigs challenged with 3 x 10^8 CFU of *M. bovis* (blue line) died within 16 days post-

627   challenge. In contrast, pigs challenged similarly with *M. tb* showed greater survival and only 1 out

628   of 6 pigs died during the 35-day period (red line). A significant difference was found between *M.*

629   *tb* and *M. bovis* challenged pigs with respect to the chance of survival post-challenge. (Log-rank

630   test: Chi-square = 10.78, p = 0.001)

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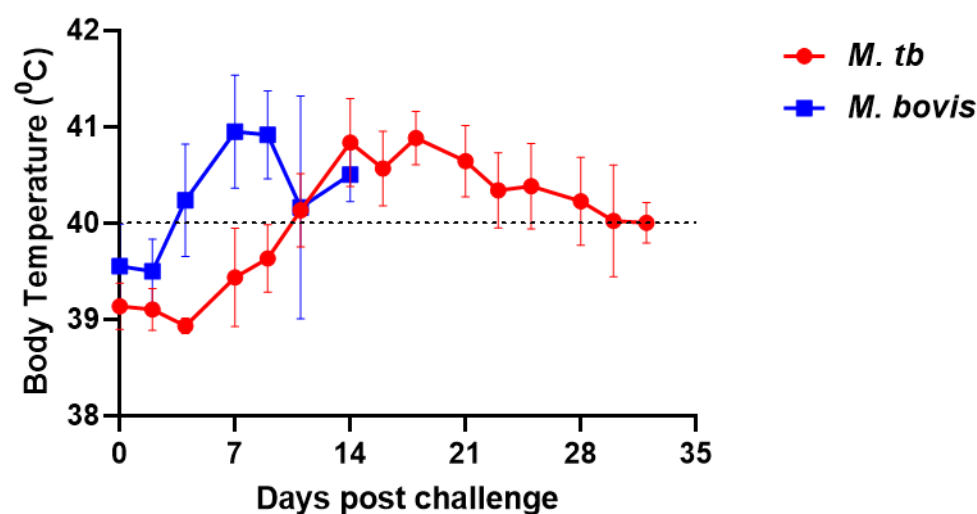
Figure 2. Differences in body temperature (mean ± SD) between *M. tb* and *M. bovis* challenged pigs by intravenous route. Earlier onset of fever was observed in pigs challenged intravenously with *M. bovis* (blue line), which peaked at 7 days post-challenge. Meanwhile, *M. tb* challenged pigs (red line) exhibited delayed onset of fever which peaked around 14 days post-challenge. Temperatures above 40^0C was considered a sign of fever - indicated by dashed line.

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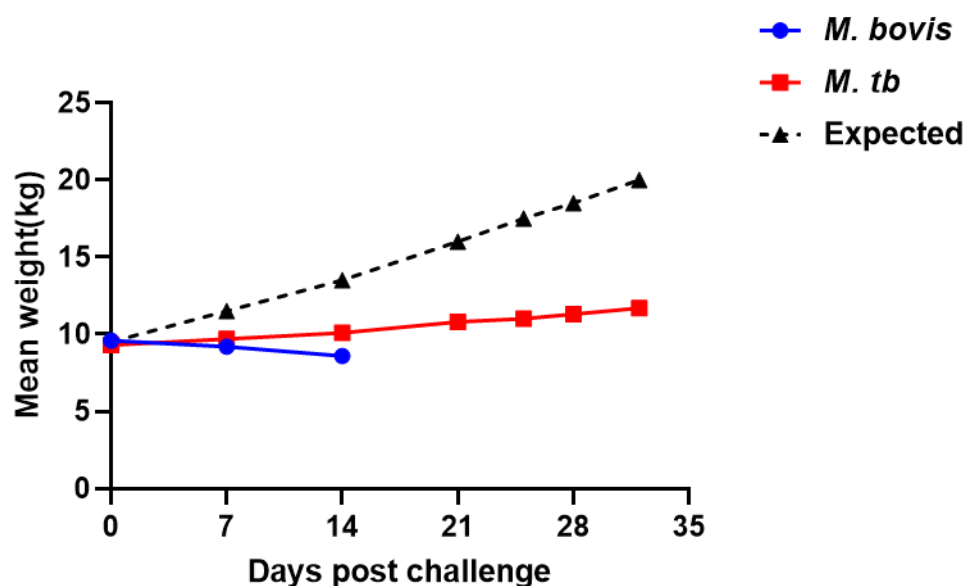
Figure 3. Mean weight (in Kg) of pigs challenge with *M. bovis* and *M. tb* by intravenous route.

M. bovis infected pigs (blue line) started losing weight immediately following the intravenous

challenge but the weight measurement could not be taken for the entire trial as the pigs either died

or reached their humane endpoint much earlier. In contrast, most *M. tb* infected pigs (red line)

gained some weight but it was not close to what is expected for pigs of that breed and age. The

expected growth curve (dashed line) is based on data supplied by the Prairie Swine Centre from

where the pigs were sourced.

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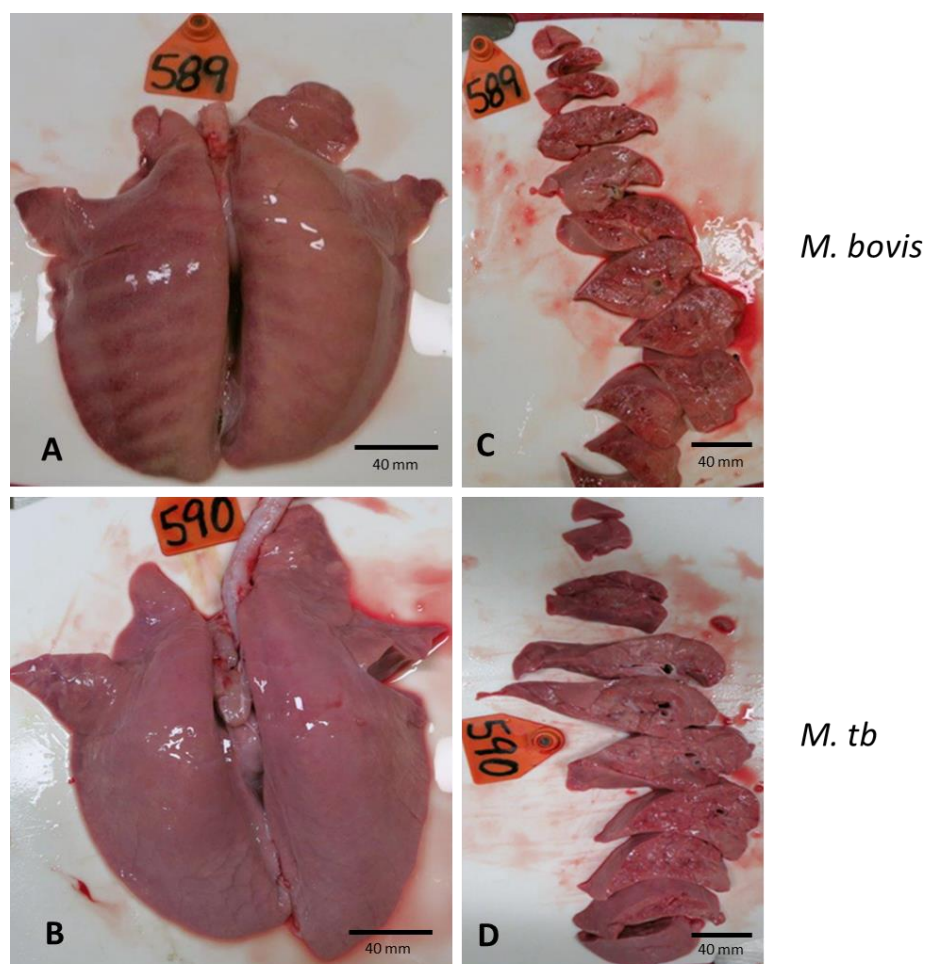
Figure 4. Gross lesions in the lungs of pigs challenged with *M. bovis* and *M. tb* by intravenous route. Lungs from both challenge groups were edematous and non-collapsing but devoid of granulomatous lesions. *M. bovis* infected lung with rib impressions on the dorsal surface (A) and cut section of the same (C). *M. tb* infected lung appears turgid due to edematous inflammation (B) and its cut section (D).

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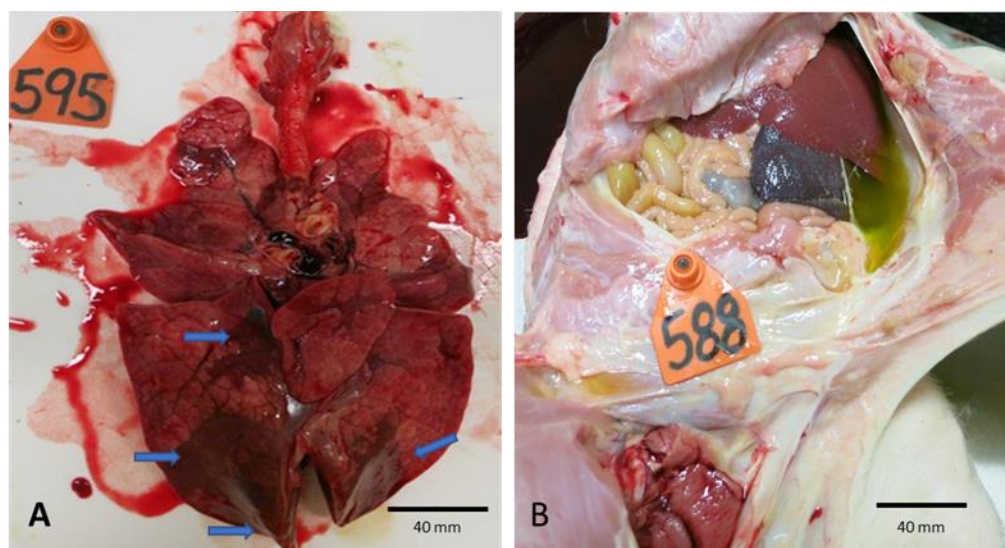
663 **Figure 5: Haemorrhages and effusion associated with *M. bovis* challenged pigs.** Some *M.*

664 *bovis* infected lung had large areas of hemorrhages (indicated by blue arrows) (A). The thoracic

665 and abdominal cavities of some pigs of the same challenge group presented with varying

666 amounts of fluids, possibly due to septicemia (B).
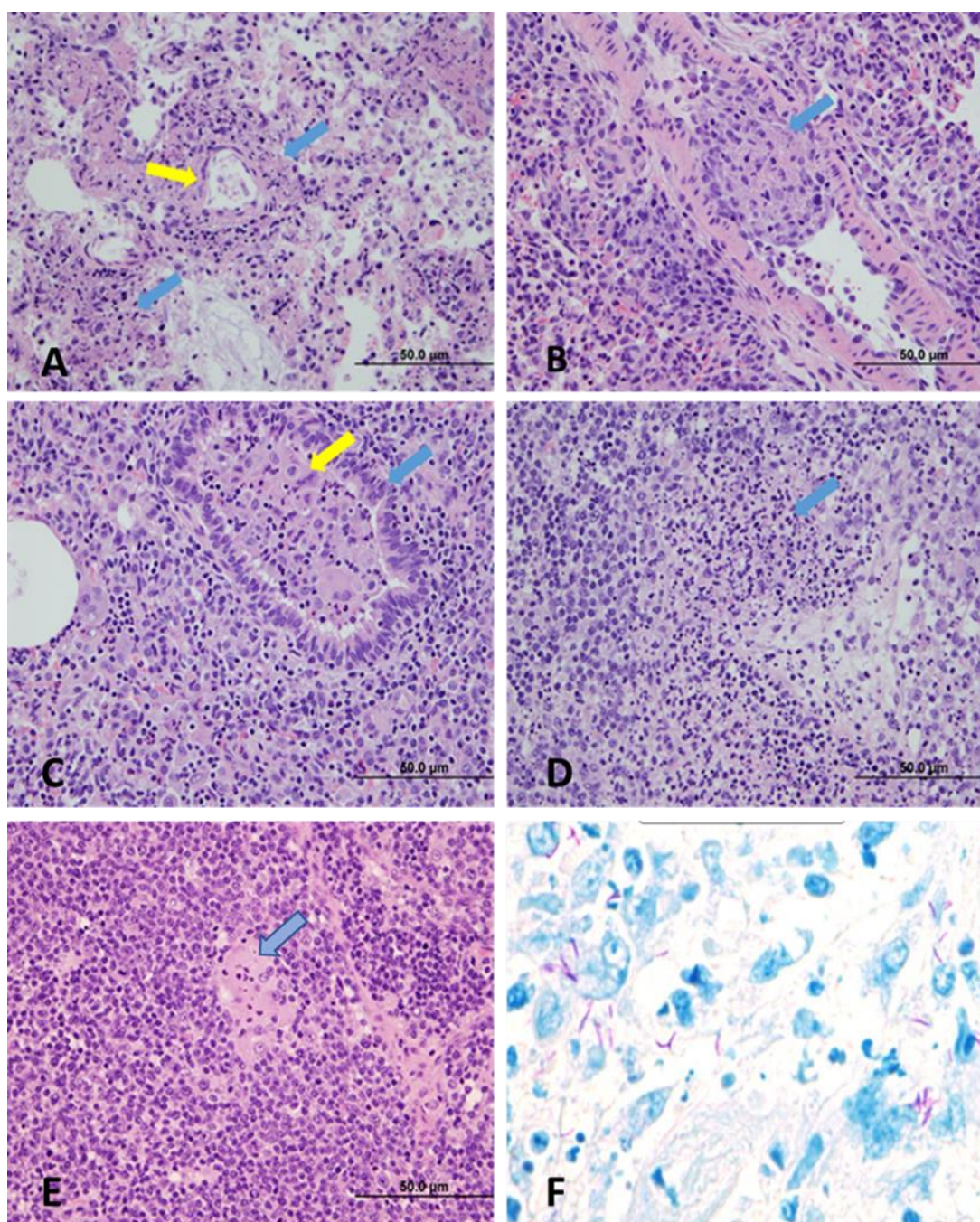
Figure 6. H&E sections of tissues of pigs challenged with *M. bovis* and *M. tb* intravenously.

M. bovis infected lung (40X magnification) with large areas of necrosis (blue arrows) in the alveolar interstitium and the wall of one vessel (yellow arrow) affected by necrosis (A). *M. bovis* infected lung (40X magnification) with an arteriole affected by vasculitis and protrusion of a

672   granuloma (blue arrow) into the lumen (B). *M. tb* infected lung (40X magnification) with a small

673   bronchiole (blue arrow) containing a mixture of neutrophils, epithelioid macrophages,

674   multinucleated giant cells (yellow arrow) (C). *M. bovis* infected lymph node (40X magnification)

675   with large areas of necrosis (blue arrow) and infiltration of neutrophils (D). *M. tb* infected lymph

676   node (40X magnification) with small pyogranuloma (blue arrow) composed of neutrophils and

677   epithelioid macrophages (E). ZN-stained section of *M. bovis* infected cranial lung lobe (F).

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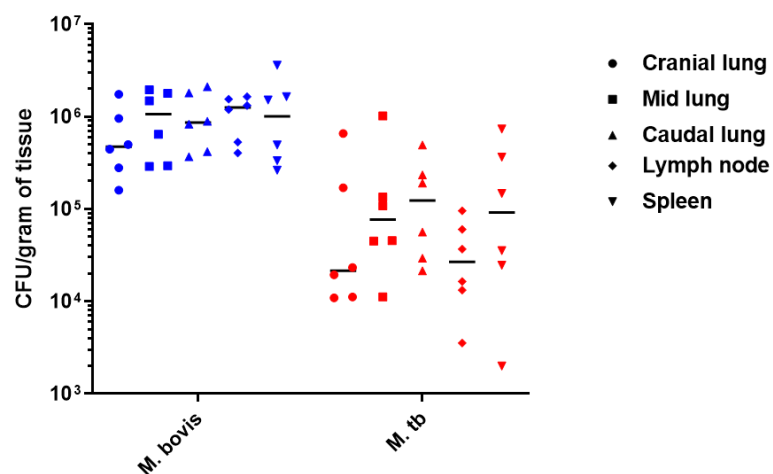
Figure 7. Bacterial burden in *M. bovis* and *M. tb* infected pigs. Each data point represents

average CFU per gram of a particular type of tissue in individual animal. Black horizontal line

represents median.

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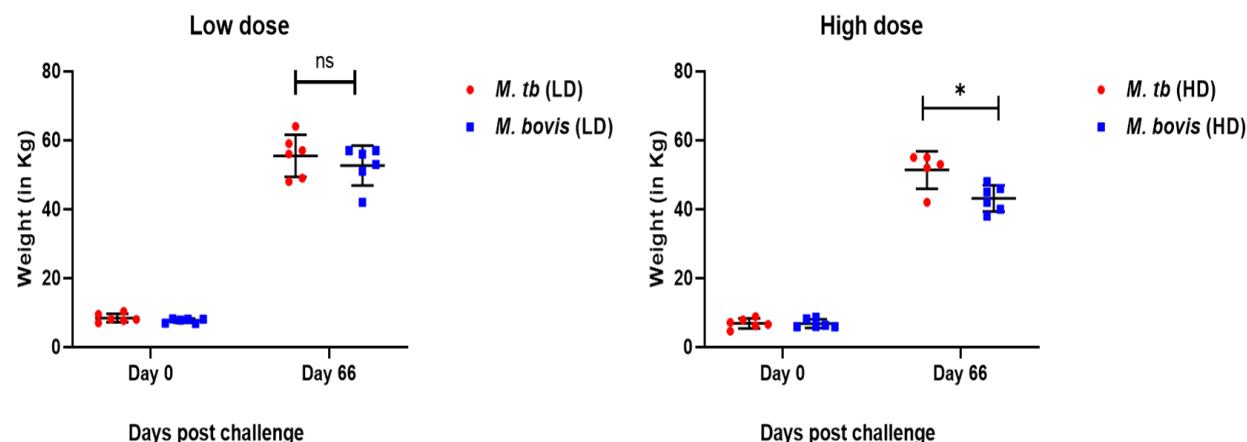
Figure 8. Differential weight gains in pigs aerosol challenged with *M. bovis* and *M. tb*. The weight gained by high dose *M. tb* infected pigs is significantly higher by approximately 8-kg than by high dose *M. bovis* infected pigs (Parametric 2-independent sample T-test: T = 2.961, df = 9, p = 0.016). No significant difference in weight gain was seen in pigs infected with low doses of *M. bovis* and *M. tb* (Each data point represents the actual weight of an individual animal).

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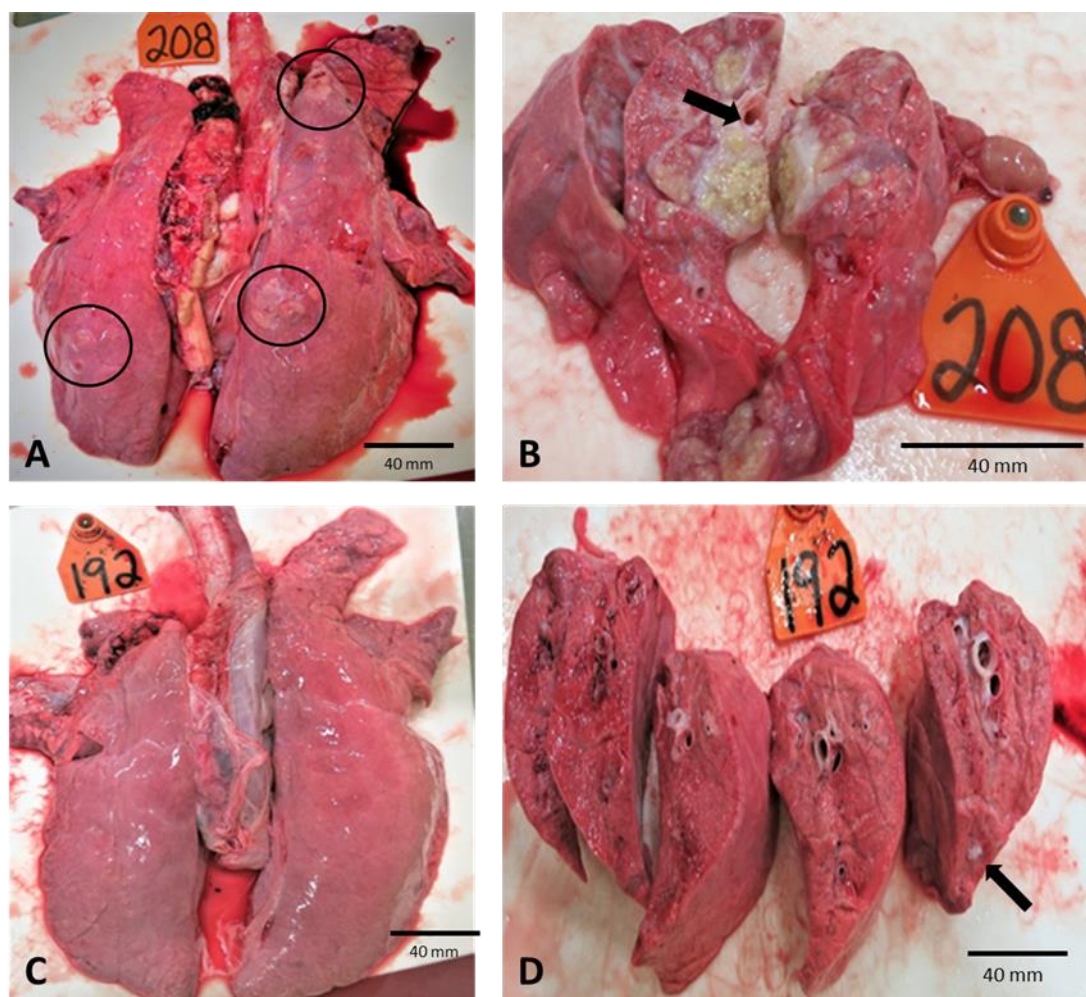
Figure 9. Gross pathology of lungs of *M. bovis* and *M. tb* infected pigs 9 weeks post-challenge.
Visible granulomas (in circles) on the surface of the lung in *M. bovis*-HD infected pigs some of which were more than 50 mm in diameter (A). Cut section of *M. bovis* infected lungs with a bronchus surrounded by caseating granulomas (black arrow) (B). *M. tb* infected lung with very few granulomas visible on the surface (C). Cut section of *M. tb* infected lung with few compact, non-necrotizing granulomas in lung parenchyma (black arrows) (D).

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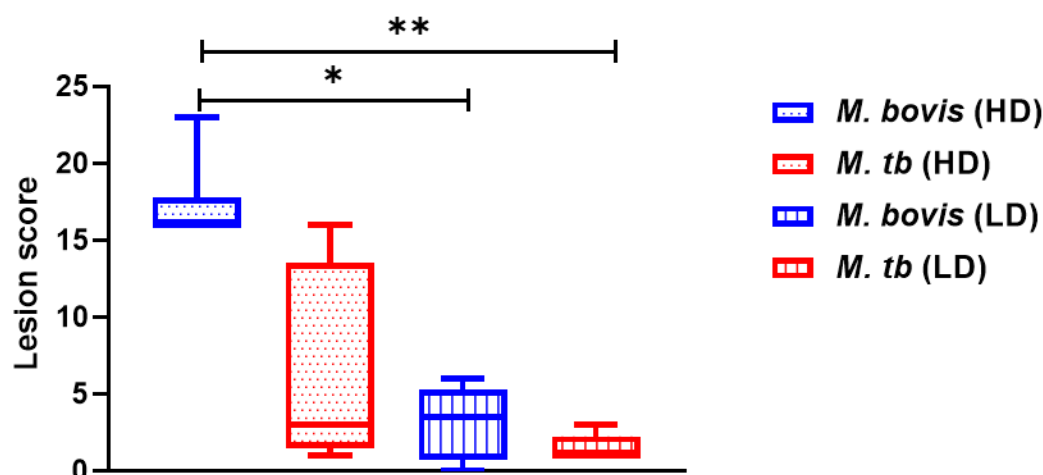
719 **Figure 10. Lung lesion scores of *M. tb* and *M. bovis* aerosol challenged pigs at necropsy 9**

720 **weeks post-challenge.** Asterisk (*) represents statistically significant difference at 0.05 level of

721 significance and double asterisk (**) represents significance at 0.005 level of significance. Lung

722 lesion scores between different treatment groups were compared by non-parametric Kruskal-

723 Wallis Test ($\alpha = 0.05$). Significant differences in lung lesion scores were found based on treatment

724 groups (Kruskal Wallis H = 14.016, df = 3, p = 0.003). Subsequently, multiple pairwise comparison

725 using Mann-Whitney U test was done to determine statistical significance between the treatment

726 groups. Lung lesion scores in *M. bovis*-HD was significantly higher than *M. bovis*-LD (Mann

727 Whitney U = 10.33, p = 0.04) as well as *M. tb*-LD (Mann Whitney U = 13.83, p = 0.002). Any

728 other pairwise combinations were non-significant. P value reported have been adjusted with

729 Bonferroni correction for multiple tests. *M. bovis*-HD infected pigs also had qualitatively more

730 severe lung pathology compared to *M. tb*-HD infected pigs. There was no significant difference

731 between the pigs infected with low doses of *M. bovis* and *M. tb*.

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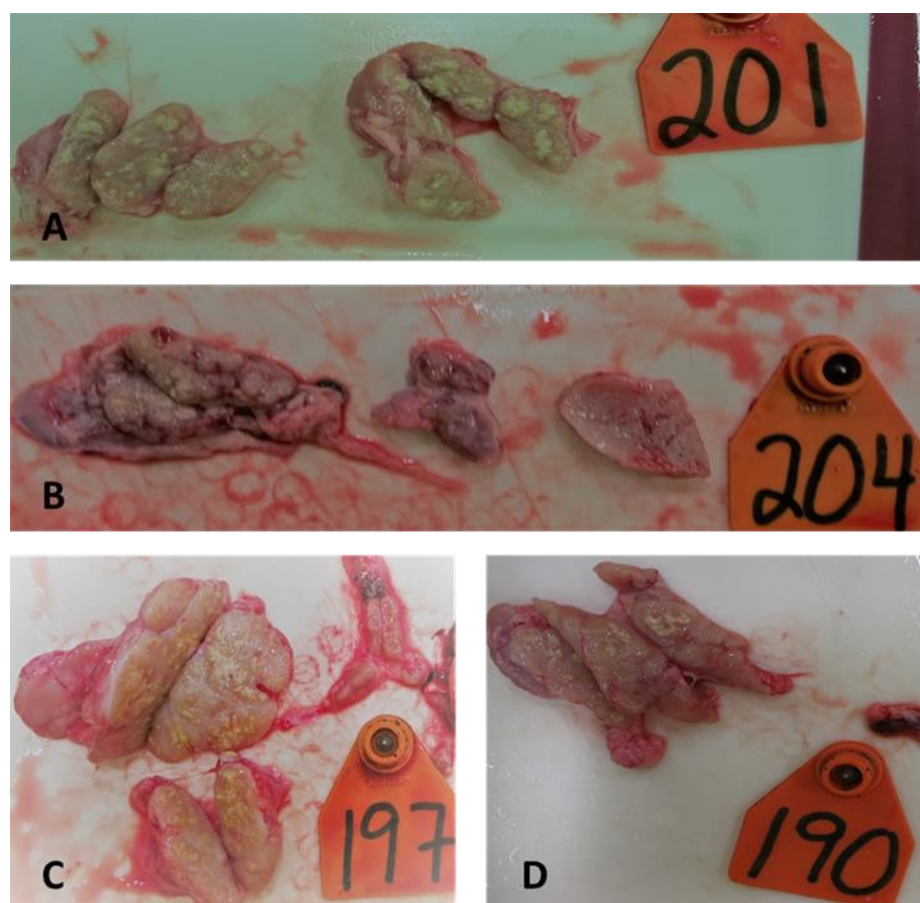
Figure 11. Gross pathological lesion in lymph nodes of *M. bovis* and *M. tb* aerosol infected pigs. Cut sections of tracheobronchial and mediastinal lymph nodes from *M. bovis*-HD infected (A), *M. tb*-HD infected pigs (B), *M. bovis*-LD infected (C) and *M. tb*-LD infected pigs (D). Note whitish yellow caseous mass in the cut sections of lymph nodes. Lesions are more abundant in *M. bovis*-HD infected pigs which corelates with higher bacterial burdens in lymph nodes of these animals.

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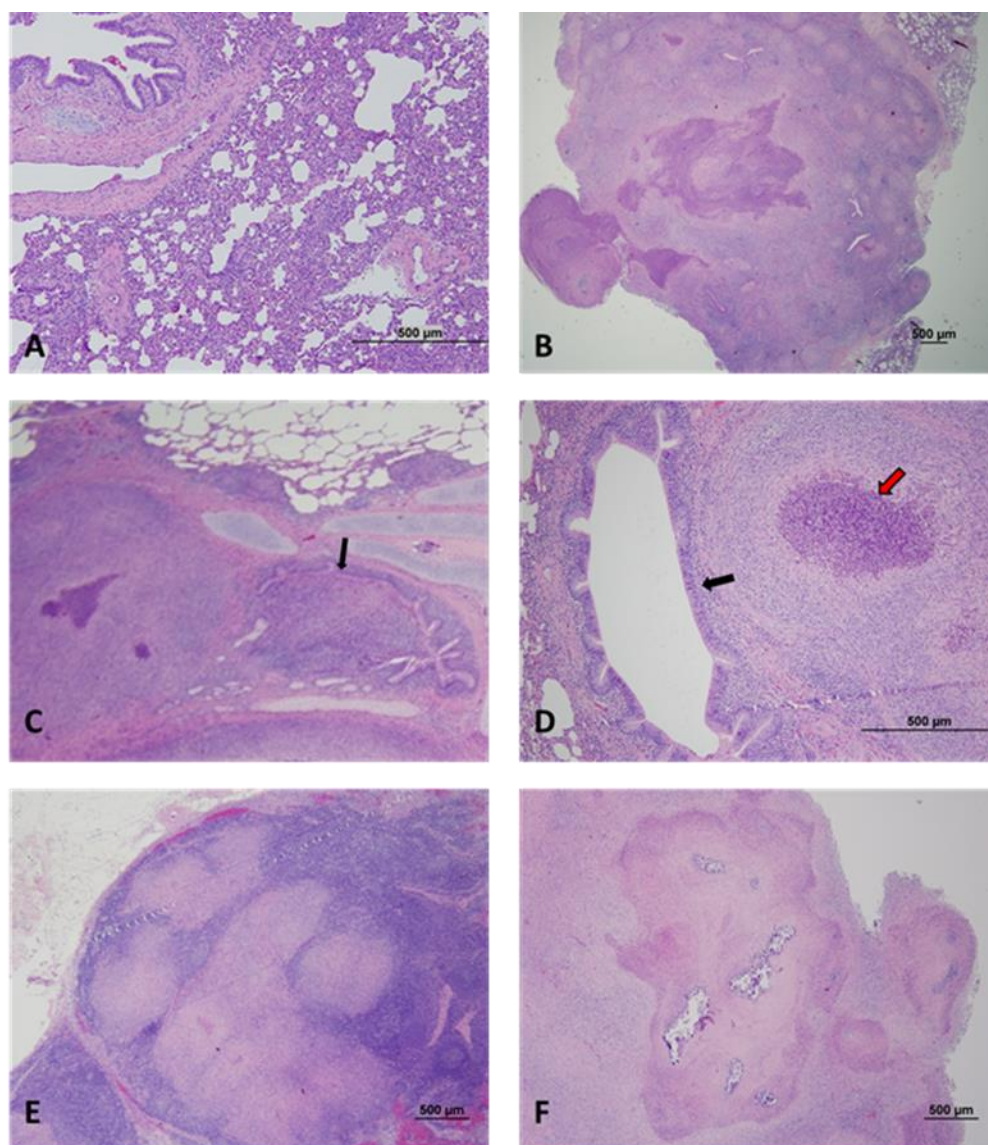
Figure 12. Histopathology of pigs infected with *M. bovis* and *M. tb*. Thickening of lung parenchyma can be seen in *M. tb*-LD infected lung (A). Multiple satellite granulomas with a follicular appearance surrounding central necrotic core of *M. bovis*-HD infected pig lung (B). *M. bovis*-HD infected lung with advanced stage granuloma with collapsed bronchiolar lumen and infiltration (arrow) (C). Another *M. bovis*-HD infected lung with stricture of bronchiole by a large granuloma (black arrow) and infiltration of neutrophils (red arrow) suggesting beginning of necrotic process (D). *M. tb*-HD infected mediastinal lymph node with a multifocal lesion and thick

752   capsule surrounding those lesions (E). *M. bovis*-HD mediastinal lymph node with advanced stage

753   lesion, amorphous mantle and liquefying core (F).

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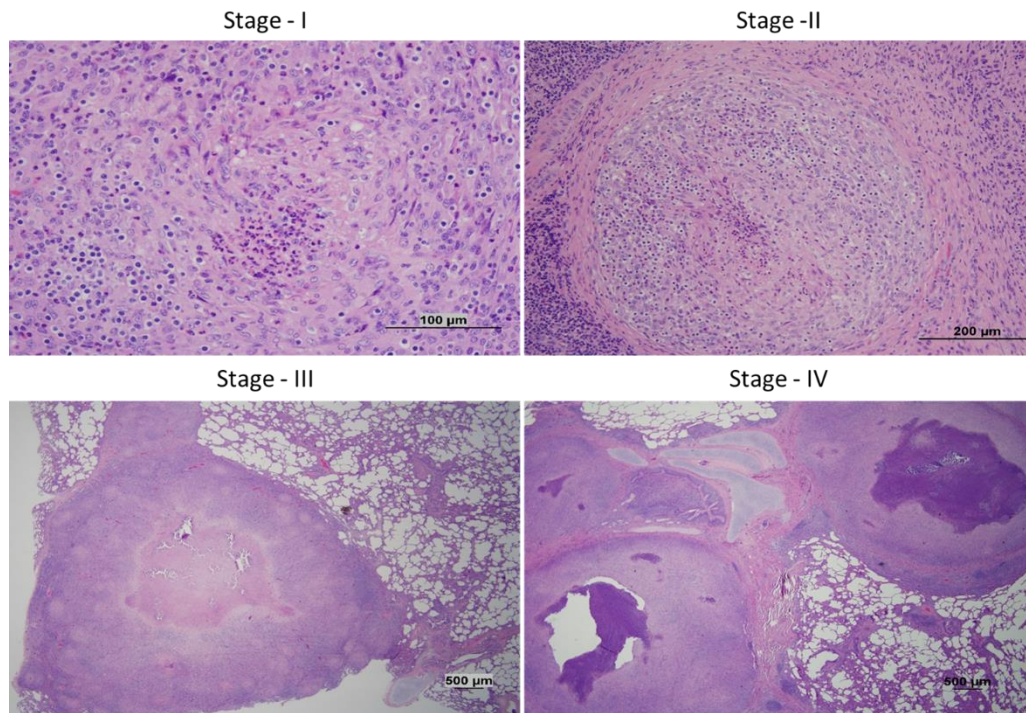
Figure 13. Different stages of granuloma in pigs infected with *M. bovis* & *M. tb*. At least one

lung H&E-stained sample from all the animals in each group were studied and the granulomas

were categorized into one of the four stages. Stage-1 lesions are the initial aggregation of

inflammatory cells such as epithelioid macrophages, lymphocytes, neutrophils. There is a lack of

fibrous capsule, and the structure is unorganized. Stage-2 granulomas appear as organized

structure with a central core of neutrophils, foamy macrophages and abundant epithelioid

macrophages, and a peripheral rim of T and B lymphocytes. A fibrous capsule demarcates the

granuloma from surrounding parenchyma. The difference between stage-2 and stage-3

granulomas is the formation of necrotic centers within the granulomas and thickening of the

capsule. Stage-4 granuloma have extensive caseous necrosis and liquefaction leading to an

768   amorphous zone in the center of the granuloma. Multiple granuloma may coalesce to form a

769   large, multifocal lesion as shown in the bottom-right panel.

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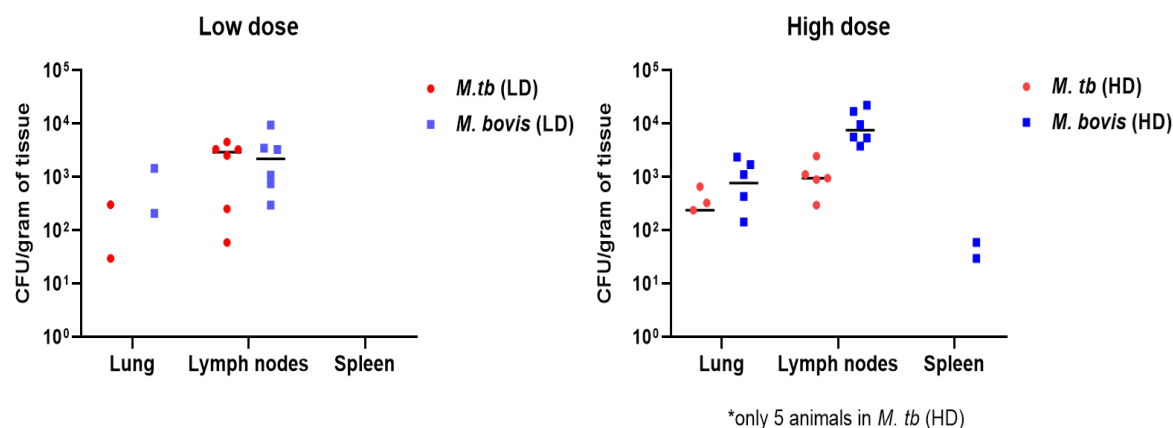
773   **Figure 14. Bacterial burden (CFU per gram of tissue) in different organs of pigs infected**

774   **with high and low doses of *M. bovis* & *M. tb***. Each point in the graph represents CFU/g of one

775   animal and the black line represents the group median. Value for some individuals is missing as it

776   was below the detection limit. Recovery of mycobacteria from spleen samples of two *M. bovis* HD

777   challenged pigs indicates post-primary dissemination.

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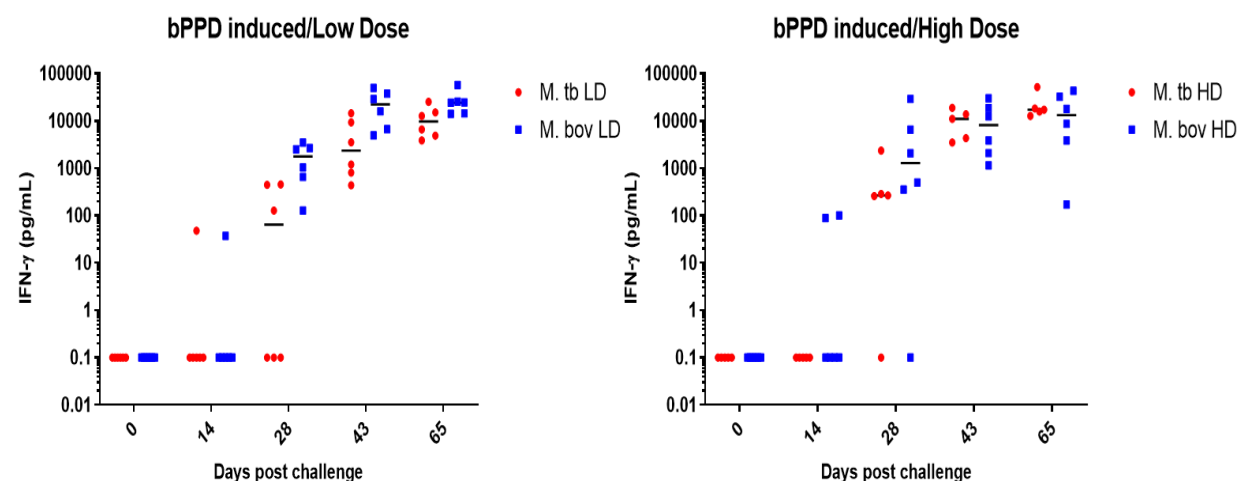
Figure 15. IFN-γ response due to bPPD stimulation of the whole blood. Each data point indicates concentration of IFN-γ in individual animal at that point after removing background concentration (PBS stimulated sample). Although few pigs had measurable IFN-γ response by day 14 post challenge, most pigs displayed IFN- γ response at day 28 post challenge. Despite challenge with different doses, the peripheral IFN-γ response is similar for *M. bovis* and *M. tb* challenge.

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800 **Table 2: Lung histopathological lesion scoring of pigs infected with *M. bovis* & *M. tb*.** At
801 least one H&E slide from each animal from all four groups was analyzed. NA -not assessed
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Groups	Animal ID	Cranial Lobe	Middle Lobe	Caudal Lobe
M. bovis HD	191	2	4	NA
	196	4	NA	4
	200	1	NA	3
	201	3	NA	NA
	208	4	4	NA
	212	4	4	NA
M. bovis LD	189	1	NA	1
	193	3	NA	1
	197	4	NA	NA
	202	2	1	NA
	207	1	NA	1
	209	3	NA	1
M. tb HD	192	4	NA	NA
	194	2	4	NA
	199	1	NA	NA
	204	3	NA	NA
	206	2	NA	NA
M. tb	190	3	NA	NA
	195	1	2	NA
	198	1	NA	NA

LD	203	1	NA	1
	205	1	NA	NA
	211	2	2	NA

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