1 Evaluation of a faecal shedding test to detect badger social groups infected with

2 *Mycobacterium bovis*

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Abstract

Bovine Tuberculosis (bTB) is an economically important disease affecting the cattle industry in England and Wales. bTB, caused by *Mycobacterium bovis*, also causes disease in the Eurasian badger (*Meles meles*), a secondary maintenance host. Disease transmission between these two species is bidirectional. Infected badgers shed *M. bovis* in their faeces. The UK Animal and Plant Health Agency (APHA) organised a comparative trial to determine the performance of tests in detecting *M. bovis* in badger faeces for the Department for Environment, Food, and Rural Affairs (DEFRA). Here we present the performance of the existing Warwick Fast24-qPCR test, and its modified version based on a high-throughput DNA extraction method (Fast96-qPCR). We found Fast24-qPCR to have a sensitivity of 96.7% (95%CI 94.5-99%, n=244) and a specificity of 99% (95%CI 97.8-100%, n=292). Fast96-qPCR requires further optimisation. Determining the disease status of badger social groups requires multiple tests per group. Therefore to increase specificity further, we independently repeated the Fast24-qPCR test on positive samples, increasing stringency by requiring a 2nd positive result. Fast24-qPCR with repeat testing had a sensitivity of 87.3% (95%CI 83.1-91.5%, n=244), and a specificity of 100% (95%CI 100-100, n=201) on an individual sample level. At the social group level, this repeat testing gives Fast24-qPCR high herd specificity, while testing multiple samples per group provides high herd sensitivity. With Fast24-qPCR we provide a social group level test with sufficient specificity and sensitivity to monitor shedding in badgers via latrine sampling, delivering a potentially valuable tool to measure the impacts of bTB control measures.

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42 **Introduction**

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44 Bovine Tuberculosis (bTB) caused by *Mycobacterium bovis* is an economically

45 important disease that is estimated to cost £100 million to the taxpayer per year (1) in

46 the United Kingdom. Prevalence of the disease in cattle herds has increased from

47 0.49% in 1979 (1) to 5.3% in England and 5.6% in Wales in 2019 (2), though this is not

48 evenly distributed, and is concentrated in the high risk area of south-west England, and

49 the surrounding edge area, in addition to south-west, and east Wales. In Britain, the

50 Eurasian badger (*Meles meles*) is considered a secondary maintenance host of bTB (3),

51 with an estimated bTB prevalence of 24.2% (4) within the high risk area of England.

52 Although cattle to cattle aerosol transmission is considered to be the predominant route

53 of infection (5), badgers are known to transmit disease to cattle (6, 7), and are estimated

54 to contribute up to 52% of individual cases within endemic areas (8), inclusive of

55 subsequent cattle-cattle transmission. Cattle are also implicated in the transmission of

56 bTB to badgers, as delays in removing infected cattle have been shown to increase bTB

57 prevalence in badgers (7).

58 The route of transmission between badgers and cattle has not been demonstrated.

59 However, several lines of evidence suggest an important role for transmission occurring

60 through contamination of the environment. Direct contact between badgers and cattle

61 are rare events (9, 10), and cattle-cattle and badger-badger transmission rates are also

62 low (11-13) despite high levels of intra-species contact in these social animals. This

63 suggests that the levels of direct contact are not high enough to explain the levels of

64 inter-specific transmission. Athough badgers avoid direct contact with cattle (14), they

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65 actively favour cattle pasture for foraging, suggesting common occurrence of shared

66 environment. *M. bovis* has been shown to persist in the environment in a number of

67 studies (15-17) and models of bTB transmission in cattle have also suggested a

68 substantial role for the environment (18) as a reservoir and route of transmission.

69 Various studies have also implicated the environment as a vector for bi-directional *M.*

70 *bovis* transmission between cattle and badgers. Badgers sampled from a natural

71 population, were shedding *M. bovis* cells in sputum, urine, and faeces (19, 20),

72 indicating that contamination of pasture with faeces and urine creates a potential source

73 of infection (21). Furthermore, cattle do not avoid areas contaminated with badger urine,

74 and will graze at badger latrines given sufficient competition for fresh pasture (22).

75 Similarly, the faeces of infected cattle contained viable *M. bovis* (23, 24), though cattle

76 were not shown to be infected from pastures contaminated with these faeces, and, as

77 these experiments were performed in the 1930s, it cannot be assumed that disease

78 progression occurs in the same fashion under modern bTB testing regimes. However, at

79 the point of detection by the single intradermal comparative tuberculin test (SICCT), the

80 test used in the UK to detect bTB in cattle (2), the disease has progressed to the extent

81 that 55.5% of positive animals have visible lesions at slaughter (25), compared to a

82 background rate of 0.63/1000 (0.063%) in negative animals (26). The spreading of

83 slurry is also considered a risk factor for bTB breakdowns (27). Finally, badgers are

84 known to forage under cattle dung (28), and earthworms have been demonstrated to

85 spread *M. bovis* BCG from spiked cattle faeces to surrounding soil (29).

86 A number of assays have been developed to diagnose bTB infection in badgers. These

87 include immunoassays such as gamma interferon (IFN-γ) (4) and BrockTB Stat-Pak

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88   assay (4), as well as culture from clinical samples (19, 30, 31). Sensitivity (Se)

89   estimates of IFN-γ range from 52-85% in adult badgers (4, 31-33) – estimates for cubs

90   are lower – compared with a range of 49-78% (dependent on severity of disease) for

91   Stat-Pak (34). Specificity (Sp) estimates for IFN-γ range from 88-94% (4, 31, 32),

92   compared with 93-97% for Stat-Pak (31, 34). Culture is very insensitive (8%), though it

93   is considered to have near perfect specificity (99.8%) (31). These tests are not

94   considered sensitive or specific enough to use alone and also neccessitate the live

95   trapping of animals, which requires intensive effort to achieve high coverage and is

96   expensive (35, 36). It has been suggested that diagnosis should be performed at the

97   social group level, using IFN-γ and Stat-Pak in parallel (33). However, in order to

98   maintain sufficient specificity at the group level, this approach requires a threshold of 2

99   badgers with positive tests to accurately identify a social group as infected. Therefore

100 this approach requires substantial trapping coverage (50%), and is unlikely to identify

101 social groups with only one positive animal. The prevalence of *M. bovis* shedding in

102 badger faeces correlates well with prevalence of infection as determined by IFN-γ and

103 Stat-Pak on contemporaneous trapped badgers at a social group level (37), though

104 social groups with similar prevalence of infection showed heterogeneity in prevalence of

105 shedding (38).

106 A culture-independent quantitative PCR (qPCR) test for detecting *M. bovis* DNA in

107 environmental samples, including badger faeces, was developed at Warwick University

108 (39, 40). Pathogen detection in faeces was thus pioneered by the use of qPCR on DNA

109 extracted from faeces and soil. It was possible to culture *M. bovis* from a proportion of

110 qPCR positive badger faeces (20), indicating pathogen viability; however, not all qPCR

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111   positive faeces provided culture positive data due to the low sensitivity of culture from

112   faecal samples. Quantification of the level of *M. bovis* genome equivalents in badger

113   faeces is likely to be a good proxy for shedding status through sputum, and thus

114   transmission of infection through the respiratory route and biting, because lesions in the

115   gut of badgers are extremely rare (41), the presence of *M. bovis* DNA in faeces likely

116   occurs via the passage of infected lung discharge through the gastro-intestinal tract.

117   Indeed, the detection of *M. bovis* DNA in both the trachea and faeces of infected

118   badgers correlates with severity of disease status (42). The test also has the advantage

119   of being non-invasive, with the potential to provide greater coverage of the population

120   than trapping based methods.

121   In this study, we assess the diagnostic sensitivity and specificity of Fast24-qPCR, our

122   existing DNA extraction and qPCR method, and Fast96-qPCR, a novel high-throughput

123   DNA extraction methodology. Fast96-qPCR uses the same qPCR, but a high-

124   throughput version of the DNA extraction based on the same chemistry as Fast24-

125   qPCR. We demonstrate that Fast24-qPCR provides a non-invasive method to detect

126   bTB infected badger social groups through latrine sampling with a high degree of social-

127   group level specificity and sensitivity. This will provide a valuable tool to enable

128   monitoring of badger social group bTB status through *M. bovis* shedding in badger

129   faeces and by extension the effects of bTB control measures.

130   **Materials and Methods**

131   *Production of the panel*

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132 In order to determine the diagnostic sensitivity and specificity of the tests involved in this

133 comparative study a panel consisting of spiked positive samples (n=245), known

134 negative samples (n=205), and putative positive samples (n=119) from 12 badger social

135 groups containing badgers known to be serological test positive was prepared by APHA.

136 APHA required minimum acceptable thresholds of social group level sensitivity and

137 specificity (50% and 80% respectively), on behalf of the Department for Environment,

138 Farming and Rural Affairs (DEFRA). These thresholds are based on the assumption of

139 a 10% within-herd prevalence (in badgers), and 10 samples analysed per social group,

140 and as such require a sample level (diagnostic) sensitivity of 50% and specificity of

141 98%. The number of known positive and negative faeces in the panels were thus

142 calculated to be able to establish these thresholds, with a 5% margin of error within 95%

143 confidence intervals in the case of sensitivity, and a 2% margin of error within 95%

144 confidence intervals in the case of specificity (43). This required a minimum of 246

145 known positive samples, and 188 known negative samples. The status of all samples

146 within this panel was blinded from all study participants until the completion of testing.

147 APHA is compliant with the Animals (Scientific Procedures) Act 1986, and, in addition,

148 all experiments involving animals are both reviewed and approved as well as subject to

149 retrospective analysis by an Ethics Committee composed of vets, animal care staff, a

150 biostatistician, scientists, and lay members of the community. The production of the

151 panel is detailed as follows.

152 Faeces (n=50) were collected from badgers of known negative status at APHA

153 Weybridge (n=25) and APHA York (n=12), and also from wild latrines at APHA

154 Woodchester (n=10), and from latrines in regions of the country where bovine TB is not

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155 endemic in cattle (n=3). Faecal samples were collected from a variety of sources in

156 order to account for factors such as faecal consistency and presence of inhibitors as

157 any test used on wild samples must be robust to these factors. APHA personnel

158 conducted the sampling and prepared spiked samples as follows: positive faecal

159 samples (n=245) were prepared by spiking 150 g pooled from the above sources with

160 20 ml of buffer containing known quantities (10^5-10^1 CFU/g) of *M. bovis* 2122/97. To

161 ensure spiked samples were homogenous the faeces were mixed with a spatula for a

162 period of 5 mins. 1 g aliquots were then frozen at -20°C in 2 ml screw cap

163 microcentrifuge tubes. Five 1g aliquots were taken from each spiked 150g faecal

164 sample, as such there were 5 technical replicates of 49 biological replicates. Full details

165 of the protocol used can be found in the Defra report (44). The dilution series chosen

166 was based on previous research within our group at Warwick University (37, 38), and

167 concentrations were determined based on CFU count.

168 Negative faeces (n=205) were aliquoted in a separate laboratory, to prevent

169 contamination, faeces were mixed with a spatula for 5 mins and aliquoting was

170 performed as described above. Five of these negatives were prepared by spiking faeces

171 as above with dilution buffer only.

172 Putative positive samples were taken from latrines connected to 12 historically positive

173 social groups at APHA Woodchester. These social groups had at least one culture-

174 positive result, and/or 4 positive IFN-γ or BrockTB StatPak test results on trapped

175 animals within the 2 year period preceding the sampling. Though this does not

176 guarantee that the social groups still contain infected animals at the time of sampling, it

177 was considered a reasonable assumption. It was aimed to sample 10 scats per social

178   group, however only 9 were available from one social group. As such the panel

179   contained 119 putative positive samples. Approximately 30 g of each scat was mixed

180   with a spatula for 5 mins. One 1 g aliquot was taken from each unique scat to make up

181   the panel, as above.

182   Samples were blinded by APHA and two replicates of the panel (one for each extraction

183   method) were sent to Warwick University using the appropriate secure transport

184   procedures (UN2814).

185   In addition to the blinded samples in the panel, known negative faeces (n=88) were

186   added at Warwick University. These faeces were also collected from badgers of known

187   negative status at APHA Weybridge. This was to provide our own internal indicator of

188   test performance. The composition of the panel is presented in Table 1.

189   ***DNA extraction from badger faeces***

190   DNA was extracted from the badger faeces using two methods, the existing Fast24-

191   qPCR extraction and the new Fast96-qPCR extraction. For Fast24-qPCR, total

192   community DNA was extracted from 0.1 g (± 0.005 g) of faeces using the FastDNA Spin

193   Kit for Soil (MP Biomedicals SKU 116560200-CF) per the manufacturer's instructions. A

194   modified ribolysis step involving two sequential homogenisation steps of 40 s at 6000

195   rpm separated by a 30 s pause was performed using a Precellys 24 homogeniser

196   (Bertin Instruments P000669-PR240-A) as previously reported (37). DNA was extracted

197   from the Fast24-qPCR panel twice in parallel by two separate operators.

198   For the Fast96-qPCR extraction total community DNA was extracted from 0.1 g of

199   faeces using the FastDNA 96 Soil Microbe DNA Kit (MP Biomedicals SKU 119696200)

200   with some alterations from the manufacturer's instructions, detailed as follows. Ribolysis

201   took place in Lysing Matrix E tubes (MP Biomedicals SKU 116914050-CF) containing

202   400 µl lysis buffer and 100 µl sterile molecular grade dH_2O. Samples were ribolysed

203   using a FastPrep-96 Instrument (MP Biomedicals SKU 116010500) at 1600 rpm for 60

204   s, and centrifuged at 16,110 x g for 10 mins. The supernatant was transferred to a 96-

205   well deep well plate, and DNA extraction then continued as per manufacturer's

206   instructions. All subsequent centrifugation steps were performed in an Eppendorf 5810R

207   using the A-2-DWP-AT rotor at 3,486 x g. DNA was extracted from the Fast96 panel

208   once.

209   *qPCR testing*

210   The RD4 qPCR assay was used as described previously (37). Briefly, samples were

211   screened using duplicate qPCR assays and those with a positive result in either

212   replicate were taken on for full quantification in triplicate. A serial dilution of genomic

213   DNA from *M. bovis* BCG Danish 1331 was used as standard. If one or more replicates

214   showed amplification in the quantification assay then samples were deemed positive,

215   otherwise samples were deemed negative. Assays were performed for inhibition of the

216   qPCR using a previously described inhibition assay (40) using the previously described

217   protocol (37), in order to detect possible false negatives. Briefly, an inhibition control

218   was previously designed with an exotic probe target (GFP) flanked by DNA

219   complementary to the RD4 primers. A known concentration of this target was added to

220   all samples; if inhibitory compounds were present in the sample, qPCR of the inhibition

221   control target was impacted in comparison with the negative control (equivalent volume

222   dH_2O). This was quantified by comparison of the threshold CT of each sample to a

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223 negative control with the difference in CT values referred to as ΔCT. Samples were

224 screened in singlet, and if ΔCT differed by >2.5 from the negative control the sample

225 was rescreened in duplicate. If the average ΔCT of the duplicates differed by >2.5 from

226 the negative control the sample was considered inhibited. Inhibited, non-positive

227 samples were excluded from analysis. All qPCR reactions were performed in an ABI

228 7500 Fast qPCR system (ThermoFischer Scientific 4351106), using 10 µl of DNA as

229 template. qPCR protocols were identical for both extraction methods.

230 ***Statistical analysis***

231 The study was designed to assess the performance of multiple tests in detecting *M.*

232 *bovis* in badger faeces. Performance was measured in terms of diagnostic sensitivity

233 and specificity at the individual sample level (DSe and DSp). Based on discrepancies

234 between observed genome equivalents within the panel and in naturally infected

235 samples (Figure 1) we also consider sensitivity within the samples spiked with the four

236 lowest concentrations of *M. bovis* separately (DSeLC) (Table 2). The quantity of *M.*

237 *bovis* genome equivalents from these four lowest spiked samples were most similar to

238 the quantities found in positive wild badger faeces in previous work (37, 38). For Fast24-

239 qPCR and Fast96-qPCR sensitivity is dependent on DNA concentration, and estimating

240 sensitivity from samples that contain *M. bovis* cell concentrations higher than those

241 found in natural positive faeces will produce a biased over-estimate of true sensitivity.

242 Despite the intention for spiked cell concentrations to cover a similar range to that found

243 in naturally infected samples, it was clear that many of the spiked samples had

244 substantially higher cell concentrations; hence the need for this sub-analysis. We

245 ascribe this discrepancy to the difference between cell number as measured by genome

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246   equivalents, and that determined by CFU. Statistical comparisons of DSe with DSeLC

247   were one-tailed as, *a priori*, we anticipated sensitivity to be lower at lower spiked *M.*

248   *bovis* concentrations. At the level of the social group, test performance was estimated

249   by calculating herd sensitivity and specificity (HSe and HSp respectively). These

250   epidemiological terms refer to the ability of the tests to accurately identify positive social

251   groups ('herds') in the case of herd sensitivity, and to accurately identify negative social

252   groups in the case of herd specificity. As it is difficult to link faeces to the animal which

253   excreted them, positive test results can only infer positivity at the social group level. The

254   performance of a test at a social group level is dependent on its sensitivity and

255   specificity at an individual level, the number of samples tested (*n*), the true within-social

256   group prevalence (TP), and the threshold value of individual positives used to classify

257   the social group as positive. Herd specificity is dependent on diagnostic specificity and

258   the number of samples tested, and herd sensitivity (based on the binomial distribution)

259   can be calculated from apparent within-social group prevalence (AP) which is calculated

260   as follows (45):

$$AP = DSe \times TP + (1 - DSp) \times (1 - TP) \quad (1)$$

$$HSe = 1 - (1 - AP^n) \quad (2)$$

$$HSp = 1 - DSp^n \quad (3)$$

261   The variance of apparent prevalence can be estimated as follows (46)

$$Var(AP) \approx TP^2 \times \frac{DSe \times (1 - DSe)}{N} + (1 - TP)^2 \times \frac{DSp \times (1 - DSp)}{M} \quad (4)$$

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262 where DSe is estimated from N known positives, and DSp is estimated from M known

263 negatives. From this 95% confidence intervals (CI) for apparent within-group prevalence

264 (AP) can be calculated as follows

$$95\% \; CI \; \approx AP \pm 1.96\sqrt{Var(AP)} \quad (5)$$

265 The post-test probability at the social group level, or the subjective probability of the

266 presence of infection within a social group, can calculated as Herd Positive Predictive

267 Value (HPPV) and Herd Negative Predictive Value (HNPV) from these estimates of

268 social group level sensitivity and specificity, as follows (45)

$$HPPV = \frac{HSe \times HP}{HSe \times HP + (1 - HSp) \times (1 - HP)} \quad (6)$$

$$HNPV = \frac{HSp \times (1 - HP)}{HSp \times (1 - HP) + (1 - HSe) \times HP} \quad (7)$$

269 where herd prevalence (HP) is the proportion of social groups that contain individuals

270 with disease. For the purposes of our analyses we have modelled *n* up to 20 as, based

271 on our field experience, this represented a reasonable upper limit for unique samples

272 taken over two sampling events (38). In addition, the range of HP (0.05-0.2) was chosen

273 based on the range of prevalence within badger social groups (37, 38).

274 We model the effects of requiring two independent DNA extractions and qPCR tests to

275 both give positive results (serial testing (47)) in order to assign a sample as positive.

276 The equations for repeat diagnostic sensitivity and specificity (DSe^R and DSp^R

277 respectively) are as follows:

$$DSe^R = DSe^2 \quad (8)$$

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$$DSp^R = 1 - (1 - DSp)^2 \quad (9)$$

278 This is possible if samples are split and stored at the point of sampling or when

279 introduced to the laboratory. False positive results are likely to be the result of

280 contamination with DNA extracted from other positive samples, most likely during the

281 DNA extraction process, as our qPCR is 100% specific for *M. bovis* DNA (38). As such,

282 re-extraction from a second aliquot of faeces would give an independent result.

283 We modelled the effects of using repeat extractions using Fast24-qPCR as it was more

284 sensitive and more specific (though not to a statistically significant degree).

285 Statistical analysis was performed in RStudio (48) using R (49). Graphics were created

286 using ggplot2 (50).

287 **Results**

288 ***Sensitivity and specificity at the sample level***

289 DNA was extracted from two replicates of a blinded panel of badger faeces containing

290 known positive and negative samples using the Fast24 (Panel 1) and Fast96 (Panel 2)

291 extraction methodology respectively, prior to qPCR screening and quantification. These

292 panels were unblinded by APHA when all data had been collected. The performance of

293 the two operators using the Fast24-qPCR method differed significantly in terms of DSe

294 (Bonferroni corrected p<0.01, two-tailed Fisher's exact test) (Table 2). DSeLC and DSp

295 were not significantly different between the two operators, though both were lower for

296 operator two. Operator one possessed the most experience with the technique at the

297 time of the study, which may explain the discrepancy.

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298 DSe was significantly higher for the Fast24-qPCR method (Operator one - Op1) than

299 the Fast96-qPCR method (Bonferroni corrected p<0.01, two-tailed Fisher's exact test,

300 respectively) (Table 2) but this was not the case for Operator Two (Op2). DSeLC was

301 not significantly different between Fast24-qPCR (Op1 vs Op2) nor between Fast24-

302 qPCR (either operator) and Fast96-qPCR. These comparisons of sub-samples are

303 comparatively statistically underpowered, however, though DSeLC was similar for both

304 operators of Fast24-qPCR. Fast24-qPCR (Op1) and Fast96-qPCR DSe was

305 significantly higher than DSeLC (p<0.01 and p<0.05, respectively, one-tailed Fisher's

306 exact test). This was not the case for Op2. Diagnostic specificity (DSp) was not

307 significantly different between the two methods or between operators. For both

308 methods, DSe meets the minimum threshold (20%) established prior to the study. In

309 terms of DSp, Fast24-qPCR as performed by Op1 meets the minimum threshold (98%),

310 though its 95% CI did drop below it, however this is not the case for Op2, though the

311 minimum threshold is within 95% CI and the difference between operators is not

312 statistically significant. Fast96-qPCR does not meet the threshold, though again the

313 threshold is within 95% CI.

314 *Sensitivity and specificity at the social group level*

315 Group level sensitivity (HSe) increased with number of samples and HP, while group

316 level specificity (HSp) decreased with number of samples for both Fast24-qPCR and

317 Fast96-qPCR (Figure 2A and 2B). To make this trade-off more favourable, two

318 amendments were modelled, based on DSp and DSe from Op1. The first was serial

319 testing i.e. requiring independent, confirmatory re-extraction and qPCR re-test of each

320 positive faeces to assign positive status to a sample. This substantially increased HSp

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321 while moderately decreasing HSe for both Fast24-qPCR and Fast96-qPCR (Figure 2C

322 and 2D). The second, increasing the threshold of positive samples required to assign

323 positive status to a social group from one to two also increased HSp but this had the

324 effect of sharply decreasing HSe (Figure S1).

325 The Fast24-qPCR datasets were also analysed to model the effects of serial testing

326 using both operators' datasets as independent repeats (Table 3) in order to compare

327 these to estimations based on DSe and DSp from Op1. When compared to the original

328 DSe and DSp data (Table 2), sensitivity decreased (93.6% compared to 96.7%), while

329 specificity increased (99.99% compared to 99.0%). DSe^R for the combined Fast24-

330 qPCR dataset was lower than estimated via equation 8 (87.3% compared to 93.6%,

331 p<0.05, two-tailed Fisher's exact test), which was explained by the lower DSe for the 2nd

332 operator. $DSeLC^R$ and DSp^R for this combined dataset are similar to the values

333 estimated by equations 8 and 9 (78.5% compared to 79.6%, and 100% compared to

334 99.99%, respectively). Repeat testing resulted in a substantially reduced decline in HSp

335 caused by increasing sample number, thus allowing HSe to be increased without

336 compromising HSp despite the reduction in DSe caused by repeat testing.

337 ***Predictive values at the social group level***

338 At low levels of HP, both Fast 24 and Fast 96 have low HPPV, but high HNPV (Figure

339 2). HPPV is increased by testing 20 samples with a threshold of 2 positives required to

340 determine herd level infection, and is increased still further by requiring positive repeats

341 when serial testing positive samples (Figure 3), which has minimal effect on HNPV.

342 These figures assume a within-herd shedding prevalence of 10%. The figures are based

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343   on estimates of HSe determined from DSeLC, though the values are similar if DSe is

344   used. HPPV increases with sample number and HP, both with and without re-testing of

345   positives (Figure S2). HNPV increase with sample number, but decrease with HP, and

346   the effect of re-testing of positives on this is minimal (Figure S3).

347

Discussion

349   The sensitivity and specificity of two tests that detect *M. bovis* in badger faeces was

350   estimated. The results presented here show that, on a per sample basis, both Fast24-

351   qPCR and Fast96-qPCR have similar or superior diagnostic specificity to existing

352   trapping-based immunological tests. All tests met the threshold criteria for diagnostic

353   sensitivity proposed in advance. Neither test consistently met the threshold criteria for

354   diagnostic specificity, with only one operator of the Fast24-qPCR method meeting this

355   threshold. However, we estimated that serial testing of positives would substantially

356   increase specificity, and through combining the datasets from both operators we

357   showed 100% specificity. Such repeats show that Fast24-qPCR can be applied to

358   multiple faecal samples from a social group in order to maximize group-level sensitivity

359   without compromising group-level specificity. We predict that similar repeat tests could

360   also improve the specificity of Fast96, though this remains to be demonstrated.

361   The Fast24-qPCR method, as performed by the first operator, was significantly more

362   sensitive than the Fast96, though this was not the case for the second operator,

363   possibly due to the difference in experience between the two operators. Regardless,

364   both extraction methods displayed high levels of sensitivity for the spiked samples

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365 analysed in this study. However, for sensitivity, comparisons with other diagnostic tests

366 should be applied with caution, as the two methods detect shedding, in contrast to

367 immunological tests which detect immune status. It is likely that there are more animals

368 that are exposed to and show immunological responses to *M. bovis* than there are

369 animals that actively shed the bacteria in their faeces, and thus a lower herd prevalence

370 is expected for faecal testing than immunological testing as previously shown by our

371 laboratory (37) . However, animals that are shedding may be both more infectious and

372 more likely to spread infection via environmental contamination than seropositive

373 animals that are not shedding. For this reason, and due to the difficulty of linking badger

374 faeces to individual animals, the tests cannot be used to determine the infection status

375 of individuals, unless faeces are taken directly from trapped animals.

376 For faeces collected from latrines, the tests can be applied at a social group level. To

377 achieve adequate social group level sensitivity (HSe) requires the testing of multiple

378 faeces; however, this comes at the cost of decreasing group level specificity (HSp). This

379 is overcome by the re-testing of any positive samples using a previously stored aliquot

380 of the same faeces. This allows up to twenty faecal samples, approximately the upper

381 limit for the quantity of unique samples that can be collected on two sampling trips, to be

382 tested with low false positive rates at the social group level. Buzdugan *et al.* (33) have

383 modelled HSe and HSp based on the parallel use of Stat-Pak and gamma interferon

384 (IFN-γ) on trapped badgers – i.e. both tests are used and the animal assigned positive

385 status if either test is positive (47). Serial testing resulted in too low DSe (30% at

386 individual animal level), (33) therefore to achieve the highest HSe and HSp Buzdugan *et*

387 *al.* (33) model the effects of parallel testing of animals with a threshold of two animals

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388 required to test positive for a social group to be considered bTB positive. Assuming 50%

389 of animals within a social group of n=15 are trapped and tested, this results in a HSp of

390 91%, with a HSe of ~60% at 25% prevalence of infection (33), though an HSp of >95%

391 is also reported if 40% of the social group is trapped and tested. Serial testing of

392 positive samples with Fast24-qPCR therefore shows a higher HSp than a trapping

393 based strategy. Given that initial data suggests that faecal qPCR can identify different

394 animals than IFN-γ and BrockTB StatPak (37), Fast24-qPCR could therefore be used to

395 complement the immunological testing model described by Buzdugan *et al.* (33).

396 Fast24-qPCR is a non-invasive sampling method that can detect the shedding of *M.*

397 *bovis* in badger faeces at the level of the social group. When performed with re-testing

398 of positives it has very high specificity and high sensitivity at the social group level. In

399 comparison to Fast24-qPCR, Fast96-qPCR increases the throughput of samples, but at

400 the expense of reduced sensitivity and specificity. The reduction in specificity can likely

401 be alleviated substantially with an independent re-test of positive faecal samples using

402 the Fast24- qPCR DNA extraction methodology, allowing testing of higher numbers of

403 faeces per social group leading to higher sensitivity at the social group level while

404 maintaining high herd positive predictive value. While re-testing of positives does

405 increase the expense of the test, it need only be applied to the proportion of samples

406 that are positive, which, based on previous research, we estimate to be in the range of

407 5-15% within regions where *M. bovis* is endemic. Given sufficient sampling effort,

408 Fast24-qPCR therefore provides a social group level test that is capable of measuring

409 the impacts of interventions designed to reduce the spread of bTB from badgers to

19

410   cattle and vice versa, by accurately measuring the shedding of *M. bovis* into the

411   environment.

Conflicts of interest

413   The authors declare no conflicts of interest.

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23

559   **Table 1** The composition of the panel used in this study. The four lowest spiked

560   concentrations (17.83-142.67 CFU/g) were used to determine diagnostic sensitivity at

561   low concentration (DSeLC).

Spiked concentration (*Mycobacterium bovis* CFU/g)	Number of samples
570666.7	5
114000	25
57066.67	25
11400	25
5706.67	20
1140	25
570.67	25
285.33	30
142.67	25
71.33	15
35.67	15
17.83	10
0	5
Negative (as part of original panel) Negative (added at Warwick) Putative positive	200 88 (Fast24-qPCR) 24 (Fast96-qPCR) 119

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24

563   **TABLE 2.** Diagnostic sensitivity (DSe), sensitivity at low concentrations (DSeLC) and

564   specificity (DSp). [ab] Pairwise comparisons within DSe P<0.05 two-tailed Fisher's Exact

565   Test (Bonferroni corrected), [*] Pairwise comparisons between DSe and DSeLC P<0.05

566   one-tailed Fisher's exact test (Bonferroni corrected).

567

Test	DSe (95% CI, N)	DSeLC (95% CI, N)	DSp (95% CI, N)
Fast24-qPCR (1st operator)	96.7%[ab*] (94.5-99.0, 244)	89.2%[*] (81.7-96.7, 65)	99.0% (97.8-100, 292)
Fast24-qPCR (2nd operator)	89.8%[a] (86.0-93.6, 245)	87.7% (79.7-95.7, 65)	96.9% (95.0-98.9, 293)
Fast96-qPCR	88.4%[b*] (84.3-92.4, 241)	75.0%[*] (64.5-85.5, 64)	97.0% (95.0-98.9, 231)

568

25

569 **TABLE 3.** Diagnostic sensitivity (DSe), sensitivity at low concentrations (DSeLC) and

570 specificity (DSp) with estimated effects of repeat testing (Fast24-/Fast96-qPCR with

571 repeat) and as measured by combining Fast24-qPCR panel results from both operators.

572 [a] Pairwise comparisons P<0.05 two-tailed Fisher's exact test.

573

Test	DSe (95% CI, N)	DSeLC (95% CI, N)	DSp (95% CI, N)
Fast24-qPCR with repeat	93.6%[a] (89.3-97.9)	79.6% (66.7-93.6)	99.99% (99.95-100)
Fast96-qPCR with repeat	85.5% (79.7-91.5)	66.9% (52.6-82.8)	99.97% (99.88-100)
Fast24-qPCR both operators	87.3%[a] (83.1-91.5, 244)	78.5% (68.5-88.5, 65)	100% (100-100, 201)

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26

575 **Figure 1**. Log10 distribution of *M. bovis* genome equivalents obtained by qPCR of

576 positive samples and comparison to a standard curve. Putative and Spiked samples are

577 from this study, SE3280 are from a previous DEFRA project SE3280 reported in King et

578 al (37, 38). Putative and SE3280 samples are taken from natural infected populations.

579 One-way ANOVA shows significant difference between the means of the three

580 populations (p<0.001). Bonferroni corrected two-tailed Welch's T-test shows significant

581 difference between SE3280 and Spiked samples (p<0.001), and between Putative and

582 Spiked samples (p<0.001), but not between SE3280 and Putative samples (p=0.90).

583

584 **Figure 2**. Relationship between Herd Sensitivity (HSe) and Herd Specificity (HSp), and

585 the number of samples tested (n). A range of Herd Prevalences (HP) are modelled. HSe

586 at HP 0.05 is shown in dark green, 0.1 (orange), 0.15 (purple), 0.2 (pink), and HSp is

587 shown in light green. (**A**) Fast24-qPCR, (**B**) Fast 96-qPCR, (**C**) Fast24-qPCR with

588 repeated positives, (**D**) Fast 96-qPCR with repeated positives. For both Fast24-qPCR

589 (**A**) and Fast 96-qPCR (**B**) HSe increase with n, however this comes at the expense of

590 HSp which decreases with n. This can be alleviated by repeat testing of positives which

591 decreases the decline in HSp with n while maintaining Hse in both Fast24-qPCR (**C**)

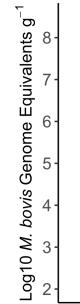592 and Fast 96-qPCR (**D**)

593

594 **Figure 3.** Relationship between Herd Positive Predictive Value (HPPV) (**A**), Herd

595 Negative Predictive Value (HNPV) (**B**) and Herd Level Prevalence (HP) – the proportion

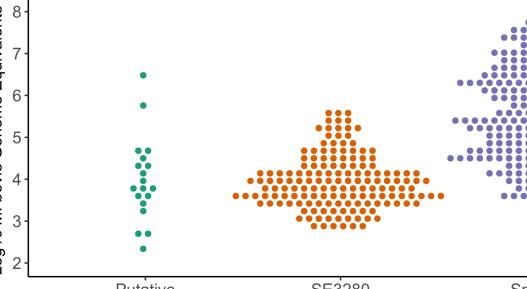596 of herds that are positive - for a variety of testing modalities. Fast24-qPCR 10, 1 (dark

27

597   green), Fast96-qPCR 10, 1 (orange), Fast24-qPCR 20, 2 (purple), Fast96-qPCR 20, 2

598   (pink), Fast24-qPCR with repeat (light green), Fast96-qPCR with repeat (yellow). The

599   numbers following the type of test show the number of samples tested, followed by the

600   number of positive samples required to assign a herd as positive. Considering Fast24-

601   qPCR 10, 1, and Fast 96 10, 1 as the baseline, HPPV is improved by doubling both the

602   number of samples tested, and the number of positive samples required, but not by as

603   much as requiring the repeat testing of positive samples (10, 1 with repeat) (**A**). HNPV

604   shows a similar relationship for all testing modalities (**B**).

28

A Fast24-qPCR

B Fast96-qPCR

C Fast24-qPCR with repeat

D Fast96-qPCR with repeat

A

B