

1 **Performance of serological antibody tests for bovine tuberculosis in cattle**
2 **from infected herds in Northern Ireland**

3 Running title: bTB serological tests performance in Northern Ireland

4 **McCallan, L.^a Brooks, C.^a, Couzens, C., Young, F.^a, Byrne, A.W.^{a,b#}, McNair, J.^a**

5 ^a *Agri-food and Biosciences Institute, Stormont, Belfast, Northern Ireland BT43SD*

6 ^a *School of Biological Sciences, Queen's University Belfast, Belfast, Northern Ireland.*

7 # Corresponding author: andrew.byrne@afbini.gov.uk; +44 2890525809

8

9 Wordcount: 3289 (not including Materials and Methods, References, tables, or figure
10 legends)

11 **Abstract**

12 The ability to accurately identify infected hosts is the cornerstone of effective disease control
13 and eradication programs. In the case of bovine tuberculosis, caused by infection with the
14 pathogen *Mycobacterium bovis*, accurately identifying infected individual animals has been
15 challenging as all available tests exhibit less than 100% discriminatory ability. Here we
16 assess the utility of three serological tests and assess their performance relative to skin test
17 (Single Intradermal Comparative Cervical Tuberculin; SICCT), gamma-interferon (IFN γ) and
18 post-mortem results in a Northern Ireland setting. Furthermore, we describe a case-study
19 where one test was used in conjunction with statutory testing.

20 Serological tests using samples taken prior to SICCT disclosed low proportions of animals as
21 test positive (mean 3% positive), despite the cohort having high proportions with positive
22 SICCT test under standard interpretation (121/921; 13%) or IFN γ (365/922; 40%) results.
23 Furthermore, for animals with a post-mortem record (n=286), there was a high proportion
24 with TB visible lesions (27%) or with laboratory confirmed infection (25%). As a result,
25 apparent sensitivities within this cohort was very low ($\leq 15\%$), however the tests succeeded in
26 achieving very high specificities (96-100%). During the case-study, 7/670 (1.04%) samples
27 from SICCT negative animals from a large chronically infected herd were serology positive,
28 with a further 10 animals being borderline positive (17/670; 2.54%). 9/17 of these animals
29 were voluntarily removed, none of which were found to be infected (-lesions/-bacteriology)
30 post-mortem; 1 serology test negative animal was subsequently lesion+ and *M. bovis*
31 confirmed at slaughter.

32 **Importance**

33 Eradication of bovine tuberculosis (bTB; caused by *Mycobacterium bovis*) has remained
34 elusive in a number of countries despite long-term coordinated test and cull programs. This

35 can partially be explained by the limitations of available statutory tests; therefore
36 supplementary test platforms that identify additional infected animals would be of significant
37 utility. Overall, during our study three serological tests did not disclose a high proportion of
38 animals as infected in high-risk cattle herds, and exhibited limited ability to disclose animals
39 that were positive to the statutory skin test, the gamma interferon test (IFN γ), or were post-
40 mortem confirmed with *M. bovis*. These serological tests could be used in a supplementary
41 fashion to the statutory tests in particular circumstances; but may be of limited advantage
42 where parallel use of IFN γ and skin testing is performed, as these tests together tended to
43 disclose the majority of animals with post-mortem evidence of infection in our study cohort.

44 **Keywords:**

45 *Mycobacterium bovis*, IDEXX, Enfer, diagnostic tests, serological tests

46

47 **1. Introduction**

48 Bovine tuberculosis is a globally distributed infectious disease. The impact of infection in
49 cattle at the national and local level can be profound (1, 2). For example, in Northern
50 Ireland legislation is in place, supported by the United Kingdom and the European Union,
51 to control this disease with the eventual aim of total eradication (3). In practical terms,
52 disease control across Northern Ireland is implemented through the single intradermal
53 comparative cervical tuberculin (SICCT) test and through carcass inspection at abattoirs
54 where cattle are slaughtered (3). Animals identified as skin test reactors, either by
55 standard or severe test interpretation, are removed for slaughter by compulsory order and
56 examined post-mortem. Furthermore, all animals slaughtered at abattoirs in Northern
57 Ireland are examined for the presence of tuberculous lesions. Clinical material collected
58 during meat inspection is cultured for the presence of acid fast bacteria with subsequent
59 identification of species and strain type (4).

60 Despite the introduction of statutory control measures to identify and remove infected
61 cattle, bovine TB is a persistent problem in Northern Ireland (5). The epidemiology of
62 disease is complicated by the presence of infection in wildlife (6, 7), and the potential
63 confounding effects of concurrent infections (8, 9, 10). Current diagnostic tests applied to
64 cattle are not sufficiently sensitive to identify all infected animals and to remove them
65 before infection is spread (11-14). This is despite the introduction and widespread use of
66 the interferon gamma release assay (IFN γ ; 15) to augment the bovine TB testing regime
67 and to support the front line tests (16). In combination, meat inspection, the skin test and
68 IFN γ tests will identify a significant number of infected cattle, but not all (17). It is
69 therefore important to investigate and validate tests or improved test strategies that will
70 broaden the capacity to identify infected animals.

71 The development of serology based assays has been very useful for diagnosis where there
72 is a Th2 type immune response. Such assays can be high throughput, relatively
73 inexpensive and blood samples can be submitted to the laboratory a substantial time after
74 they have been taken from the animal. However, with certain diseases a Th1 type immune
75 responses predominates and antibody tests are largely inappropriate. This is usually the
76 case with bovine TB when following infection, the immune response is influenced by T-
77 cells that direct and maintain a response dominated by IFN γ release (18). Should disease
78 progress and the burden of infection increase then the immune response changes subtly to
79 a Th2 type where B-cells release antibody (19). In this situation and in the absence of cell
80 mediated responses that can be exploited using the skin test or the IFN γ assay, an
81 antibody assay may prove useful in the diagnosis of disease. In order to assess the role of
82 antibody tests within a disease control programme that is already based on cell mediated
83 responses, we instigated a study that was centred on bovine TB diseased cattle and at-risk
84 herds. In the study reported here, we compared results from two blind tested antibody
85 assays with the skin test, post-mortem examination, culture confirmation and the IFN γ
86 assay in order to define the utility of serology as a potential diagnostic test. We also
87 report on a case-study where one of the serological tests was used in a large herd where
88 there was a recent chronic history of bTB, and where statutory tests were failing to clear
89 infection.

90

91

92 2. Materials and Methods

93 2.1 Study cohort

94 Samples intended for analysis were taken from cattle selected from Northern Ireland herds
95 that were deemed to have a bovine TB problem and were eligible for inclusion in the IFN γ
96 testing scheme operated by the Department for Agriculture, Environment and Rural Affairs
97 (DAERA), Northern Ireland (see 16, 17). Individual blood samples were taken just prior to
98 the inoculation of tuberculins on day one of the skin test and were submitted to the laboratory
99 within 8 hours of collection. Whole blood was removed and stimulated with antigens, to be
100 tested later for IFN γ release. Residual whole blood was centrifuged for 15 minutes to separate
101 plasma from blood cells. Clarified plasma samples were removed individually and stored at -
102 20°C.

103 The skin test and carcass inspection at abattoir

104 All animals included in the study were skin tested under Annex A, Council Directive
105 64/432/EEC using Prionics tuberculins (PPD_{bovis} and PPD_{avium}). Each tuberculin (0.1mL) was
106 injected intradermally at 3000 IU (PPD_{bovis}) or 2500 IU (PPD_{avium}) on day one of the test.
107 Skin thickness measurement, pre- and 72 hours post-injection was used to calculate increased
108 skin thickness and to indicate the diagnostic outcome of the test. Skin test positive cattle were
109 submitted for slaughter at a designated abattoir in Northern Ireland where carcass inspection
110 was carried out to reveal the presence or absence of tuberculous lesions. Tissue samples were
111 taken from tissues with and without tuberculous-like lesions and submitted to the culture
112 laboratory. Information pertinent to the skin test, and abattoir inspection as well as laboratory
113 test data was recorded onto the Animal and Public Health Information System (APHIS)
114 operated by DAERA.

115

116 **2.2 Laboratory procedures**

117 **Blinded approach to laboratory tests**

118 Sample testing was conducted using a single blind study design in which sample information,
119 including herd number, ear tag, other laboratory test results, was withheld from technical
120 staff. This was achieved by assigning arbitrary codes to plasma samples upon collection. The
121 arbitrary codes and corresponding sample information was stored in a database which was
122 controlled by a senior technician. In compliance with data protection, information relating to
123 herds, animals, or samples was withheld.

124 **The Interferon gamma (IFN γ) test**

125 Whole blood samples were tested for IFN γ release using the Bovigam assay (Prionics,
126 Switzerland) accredited by the United Kingdom Accreditation Service (UKAS). The
127 methodology has been described previously (15). Briefly, whole blood samples were received
128 into the laboratory within eight hours of removal from the animal, stimulated overnight with
129 Pokeweed mitogen (2ug/ml), phosphate buffered saline, PPD_{bovis}, PPD_{avium} (both at 2ug/ml)
130 and ESAT-6 (0.5ug/ml). After overnight culture at 37°C, plasma supernatant fluids were
131 removed and stored prior to test by ELISA. The ELISA was carried out according the
132 manufacturer's protocol with regards to reagent dilutions, incubation times and plate wash
133 regimes. Individual sample results were recorded if reagent control and quality assurance
134 standards were met. Those samples with a net optical density (OD) index of 0.1 or greater
135 were positive (net PPD_{bovis} – net PPD_{avium}) and those less than 0.1 OD units were negative.

136 **Selection of serological tests**

137 Tests to be evaluated were based on commercial availability and/or through fulfilling the
138 tender to test samples via a public tender established by AFBI. Two test providers were
139 identified (see below) who satisfied the tender requirements.

140 **The IDEXX ELISA for antibodies**

141 IDEXX *M. bovis* ELISA kits were purchased from the manufacturer and the assay was
142 carried out according to the manufacturer's protocol. The IDEXX ELISA is a commercially
143 available kit. This ELISA has a 96 well microtitre plate format that detects antibodies to two
144 *Mycobacterium tuberculosis* complex antigens (MPB70 and MPB83) known to be serological
145 targets in *Mycobacterium bovis* (*M. bovis*) infections. Briefly, plasma samples were diluted to
146 1 in 50 in PBS and tested in duplicate. One hundred microliters of reagents were added to
147 wells in duplicate and incubated for 60 minutes then washed 6 times. Assay positive and
148 negative test control reagents were used to validate each microtitre plate and provided data to
149 calculate the test result [sample / positive ratio (S/P ratio)]. Test results were interpreted as
150 follows: an S/P ratio greater or equal to 0.30 was considered positive and a ratio less than 0.3
151 was negative.

152 **The Enfer provisioned antibody assay**

153 An Enfer provisioned assay was carried out by Enfer staff at their Naas laboratories (Enfer
154 ltd, Naas, Co Kildare). All tests were blinded, with no information on the epidemiological
155 situation (e.g. within herd prevalence) from which animals were selected provided to Enfer. It
156 should be noted that this Enfer multiplex antibody assay is not a commercially available as a
157 standalone kit, but testing was provided in fulfilment of commercial services as part of a
158 commercial tender to AFBI. The basis for this assay methodology have been described
159 previously (20). For this project, the defined antigens used in this assay were MPB83, ESAT-
160 6, CFP-10 and MPB70. Two antigen combinations were assessed; these combinations were

161 positive to either MPB70 with MPB83 (Enfer-A), or MPB70, MPB83, ESAT-6 and CFP10
162 (Enfer-B). Enfer scientific printed the bespoke multiplex according to the tender
163 requirements, and carried out the screening, utilising bespoke software to read the multiplex
164 plates (20). It should also be noted that the antibody test set-up did not include protein fusions
165 and cocktails, which may be used during other Enfer tests (J. Clarke, pers. comm.). Plasma
166 samples were diluted to 1 in 250 (in Enfer sample buffer A) and added to each well and
167 incubated and agitated for 30 minutes. After washing, horseradish conjugated anti-bovine
168 immunoglobulin was added, incubated and washed again. Substrate was added and signals
169 were captured during a 45 second exposure stored as relative light units. The manufacturer
170 recommends that a positive result is recorded when a minimum of any two antigens are test
171 positive.

172 **Laboratory confirmatory tests for Mycobacteria**

173 Clinical samples removed from animals at slaughter were submitted to the containment level
174 3 laboratory for preparation, decontamination and inoculation onto solid and liquid media.
175 Culture procedures at the Statutory TB Laboratory at the Agri-food and Biosciences Institute
176 have been described extensively previously (e.g.17, 21). Tissue structure was disrupted using
177 either ribolysation or grinding with sterile sand in a pestle and mortar. Prior to inoculation,
178 clinical samples were decontaminated using 5% oxalic acid for a maximum of 30 min and
179 washed twice with sterile PBS. Samples were then inoculated onto Lowenstein-Jensen and
180 Stonebrink slopes, as well as into MGIT culture vessels containing PANTA. At 56 days post
181 inoculation, cultures were examined for the presence of acid fast mycobacteria and if present
182 were further analysed using a spoligotype method (22) to identify mycobacterial species and
183 sub-type. Also, a selection of tissues that were lesion positive were fixed in neutral buffered
184 formalin solution and prepared for additional histological examination.

185

186 **2.3 Analysis**

187 The relationship between the test status and the independent variables was modelled
188 throughout using binary logit regression models. A random effect for herd id (to account for
189 potential clustering effects) was included if significant and was tested using a likelihood ratio
190 test.

191 Throughout we estimated the Area Under the receiver operator Curve (AUC) as an
192 assessment of the ability of the serological test to discriminate between (apparent) infection
193 states. The AUC is measured on a continuous scale from 0 to 1; an AUC of 0.5 is no better
194 than random, with values >0.7 considered an “adequate” diagnostic (23). Apparent
195 sensitivity, specificity, positive predictive value and negative predictive value was calculated
196 and reported against non-gold standards of infection status.

197 Each diagnostic was compared against the skin test (SICCT) result, IFN γ test result and post-
198 mortem status of the animal, giving apparent/relative performance indices. We also used the
199 definition adopted by Whelan et al, (24) to define “true” infection status. In this case,
200 infection was defined by an animal being positive to the skin test (SICCT standard
201 interpretation), having a visible lesion at slaughter and having a bacteriological confirmation
202 result (positive to histology and/or microbiological culture). Being free of infection, negative
203 animals were negative to SICCT, without lesions at slaughter and without post-mortem
204 bacteriological confirmation. In addition, we used a combination of IFN γ , SICCT, VL and
205 confirmation, to assess the relative performance of the serology tests.

206 We used binary logit models to assess whether there was any association between animal sex,
207 age at blood test sample, breed type and the probability of a positive serological test results
208 being disclosed. Age was modelled both as a raw continuous variable and as a log-
209 transformed predictor.

210 Throughout, the dataset was organised using Microsoft excel, while all statistical analysis
211 was undertaken using Stata version 14 (Stata Corp., Texas, USA, 2015).

212 **2.4 A problem herd based case study**

213 A case study centred on a relatively large (approximately 1000 cattle over the period) dairy
214 herd was carried out to assess the utility of antibody detection where animals were known to
215 be infected and resolution of the problem was proving to be difficult. This particular herd had
216 a seemingly intractable chronic bovine TB problem which originated between 2002 and
217 2004. Initially, a relatively small number of bovine TB breakdowns were recorded with
218 subsequent confirmation of infection caused by *Mycobacterium bovis*. From 2008 onward,
219 the rate of skin test positive cattle increased significantly with up to 148 skin test positive
220 animals identified as well as 2 cases of lesions at routine slaughter, i.e. skin test negative
221 cattle sent for slaughter with confirmed tuberculous lesions disclosed during carcass
222 inspection. Given the disease history of this herd following routine TB diagnostic
223 investigations, high risk cohorts of cattle within this herd were blood sampled and tested for
224 the presence of antibodies to *M. bovis* using IDEXX serology (OIE approved) in 2016. The
225 fundamental rationale was that detecting antibody in cattle that were skin test negative may
226 indicate the presence of infection in animals that were considered to be anergic, that is,
227 unresponsive to cell mediated tests such as the skin test and IFN γ assay.

228 **Data availability**

229 All data was provided through the APHIS dataset, for which the data controller is DAERA.
230 All data from which inferences were made are provided within the paper, raw test data has
231 been deposited in an online repository (25). Additional information on these data is available
232 from DAERA, Northern Ireland (<https://www.daera-ni.gov.uk/access-information-0>;

233 daera.informationmanager@daera-ni.gov.uk) and would be subject to appropriate Data

234 Protection regulations (UK) in relation to individual farmers/herds.

235 3. Results

236 *Agreement and comparison*

237 Overall, there were 922 animals with test result data; all animals had test results for IFN γ and
238 IDEXX, 921 had SICCT, Enfer-A and Enfer-B results, while 286 animals had a post-mortem
239 result. These animals came from 64 herds with recent bTB breakdowns, with a mean of 14.39
240 animals sampled per herd (Median: 9.5; Std. Dev.: 13.39; Range: 1-76).

241 There was significant ($p < 0.001$) moderate agreement between the three tests ranging from a
242 kappa of 0.40 (IDEXX and Enfer-B) to 0.55 (Enfer-A and Enfer-B). Of the animals with
243 visible lesions found at post-mortem, the proportions deemed positive were not significantly
244 different between the serological test types (McNemar's test: Enfer-A vs. IDEXX: $p = 0.65$;
245 Enfer-B vs. IDEXX: $p = 0.16$; Enfer-B vs Enfer-A: $p = 0.18$). Similarly, there were no
246 difference between test types, when using bacteriological confirmation as the infection status
247 diagnostic ($p > 0.25$).

248 *Serology test performance in comparison with single or combined diagnostic techniques*

249 The relative performance of the serological tests in comparison with single ante-mortem
250 diagnostics (Table 1), post-mortem diagnostics (Table 2) and combined tests (Table 3 and
251 Table 4) are presented below. Relative to single ante-mortem tests (mean test prevalence
252 27%; Table 1), the serological tests did not disclose a high proportion of test-positive animals
253 (mean 3% positive). This resulted in the tests exhibiting low apparent sensitivities, averaging
254 5.73% (range: 4.13% - 9.09%). However, the apparent specificities were always very high,
255 with a mean of 97.82% (96.40% - 99.50%). While there was a significant positive
256 relationship between serological test result and statutory ante-mortem outcome, the
257 discriminatory ability of the tests were always poor (mean AUC: 0.52).

258 Similar results were found when post-mortem diagnostic techniques were used as the
259 apparent infection status (Table 2). Due to the low sensitivity of the serological antibody
260 tests, the mean test prevalence was always low (mean test prevalence 4.92%) relative to the
261 proportion of animals with lesions or post-mortem confirmed infection (mean prevalence
262 26%).

263 Using similar criteria to Whelan et al. (24) to define animals as “truly” infected and non-
264 infected, we found that the serological tests exhibited poor sensitivity (9.09% - 13.64%;
265 Table 3). Utilising IFN γ test results as an additional criterion (Table 4), suggested again that
266 the serological tests exhibited low sensitivities, however the three tests achieved 100%
267 apparent specificities.

268 Table 5 gives the breakdown of animal ante-mortem test results in relation of each serological
269 test result. Overall, 8 (8/505; 1.56%), 2 (2/513; 0.39%), and 17 (17/514; 3.31%) animals were
270 ante-mortem test negative, that were deemed serologically test positive to Enfer-A, Enfer-B
271 and IDEXX respectively.

272 Table 6 gives a breakdown of animals with post-mortem confirmed *M. bovis* infection, that
273 were skin-test, IFN γ , or either skin-test/ IFN γ negative. ENFER-A and IDEXX both disclosed
274 as positive 3/19 (15.79%) SICCT false-negative animals. The ENFER-B test disclosed two
275 animals of these 19 animals as positive. However, none of the 14 post-mortem confirmed
276 animals that were as IFN γ negative were found to be serologically positive. Overall, 6 of the
277 animals with confirmed infection were missed by both SICCT and IFN γ
278 tests (6/286; 2.10%), and none of these were disclosed using any of the serological antibody
279 tests.

280

281 *Sex, age and breed associations with serological test results*

282 There was no evidence of a significant effect of sex on the probability of an animal disclosing
283 as serological positive across all tests ($p>0.1$). Similarly, there was no evidence of an age
284 effect on the probability of animals disclosing with serological positive test ($p>0.08$). There
285 was no relationship between the breed and either Enfer-A ($p=0.617$) or IDEXX ($p=0.457$)
286 positivity. However, there was significant variation in the probability of a positive disclosure
287 with Enfer-B ($n= 683$; $p=0.005$; Figure 1). This model included five breed types with enough
288 samples and variation to allow the model to fit ($n= 26$ to 378 per breed type; Figure 1).
289 Friesian breed cattle exhibited significantly lower odds of disclosing as an Enfer-B test
290 positive relative to Charolais ($p=0.025$), Hereford ($p=0.006$) and Limousine ($p=0.012$)
291 animals, respectively, however the difference was not significant relative to Aberdeen Angus
292 cattle ($p=0.116$; see supplementary material for figure of linear predictions with associated
293 95%CI).

294 **A problem herd based case study**

295 In total, 670 samples from cattle were blood sampled having been selected on the basis of
296 being high risk cohorts of animal where the infection was most prevalent (ante-mortem
297 negative in contact animals). Using the manufacturer's recommended S/P ratio cut-off value
298 of 0.3, seven samples were positive (≥ 0.3) and 663 samples were negative (≤ 0.3). Five
299 samples were clearly positive (> 0.3), two samples were just above the threshold (0.340 and
300 0.331) and all the remaining samples were negative. However, 17 samples had S/P ratios just
301 below the cut-off value, ranging between 0.270 to 0.100 (Table 7).

302 Following release of the serology results and discussions with the herd keeper, nine animals
303 were voluntarily surrendered for slaughter. At post-mortem examination, all cattle were
304 designated non-visibly lesioned and clinical samples from the lung associated lymph nodes

305 were submitted for laboratory tests. All samples were culture negative for *M. bovis*.
306 Subsequent to this serology test based investigation, one animal which was serology negative
307 and submitted for voluntary slaughter, was examined and found to be visibly lesioned.
308 Subsequently, clinical samples were culture positive with *M. bovis* confirmed by spoligotype.

309

310 4. Discussion

311

312

313 During the present study, we tested three serological tests (2 versions of the ENFER
314 multiplex, and the IDEXX AB test) for their relative performance in at-risk herds in Northern
315 Ireland. Overall, our results suggested that the tests can achieve very high levels of apparent
316 specificity. However, our results suggested that these tests failed to identify many animals
317 with pathology or confirmed *M. bovis* infection post-mortem. These results appear at odds
318 with some previous studies in other populations (24, 26), with serological antibody tests
319 being suggested as a potential useful diagnostic in certain situations (27-30). However, there
320 has been reported lower sensitivity estimates elsewhere (e.g. 27, 31).

321 In comparison with previous work by our group (14), serology samples were taken prior to
322 the SICCT tuberculin test. In work from Spain, when serology tests were evaluated prior to
323 the tuberculin test, their performance was reduced relative to tests undertaken with samples
324 after the tuberculin test (27). Samples taken from a cohort of animals prior to skin testing
325 suggested that the serology tests examined exhibited a sensitivity of 23.9%-32.6% (*M. bovis*
326 Ab Test (IDEXX) & Enferplex TB assay, respectively; 27). For animals sampled post-skin
327 test, the beneficial anamnestic effect was most pronounced 15 days post-intradermal testing,
328 achieving sensitivity estimates of 66.7%-85.2% (27). The effect was apparent by the number
329 of animals disclosed as serology test positive when tested prior to skin testing (10.7%; 6/56),
330 72hrs after skin testing (7.1%; 4/56) and 15 days after testing (57.1%; 32/56). In the current

331 study, a small proportion of animals were disclosed as serology positive (mean 3% positive).
332 However, during another study in Northern Ireland, we found a higher proportion of animals
333 were disclosed as positive when prevalence was higher (86% SICCT test reactors) and testing
334 occurred after skin testing (14). The proportion serology positive in that cohort was 39.02-
335 62.20% positive, with apparent sensitivities relative to post-mortem confirmed infection
336 estimated to be 68-82%. These results suggest that maximising the beneficial effects of
337 serology testing may occur if samples are taken after skin testing. Such boosting/priming
338 effects have been described before in cattle in a number of studies (27, 28, 32-35) and in
339 other species also (see 36). Two antigens used in the tests assessed during the present study
340 are known to be boosted by skin testing (MPB83 and MPB70; 35). Such effects have led to
341 some authorities to require follow-up serology testing during statutory tests, for example with
342 camelids in Wales (36). However, this effect reduces the utility of such a test in cattle where
343 proactive eradication programs are in place, as skin test positive animals in Northern Ireland
344 are routinely culled under legislation (3). Furthermore, in problem herds judicious use of
345 severe interpretation of the SICCT (this increases sensitivity, at the cost of specificity (13)) is
346 employed, and also INF γ tests (16; and see below), where positive animals are predominately
347 culled to clear infection.

348 In the present study, a small proportion of infected but SICCT negative animals that were
349 identified by the serological tests (2-3/19 animals; 10.53%- 15.79%). This suggests that, in
350 the absence of other ancillary testing, serological tests could be useful to identify part of this
351 subpopulation (Whelan et al. (24)). Previous research found that of 60 truly infected SICCT
352 negative or inconclusive animals, 53 (88.3%) were disclosed as positive using a multiplex
353 ELISA test (24). It is hard to account for the relatively poorer detection rate in our study
354 relative to Whelan et al. (24), but the discrepancy can partly be explained by the relatively
355 small number of SICCT negative, *M. bovis* confirmed animals available in the present study.

356 Employing exact confidence intervals around the proportion, suggests significant uncertainty
357 in our estimate (exact CI: 3.38% - 39.58%). In Northern Ireland, IFN γ is routinely used in
358 herds with problems clearing infection (e.g. see 16, 17). We found in this study, that when
359 IFN γ was used instead of, or in parallel with, SICCT, there were no *M. bovis* confirmed
360 animals identified by the serological tests employed. This suggests, where both SICCT and
361 IFN γ are used together, there may be limited opportunities to detect additional missed
362 infected animals using serological tests. Casal et al. (29), however, suggests that in very high
363 prevalence regions there may be value in parallel interpretation of cellular and antibody
364 detection techniques to maximise sensitivity.

365 During the case study presented, we found that few animals were disclosed as serologically
366 positive from a large herd with a substantial chronic bTB problem. Even with liberal
367 interpretation of one of the serology test (IDEXX) data, few animals were removed, and all
368 those culled were found to have no visible lesions nor could *M. bovis* be isolated from
369 samples taken from these animals. One animal that was serologically tested, and found
370 negative, was subsequently found to have visible lesions and confirmed for *M. bovis* at post-
371 mortem. This field application of the test in a particular problematic herd appears to
372 corroborate our findings from the prospective study results. However, other case-studies have
373 highlighted benefits of serology as ancillary tests in eradicating TB. For example, a red deer
374 herd in England with a TB outbreak was cleared of infection with the use of both tuberculin
375 testing and serological testing over a 2 year period (30). The authors suggest that without the
376 additional removal of serologically test positive, the time to eradication may have been
377 significantly increased as well as contributing to maintenance and potential transmission to
378 local wildlife. O'Brien et al. (28) also describes a case-study in a goat herd where skin tests
379 failed to identify all infected animals, with 6/20 slaughtered animals having visible lesions
380 and positive to six *M. bovis* antigens.

381 Serological tests could be strategically useful in the case of anergic animals, where advanced
382 and generalised infection is present leading to failure to respond to SICCT due to an impaired
383 Cell mediated immunity (CMI) response (12). However, currently there is limited data on the
384 proportion of animals that could be deemed anergic in Northern Ireland farms. Potentially,
385 the repeated application of SICCT testing over an animal's lifetime could lead to
386 desensitisation (12, 37), again resulting in false negatives. When we looked at the impact of
387 age on the probability of disclosure, we found no significant variation in our cohort.
388 However, we did find some evidence for variation in disclosure depending on breed-type,
389 with Friesian cattle exhibiting significantly lower probability of disclosing serology positive
390 on one of the tests. Further research is required to ascertain whether this is a robust finding –
391 there is significant uncertainty with the current study given the very small numbers of
392 animals serologically test-positive. However, previous research has suggested that there may
393 be significant variation in *M. bovis* susceptibility and pathology across breeds (38, 39), which
394 could be partially attributed to immunological or genetic variation (40).
395 One potential reason for the differing outcomes from this study and some other studies using
396 the Enfer test platform, is that there was a limited set of antigens used across the two test
397 types (Enfer-a and Enfer-b), namely MPB70, MPB83, ESAT-6 and CFP10. The Enfer
398 multiplex can detect antibody activity to 25 antigens in a single well in a 96-well plate array
399 format (20). However, to make cross-comparisons, only the most commonly used antigens
400 were used during the present study. However, such issues do not arise with the IDEXX-ab
401 test, as it is a standard commercial kit.

402

403 **Conclusions**

404 We have shown that three available serological tests, when applied to cattle populations with
405 moderate prevalence and with samples taken prior to tuberculin testing, can exhibit limited

406 apparent sensitivities but very high specificities. Serological tests can disclose additional test-
407 positive animals when used in parallel with the skin tuberculin test. However, we found in
408 this study, that when IFN γ was used instead of, or in parallel with, SICCT, there were no *M.*
409 *bovis* confirmed animals identified by the serological tests employed. This suggests, where
410 both SICCT and IFN γ are used together, there may be limited opportunities to detect
411 additional missed infected animals via the serological tests examined when samples were
412 taken prior to skin testing. From a perspective of a country with an ongoing extensive
413 eradication scheme, future strategic use of serology may be limited to: 1. extreme cases of
414 very large breakdowns within herds leading to high within herd bTB prevalence, 2. in
415 problem herds where IFN γ testing is unavailable, and 3. chronically infected herds where
416 blood samples are taken after tuberculin testing to maximise sensitivity gained from any
417 anamnestic effects.

418

419

420 **Acknowledgements**

421 This study was funded by the Department of Agriculture, Environment and Rural Affairs
422 (DAERA) as part of the Evidence and Innovation Strategy under the grant “An assessment of
423 commercially available serological tests for the detection of cattle infected with bovine
424 tuberculosis” (grant no.: 15/3/09; Project Leaders: Dr. J. McNair and Dr. A. Byrne).

425 5. **References**

- 426 1. Zinsstag J, Schelling E, Roth F, Kazwala RR. 2006. Economics of bovine tuberculosis. *In*
427 Thoen CO (Ed.), *Mycobacterium bovis* infection in animals and humans. 2nd edition.
428 Blackwell Publishing, pp. 68-83
429
- 430 2. O'Reilly LM, Daborn CJ 1995. The epidemiology of *Mycobacterium bovis* infections in
431 animals and man: a review. *Tuber Lung Dis* 76:1-46.
432
- 433 3. Abernethy DA, Denny GO, Menzies FD, McGuckian P, Honhold N, Roberts AR. 2006.
434 The Northern Ireland programme for the control and eradication of *Mycobacterium bovis*.
435 *Vet Microbiol* 112:231-237.
- 436 4. Skuce RA, McDowell SW, Mallon TR, Luke B, Breadon EL, Lagan PL, McCormick CM,
437 McBride SH, Pollock JM. 2005. Discrimination of isolates of *Mycobacterium bovis* in
438 Northern Ireland on the basis of variable numbers of tandem repeats (VNTRs). *Vet Rec*
439 157:501.
- 440 5. Abernethy DA, Upton P, Higgins IM, McGrath G, Goodchild AV, Rolfe SJ, Broughan
441 JM, Downs SH, Clifton-Hadley R, Menzies FD, De la Rua-Domenech R. 2013. Bovine
442 tuberculosis trends in the UK and the Republic of Ireland, 1995–2010. *Vet Rec* 172:312-312.
- 443 6. Palmer MV. 2007. Tuberculosis: a reemerging disease at the interface of domestic animals
444 and wildlife. *In* *Wildlife and Emerging Zoonotic Diseases: The Biology, Circumstances and*
445 *Consequences of Cross-Species Transmission* (pp. 195-215). Springer Berlin Heidelberg.
446
- 447 7. Byrne AW, White PW, McGrath G, O’Keeffe J, Martin SW. 2014. Risk of tuberculosis
448 cattle herd breakdowns in Ireland: effects of badger culling effort, density and historic large-
449 scale interventions. *Vet Res* 45:1.
- 450 8. Claridge J, Diggle P, McCann CM, Mulcahy G, Flynn R, McNair J, Strain S, Welsh M,
451 Baylis M, Williams DJ. 2012. *Fasciola hepatica* is associated with the failure to detect
452 bovine tuberculosis in dairy cattle. *Nature Comm* 3:853.
- 453 9. Byrne AW, Guelbenzu-Gonzalo M, Strain SAJ, McBride S, Graham J, Lahuerta-Marin A,
454 Harwood R, Graham DA, McDowell S. 2017. Assessment of concurrent infection with
455 bovine viral diarrhoea virus (BVDV) and *Mycobacterium bovis*: a herd-level risk factor
456 analysis from Northern Ireland. *Preventive Vet Medicine*.
- 457 10. Álvarez J, De Juan L, Bezos J, Romero B, Sáez JL, Marqués S, Domínguez C, Mínguez
458 O, Fernández-Mardomingo B, Mateos A, Domínguez L. 2009. Effect of paratuberculosis on
459 the diagnosis of bovine tuberculosis in a cattle herd with a mixed infection using interferon-
460 gamma detection assay. *Vet Microbiol* 135:389-393.
- 461 11. Pollock JM, Welsh MD, McNair J (2005) Immune responses in bovine tuberculosis:
462 towards new strategies for the diagnosis and control of disease. *Vet Immunol Immunopathol*
463 108:37–43
464

- 465 12. de la Rua-Domenech R, Goodchild AT, Vordermeier HM, Hewinson RG, Christiansen
466 KH, Clifton-Hadley RS. 2006. Ante mortem diagnosis of tuberculosis in cattle: a review of
467 the tuberculin tests, γ -interferon assay and other ancillary diagnostic techniques. Res Vet Sci
468 81:190-210.
- 469 13. Lahuerta-Marin A, Milne G, McNair J, Skuce R., McBride S., Menzies F. , McDowell
470 S.J.W., Byrne A.W., Handel I.G., Bronsvort M.B.C. 2018. Bayesian Latent Class
471 estimation of sensitivity and specificity parameters of diagnostic tests for bovine tuberculosis
472 in chronically infected herds in Northern Ireland Bayesian Latent Class estimation of
473 Sensitivity and Specificity parameters of diagnostic tests for bovine tuberculosis in chronic
474 herds-Northern Ireland. Vet J in press.
- 475 14. McCallan L, Brooks C, Couzens C, Young F, McNair J, Byrne AW. 2017. Assessment of
476 serological tests for diagnosis of bovine tuberculosis. Vet Rec 181: 90
- 477 15. Welsh MD, McNair J, McDowell SWJ, Buchanan J, Hill R, McBride SH, Clarke N.
478 2008. The Northern Ireland Interferon-gamma (IFN-g) Testing Programme. Cattle
479 Practice 16:136-139
- 480 16. Lahuerta-Marin A, Gallagher M, McBride S, Skuce R, Menzies F, McNair J, McDowell
481 SW, Byrne AW. 2015. Should they stay, or should they go? Relative future risk of bovine
482 tuberculosis for interferon-gamma test-positive cattle left on farms. Vet Res 46:1.
- 483 17. Lahuerta-Marin A, McNair J, Skuce R, McBride S, Allen M, Strain SA, Menzies FD,
484 McDowell SJ, Byrne AW. 2016. Risk factors for failure to detect bovine tuberculosis in cattle
485 from infected herds across Northern Ireland (2004–2010). Res Vet Sci 107:233-239.
- 486 18. Welsh MD, Cunningham RT, Corbett DM, Girvin RM, McNair J, Skuce RA, Bryson DG,
487 Pollock JM. 2005. Influence of pathological progression on the balance between cellular and
488 humoral immune responses in bovine tuberculosis. Immunol 114:101-111.
- 489 19. Pollock JM, Neill SD. 2002. *Mycobacterium bovis* infection and tuberculosis in cattle.
490 Vet J 163:115-127.
- 491 20. Whelan C, Shuralev E, O'Keeffe G, Hyland P, Kwok HF, Snoddy P, O'Brien A, Connolly
492 M, Quinn P, Groll M, Watterson T. 2008. Multiplex immunoassay for serological diagnosis
493 of *Mycobacterium bovis* infection in cattle. Clin Vac Immunol 15:1834-1838.
- 494 21. Stewart LD, McNair J, McCallan L, Gordon A, Grant IR. 2013. Improved detection of
495 *Mycobacterium bovis* infection in bovine lymph node tissue using immunomagnetic
496 separation (IMS)-based methods. PloS one 8:e58374.
- 497 22. Kamerbeek J, Schouls LE, Kolk A, Van Agterveld M, Van Soolingen D, Kuijper S,
498 Bunschoten A, Molhuizen H, Shaw R, Goyal M, Van Embden J. 1997. Simultaneous
499 detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and
500 epidemiology. J Clin Microbiol 35:907-14.
- 501 23. Hosmer DW, Lemeshow S. 2004. Applied logistic regression. John Wiley & Sons.

- 502 24. Whelan C, Whelan AO, Shuralev E, Kwok HF, Hewinson G, Clarke J, Vordermeier HM.
503 2010. Performance of the Enferplex TB assay with cattle in Great Britain and assessment of
504 its suitability as a test to distinguish infected and vaccinated animals. *ClinVaccine Immunol*
505 17:813-817.
- 506 25. McCallan L, Brooks C, Couzens C, Young F, McNair J, Byrne AW. 2017. Data from
507 Performance of serological antibody tests for bovine tuberculosis in cattle from infected herds
508 in Northern Ireland. Figshare. <https://figshare.com/s/db7f5956d1094066180a> DOI:
509 10.6084/m9.figshare.5705893
- 510 26. Waters WR, Buddle BM, Vordermeier HM, Gormley E, Palmer MV, Thacker TC,
511 Bannantine JP, Stabel JR, Linscott R, Martel E, Milian F. 2011. Development and evaluation
512 of an enzyme-linked immunosorbent assay for use in the detection of bovine tuberculosis in
513 cattle. *ClinVaccine Immunol* 18:1882-1888.
- 514 27. Casal C, Infantes JA, Rivalde MA, Díez-Guerrier A, Domínguez M, Moreno I, Romero B,
515 de Juan L, Sáez JL, Juste R, Gortázar C. 2017. Antibody detection tests improve the
516 sensitivity of tuberculosis diagnosis in cattle. *Res Vet Sci* 112:214-21.
- 517 28. O'Brien A, Whelan C, Clarke JB, Hayton A, Watt NJ, Harkiss GD. 2017. Serological
518 Analysis of Tuberculosis in Goats by Use of the Enferplex Caprine TB Multiplex Test. *Clin*
519 *Vaccine Immunol* 24:e00518-16.
- 520 30. Busch F, Bannerman F, Liggett S, Griffin F, Clarke J, Lyashchenko KP, Rhodes S. 2017.
521 Control of bovine tuberculosis in a farmed red deer herd in England. *Vet Rec* 180:68.
- 522 31. Wood PR, Corner LA, Rothel JS, Ripper JL, Fifis T, McCormick BS, Francis B, Melville
523 L, Small K, De Witte K, Tolson J. 1992. A field evaluation of serological and cellular
524 diagnostic tests for bovine tuberculosis. *Vet Microbial* 31:71-79.
- 525 32. Waters WR, Palmer MV, Thacker TC, Bannantine JP, Vordermeier HM, Hewinson RG,
526 Greenwald R, Esfandiari J, McNair J, Pollock JM, Andersen P. 2006. Early antibody
527 responses to experimental *Mycobacterium bovis* infection of cattle. *Clin Vaccine Immunol*
528 13:648-54.
- 529 33. Waters WR, Maggioli MF, McGill J, Lyashchenko KP, Palmer MV. 2014. Relevance of
530 bovine tuberculosis research to the understanding of human disease: Historical perspectives,
531 approaches and immunologic mechanisms. *Vet Immunol Immunopathol* 159:113-132.
- 532 34. Waters WR, Thacker TC, Nelson JT, DiCarlo DM, Maggioli MF, Greenwald R,
533 Esfandiari J, Lyashchenko KP, Palmer MV. 2014. Virulence of two strains of *Mycobacterium*
534 *bovis* in cattle following aerosol infection. *J Com Pathol* 151:410-419.
- 535 35. Waters WR, Palmer MV, Stafne MR, Bass KE, Maggioli MF, Thacker TC, Linscott R,
536 Lawrence JC, Nelson JT, Esfandiari J, Greenwald R. 2015. Effects of serial skin testing with
537 purified protein derivative on the level and quality of antibodies to complex and defined
538 antigens in *Mycobacterium bovis*-infected cattle. *Clin Vaccine Immunol* 6:641-649.

- 539 36. de la Rúa-Domenech R, Rhodes S, Rolfe S, Vordermeier M. 2017. The anamnestic boost
540 effect of the skin test on antibody responses to *Mycobacterium bovis* in camelids – summary
541 of evidence.
542 [http://www.alpacatb.com/anamnestic%20antibody%20response%20\(scientific%20evidence\)](http://www.alpacatb.com/anamnestic%20antibody%20response%20(scientific%20evidence)%20(2).pdf)
543 [%20\(2\).pdf](http://www.alpacatb.com/anamnestic%20antibody%20response%20(scientific%20evidence)%20(2).pdf)
- 544 37. Monaghan ML, Doherty ML, Collins JD, Kazda JF, Quinn PJ. 1994. The tuberculin test.
545 Vet Microbiol 40:111-24.
- 546 38. Ameni G, Aseffa A, Engers H, Young D, Gordon S, Hewinson G, Vordermeier M. 2007.
547 High prevalence and increased severity of pathology of bovine tuberculosis in Holsteins
548 compared to zebu breeds under field cattle husbandry in central Ethiopia. Clin Vaccine
549 Immunol 14:1356-1361.
- 550 39. Wright DM, Allen AR, Mallon TR, McDowell SW, Bishop SC, Glass EJ, Bermingham
551 ML, Woolliams JA, Skuce RA. 2013. Field-isolated genotypes of *Mycobacterium bovis* vary
552 in virulence and influence case pathology but do not affect outbreak size. PloS one 8:e74503.
- 553 40. Allen AR, Minozzi G, Glass EJ, Skuce RA, McDowell SWJ, Woolliams JA, Bishop SC.
554 2010. Bovine tuberculosis: the genetic basis of host susceptibility. P R Soc Lond B: Biol Sci
555 rspb20100830.

556 **Table 1: The relative performance of serological tests against statutory ante-mortem tests. Skin**
 557 **test = SICCT standard interpretation.**

Test type	n	Comparator	Odds ratio	P-value	AUC	Sens	Spec	PPV	NPV	Prev. (comparator)	Test prev.
ENFER-A	919	Skin test	2.97	0.008	0.52	7.44%	97.40%	30.00%	87.40%	13%	3.26%
ENFER-A	920	IFN γ	2.73	0.009	0.52	5.22%	98.00%	63.30%	61.20%	40%	3.26%
ENFER-B	919	Skin test	4.26	0.012	0.52	4.13%	99.00%	38.50%	87.20%	13%	1.41%
ENFER-B	920	IFN γ	5.21	0.013	0.51	2.75%	99.50%	76.90%	61.00%	40%	1.41%
IDEXX	921	Skin test	2.66	0.008	0.53	9.09%	96.40%	27.50%	87.50%	13%	4.34%
IDEXX	922	IFN γ	1.73	0.091	0.51	5.75%	96.60%	52.50%	61.00%	40%	4.34%
Mean	920		3.26	0.024	0.52	5.73%	97.82%	48.12%	74.22%	27%	3.00%

558

559

560 **Table 2: The relative performance of serological tests against statutory post-mortem diagnostic**
 561 **techniques.**

Test type	n	Comparator	Odds ratio	P-value	AUC	Sens	Spec	PPV	NPV	Prev. (comparator)	Test prev.
ENFER-A	283	Visible lesion	5.88	0.002	0.55	12.80%	97.60%	66.70%	74.60%	28%	5.30%
ENFER-A	285	Confirmed	5.03	0.003	0.55	12.70%	97.20%	60.00%	77.00%	25%	5.26%
ENFER-B	283	Visible lesion	6.63	0.007	0.54	8.97%	98.50%	70.00%	68.40%	28%	3.53%
ENFER-B	285	Confirmed	7.69	0.004	0.54	9.86%	98.60%	70.00%	76.50%	25%	3.51%
IDEXX	284	Visible lesion	5.47	0.001	0.56	14.10%	97.10%	64.70%	74.90%	27%	5.99%
IDEXX	286	Confirmed	4.87	0.002	0.55	14.10%	96.70%	58.80%	77.30%	25%	5.94%
Mean	284		5.93	0.003	0.55	12.09%	97.62%	65.03%	74.78%	26%	4.92%

562

563

564 **Table 3: The relative performance of serological tests against a combination of statutory ante-**
 565 **mortem and post-mortem diagnostic techniques. Positive status animals were positive to SICCT,**
 566 **had a visible lesion (VL) at slaughter and had bacteriologically confirmed infection; negative**
 567 **status animals were negative to SICCT, VL and were not confirmed at slaughter.**

Test type	n	Comparator	Odds ratio	P-value	AUC	Sens	Spec	PPV	NPV	Prev. (comparator)	Test prev.
ENFER-A	187	SICCT + VL + CONFIRM	14.20	0.019	0.54	9.09%	99.30%	80.00%	78.02%	24%	2.67%
ENFER-B	187	SICCT + VL + CONFIRM	10.39	0.045	0.53	6.82%	99.30%	75.00%	77.60%	24%	2.14%
IDEXX	188	SICCT + VL + CONFIRM	7.42	0.006	0.56	13.64%	97.92%	66.67%	78.77%	31%	6.25%
Mean	187		10.67	0.023	0.54	9.85%	98.84%	73.89%	78.13%	26%	3.69%

568

569

570 **Table 4: The relative performance of serological tests against a combination of statutory ante-**
 571 **mortem and post-mortem diagnostic techniques. Positive status animals were positive to SICCT,**
 572 **Interferon-G, had a visible lesion (VL) at slaughter and had bacteriologically confirmed**
 573 **infection; negative status animals were negative to SICCT, IFN γ , VL and were not confirmed at**
 574 **slaughter.**

Test type	n	Comparator	Odds ratio	P-value	AUC*	Sens	Spec	PPV	NPV	Prev. (comparator)	Test prev.
ENFER-A	68	SICCT + IFN γ + VL + CONFIRM	NA	NA	0.55	10.00%	100.00%	100.00%	43.75%	59%	5.88%
ENFER-B	68	SICCT + IFN γ + VL + CONFIRM	NA	NA	0.54	7.50%	100.00%	100.00%	43.08%	59%	4.41%
IDEXX	68	SICCT + IFN γ + VL + CONFIRM	NA	NA	0.58	15.00%	100.00%	100.00%	45.16%	59%	8.82%
Mean	68				0.55	10.83%	100.00%	100.00%	44.00%	59%	6.37%

575

576

577 **Table 5: Tabulation of the relationship between serological test result, gamma interferon (IFN γ)**
 578 **status and skin test status. Numbers italicised represent ante-mortem negative animals that**
 579 **were serologically test positive.**

		IFNγ-	IFNγ+		IFNγ-	IFNγ+		IFNγ-	IFNγ+
SICCT-	Enfer-A-	505	272	Enfer-B-	511	279	IDEXX-	497	274
	Enfer-A+	8	13	Enfer-B+	2	6	IDEXX+	17	12
SICCT+	Enfer-A-	39	73	Enfer-B-	41	75	IDEXX-	40	70
	Enfer-A+	3	6	Enfer-B+	1	4	IDEXX+	2	9

580

581 **Table 6: Proportion of confirmed infected animals with positive serological test results, which were**
582 **missed by SICCT, IFN γ , or both ante mortem bovine TB tests.**

Confirmed infection	ENFER- A	ENFER- B	IDEXX
SICCT- (n)	3/19	2/19	3/19
(% serology positive)	15.79%	10.53%	15.79%
IFNγ -	0/14	0/14	0/14
(% serology positive)	0%	0%	0%
SICCT or IFNγ -	0/6	0/6	0/6
(% serology positive)	0%	0%	0%

583

584

585 **Table 7. Distribution of S/P ratios for 670 samples tested for antibodies using the IDEXX**
586 **ELISA for *M. bovis* in one chronically infected case-study farm in Northern Ireland.**

S/P \geq 0.3	S/P < 0.3 to 0.1	< 0.1 to 0.0	< 0.0 to -0.094
Positive	Negative	Negative	Negative
7	17	55	591

587

588

589

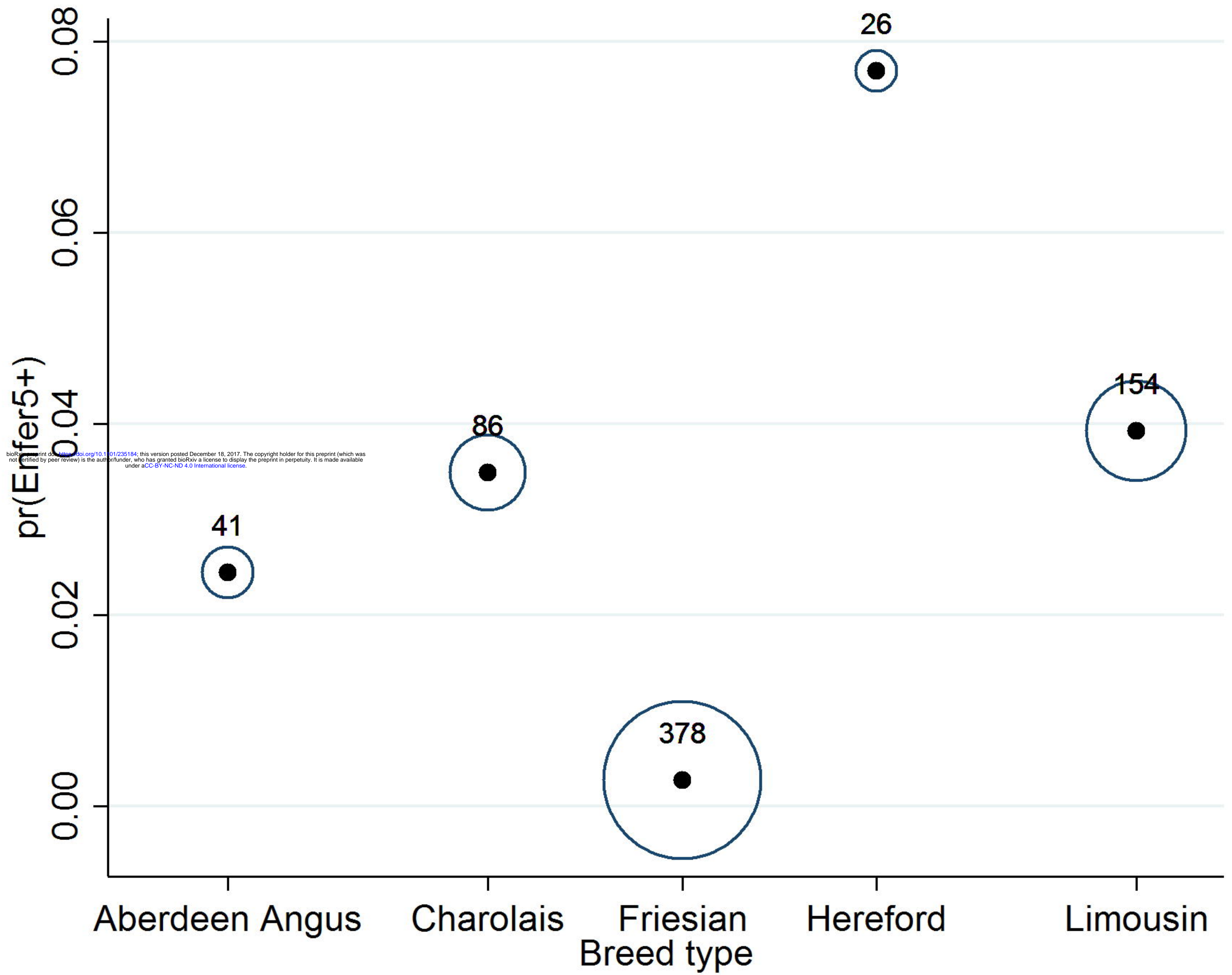
590

591

592 **Figure 1:** The predicted probability of an animal testing positive to the Enfer-B serological test
593 depending on breed type. The size of the circles represents the weighted sample size for each breed
594 type modelled; numbers represent the number of observations for each breed type.

595

596



bioRxiv preprint doi: <https://doi.org/10.1101/235184>; this version posted December 18, 2017. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.