

1 Experimental *Fasciola hepatica* infection alters responses to tests used for diagnosis of  
2 bovine tuberculosis

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10 Running Title: *Fasciola hepatica* and diagnosis of bovine tuberculosis

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Abstract

*Fasciola hepatica* is a prevalent helminth parasite of livestock. Infection results in polarization of the host's immune response and generation of type 2 helper (Th2) immune responses are generated, which are known to be inhibitory to Th1 responses. Bovine tuberculosis (BTB) is a bacterial disease of economic and zoonotic importance. Control policies for this disease rely on extensive annual testing and a "test and slaughter" policy. The correct diagnosis of BTB relies on cell-mediated immune responses. We established a model of co-infection of *F. hepatica* and *Mycobacterium bovis* BCG to examine the impact of helminth infection on correct diagnosis. We found the predictive capacity of tests to be compromised in co-infected animals, and that *F. hepatica* infection altered macrophage function. IL - 4 and IFN -  $\gamma$  expression in whole blood lymphocytes re-stimulated *in vitro* with *M. bovis* antigen were also altered in co-infected animals. These results raise the question of whether *F. hepatica* infection can affect the predictive capacity of tests for diagnosis of BTB and possibly, also influence susceptibility to BTB and other bacterial diseases. Further studies on the interplay between helminth infection and BTB are warranted.

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## 50 1. Introduction

51 The helminth parasite *Fasciola hepatica* is the causative agent of fasciolosis, a  
52 prevalent disease of livestock in temperate regions. Fasciolosis causes losses to agri-  
53 business estimated at around US\$2 billion per year (32). The parasite has a complex  
54 lifecycle utilising the mud snail, *Galba truncatula*, as an intermediate host, while sheep  
55 and cattle, as well as various wildlife species, serve as the definitive hosts. At present,  
56 chemotherapy is the only treatment available (24), however much effort is being focused  
57 on the production of a recombinant vaccine (12). The prevalence of fasciolosis is  
58 increasing, due in part perhaps to climatic changes (30), and a recent abattoir study  
59 indicates that 65% of cull cows in Ireland are infected (26),

60 The immune response to *F. hepatica* is skewed towards type 2 helper (Th2)  
61 dominance, characterised by interleukin (IL) - 4 production, eosinophilia, and a specific  
62 IgG1 response with little or no specific IgG2 (5, 8, 25). Studies on the response in cattle  
63 suggest that animals experience a downregulation of Th1 responses, including interferon  
64 gamma (IFN -  $\gamma$ ) production and lymphocyte responsiveness by week four of infection (8,  
65 9). These results are similar to those emerging from studies carried out using a murine  
66 model. Here, a complete downregulation of Th1 responses was seen to occur with an  
67 upregulation of the Th2 cytokines IL - 4 and IL - 5, with the magnitude of the effect  
68 dependent on the parasite burden (27). In murine models of co-infection, *F. hepatica*  
69 delays bacterial clearance and inhibits bacterial specific IFN -  $\gamma$  production (4). These

70 results suggest that *F. hepatica*, like many other helminths, modulates the host's immune  
71 system in order to evade detection, damage, or ultimately expulsion (22). A study of *F.*  
72 *hepatica* infection in the murine model has also revealed the presence of alternatively-  
73 activated macrophages in infected animals (14). These macrophages were seen to  
74 produce increased levels of IL - 10 and TGF -  $\beta$ , both of which are regulatory cytokines,  
75 have anti-inflammatory properties and could be responsible for reduced lymphocyte  
76 proliferation. Alternatively-activated macrophages also have reduced microbicidal  
77 properties and could result in poor innate defence against bacterial infection (19). These  
78 immunomodulatory effects may also be responsible for increased susceptibility to  
79 secondary bacterial diseases. De-worming of a human study group has been shown to  
80 lead to enhanced cellular responses to *Mycobacterium tuberculosis* antigens (17). In  
81 another study, *Schistosoma mansoni* and hepatitis C virus (HCV) co-infection revealed  
82 that cellular immunity and cytokine production directed at HCV was inhibited (18).  
83 Vaccination against experimental *M. tuberculosis* infection in mice using BCG was found  
84 to be less effective in cases where co-infection occurred with *S. mansoni* (15)

85 Bovine tuberculosis (BTB), caused by *M. bovis*, is one of the most intractable  
86 diseases of livestock (13). Eradication schemes for BTB in the UK and Ireland rely on a  
87 "test and slaughter" policy that uses the single comparative intradermal tuberculin test  
88 (SCITT). This test detects animals that specifically react to intradermal inoculation of a  
89 specific antigen - purified protein derivative B (PPD - B) - derived from *M. bovis* with a  
90 response greater than that directed against its equivalent, PPD - A, derived from *M.*  
91 *avium*. The development of a reaction to the SCITT test relies on the animal forming a  
92 delayed-type hypersensitivity (DTH) response to PPD - B (23). Because of issues with

93 low sensitivity in some situations, in Ireland, a second-line test, the whole blood IFN -  $\gamma$   
94 assay was introduced, to be used in problem areas. This test relies on measuring the  
95 differences in IFN -  $\gamma$  production *in vitro* in response to PPD - B and PPD - A (20). The  
96 influence of other intercurrent infections on the performance characteristics of these tests  
97 is largely unknown. One previous study has demonstrated that acute bovine viral  
98 diarrhoea virus infection could generate false negative responses to both tests in BTB -  
99 infected calves (6). The possible influence of *F. hepatica* or other common helminths on  
100 the outcome of the SCITT or the whole blood IFN -  $\gamma$  assay is relevant to eradication and  
101 testing policies for BTB and also to the wider question of co-infection.

102 The objective of this study was to examine the influence of *F. hepatica* infection  
103 on the outcome of the two BTB diagnostic assays routinely used in control schemes. We  
104 also sought to determine if the timing of helminth infection had an influence on the  
105 response to tests and to outline possible mechanisms that could be responsible for altered  
106 responses in co-infected animals. We established a co-infection model using *F. hepatica*  
107 and *M. bovis* BCG, an avirulent strain of *M. bovis* that is commonly used for vaccination  
108 in man. Vaccination with *M. bovis* BCG is known to induce a positive reaction on BTB  
109 diagnosis in cattle (34). Hence, it is useful model to use when examining the effect of co-  
110 infection on the predictive capacity of tests used in the diagnosis of BTB. However, it is  
111 also important to bear in mind when interpreting these results that the model does not  
112 replicate in full the host-pathogen relationship developing in BTB, which involves a  
113 virulent, persistent organism that continues to multiply within the host.

114

115 2. Materials and methods

116

## 117 2.1 Experimental design.

118 Experimental animals were male, castrated Friesian calves aged between 6 and 9  
119 months of age, purchased from herds free of BTB as determined by a negative result on  
120 the annual SCITT carried out as part of the Irish Bovine Tuberculosis Eradication  
121 Scheme. The most recent SCITT on this herd, which included these experimental calves,  
122 was carried out three months prior to the start of the experiment. The calves were free  
123 from exposure to *F. hepatica* infection as determined by history, serum antibody levels,  
124 and faecal examination. They were group housed in slatted pens with good ventilation  
125 and fed good quality grass silage *ab libitum*. Animals were randomly assigned to one of  
126 four treatment groups as shown in Table 1. At week 0, animals in Group 1 and 2 were  
127 infected with 400 *F. hepatica* metacercariae, (obtained from Dr. G. Coles, Dept of  
128 Veterinary Clinical Sciences, University of Bristol), contained within a 5% gelatin bolus,  
129 as previously described (7). Animals in Groups 3 and 4 were inoculated subcutaneously  
130 in the right shoulder with  $10^6$  -  $10^7$  colony forming units (CFU), of *M. bovis* BCG,  
131 Danish strain 1331 (Statens Serum Institute, Denmark). Four weeks after the initial  
132 infection animals in Group 2 were inoculated with BCG as above, while animals in  
133 Group 3 were infected with *F. hepatica*. Animals were necropsied 23 weeks following  
134 initial infection or inoculation. All experiments were approved by the University ethics  
135 committee and were performed under Licence from the Irish Department of Health and  
136 Children.

137

## 138 2.2 Antigens.

139 Excretory - Secretory (ES) products were produced as previously described (14).  
140 To prepare endotoxin-free ES, phase separation was employed (1). Briefly proteins,  
141 adjusted to 1mg/ml in sterile endotoxin free PBS, were vortexed with 5% Triton X - 114,  
142 and incubated on ice for 5 min, then at 37°C for 5 min. Following the last incubation  
143 solutions were centrifuged at 5000 x g for 7s at 37°C. Following centrifugation, the upper  
144 phase of the solution, containing the endotoxin-free proteins, was collected. Protein  
145 concentration was quantified using the BCA assay (Pierce) using bovine serum albumin  
146 (BSA) as a standard. Proteins were determined to be endotoxin-free by testing with  
147 Cambrex QCL-1000 Chromogenic LAL Endpoint Assay as per manufacturer's  
148 instructions. Only ES found to contain no endotoxin was used for macrophage  
149 stimulation.

### 151 2.3 ELISA for *F. hepatica* specific antibodies

152 Blood was collected by venepuncture of the coccygeal vein and allowed to clot.  
153 Serum was removed and stored at -20°C prior to analysis. Specific antibodies to *F.*  
154 *hepatica* were measured by ELISA using recombinant *F. hepatica* cathepsin L1 (rFhCL1)  
155 as antigen. This is a recombinant version of a molecule secreted by the parasite, shown  
156 to be useful in detecting animals exposed to *F. hepatica* infection (10). Briefly, 96-well  
157 plates (Sartstedt) were coated overnight at 36°C with 1µg/ml of antigen in carbonate  
158 /bicarbonate coating buffer. Plates were washed in phosphate-buffered saline (PBS)  
159 containing 0.05% Tween 20 (PBST), and blocked with 1% BSA-PBS. Samples diluted  
160 1/200 in 1% BSA-PBS were loaded onto the plate (100µl/well) with appropriate positive  
161 and negative controls and doubling serial dilutions performed. Following incubation, (30

162 min at 37°C) plates were washed 3 times in PBST and monoclonal mouse anti - bovine  
163 IgG1 (1/4000) (Cedi Diagnostics) was added. Following incubation and washing,  
164 polyclonal rabbit anti - mouse IgG:HRP (1/2000) (Dako) was added and incubated as  
165 above. Following a final wash, 100µl of TMB (Sigma-Aldrich) was added and the  
166 reaction stopped after 10 minutes by addition of 100µl of H<sub>2</sub>SO<sub>4</sub>. Plates were read at a  
167 wavelength of 450nm, on a Dynatec 4500 plate reader and results were expressed as log<sub>10</sub>  
168 antibody titers.

169

#### 170 2.4 BTB Diagnostic Tests.

171 Two tests used in the diagnosis of BTB were employed in this experiment, the  
172 SCITT and the whole blood IFN - γ assay. The SCITT was carried out and interpreted  
173 using standard procedures as outlined in EU directive 80/219 EU, by a qualified  
174 veterinary surgeon who was unaware of the treatment groups to which animals belonged.  
175 Hair was clipped from a site over the neck region. Using McClintock syringes, avian  
176 tuberculin (0.5mg/ml, Leystad) was injected intradermally at one point on the neck and  
177 bovine tuberculin (1mg/ml, Leystad) at a distinct site directly below. Skin thickness (mm)  
178 at both sites was measured before injection and again 72 hours later. For the whole blood  
179 IFN - γ assay, heparinised blood was collected and divided into 1.5ml aliquots in 12-well  
180 tissue culture plates. These aliquots were stimulated in duplicate with PPD - A and PPD -  
181 B both at 20µg/ml at 37°C, 5% CO<sub>2</sub>. Twenty-four hours later plasma was collected and  
182 tested for the presence of IFN - γ using the Bovigam enzyme immunoassay (CSIRO) as  
183 per manufacturer's instructions. Results were interpreted as previously described (20).  
184 Further aliquots of the supernatant (plasma) from this assay were used for IL-4



185 measurement, as described below.

186

## 187 2.5 Macrophage isolation and phenotyping

188 Peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood  
189 taken 14 weeks post BCG-inoculation by centrifugation over Ficoll-Histopaque (Sigma).  
190 Monocytes were then further isolated from PBMC using anti-CD14 microbeads (Miltenyi  
191 Biotec) on a magnetic separation column (21). Adherent cells were incubated for 24 or  
192 48 hours in tissue-culture flasks at 37°C, 5% CO<sub>2</sub>, in D-MEM medium (Invitrogen)  
193 containing 10% FCS (Sigma), 200U/ml penicillin (Sigma) and 200µg/ml streptomycin  
194 (Sigma), after which they were tested for nitric oxide (NO) production and intracellular  
195 arginase levels. To generate macrophages from naïve monocytes, blood was collected in  
196 heparinised tubes and PBMC and CD14+ cells isolated as above. Adherent cells were  
197 cultured for 10 days, with media changes every 2 days until mature macrophages had  
198 formed. Cells were collected in Trypsin/EDTA without Mg<sup>+2</sup> Ca<sup>+2</sup> and plated at a density  
199 of 10<sup>5</sup> cells per well for stimulation. Cell lysates and supernatants were then examined as  
200 detailed below.

201

## 202 2.6 Nitric Oxide measurement.

203 Cell culture supernatants were tested in duplicate for NO using the Griess reagent  
204 system (Promega). Briefly, 50µl of supernatant was added to wells of a 96-well  
205 microtiter plate (Sarstedt). To this, 100µl of sulfanamide solution was added and the  
206 plate was incubated for 10 minutes in the dark at room temperature. Following this, 100µl  
207 of N - 1 - naphylethylenediamine dihydrochloride (NED) solution was added and the

208 plate incubated as before. Readings were taken at 570nm and NO concentration was  
209 determined by comparison with a standard curve using serial dilutions of a 100µM  
210 solution of nitrate.

211

## 212 2.7 Arginase activity.

213 Cell lysates were prepared by addition of 400µl of 1% Triton X-100 (Sigma), and  
214 incubation on a rocking platform for 40 minutes. Lysate (50µl) was added to 50µl of  
215 Tris-HCl buffer, pH 7.5, and incubated at 55°C for 10 minutes to allow for enzyme  
216 activation. Following this, 25µl of the activated lysate was added to 25µl of arginine  
217 substrate at a concentration of 0.5M (pH 9.7). This mixture was incubated at 37°C for 1  
218 hour. The reaction was stopped by addition of 400µl of acid stop solution, comprising  
219 H<sub>2</sub>SO<sub>4</sub> (96%), H<sub>3</sub>PO<sub>4</sub> (85%), and H<sub>2</sub>O in a ratio of 1:3:7. Colour was developed by adding  
220 25µl of 9% isonitrosopriophenone (Sigma) and heating to 100°C for 45 minutes (10). A  
221 1:20 dilution of beef liver homogenate was used as a positive control.

222

## 223 2.8 IL - 4 detection.

224 IL - 4 produced in response to PPD - B, in plasma derived from the IFN - γ whole  
225 blood assays, was measured using a commercial ELISA (Endogen, USA) as per  
226 manufacturer's instructions. Briefly, recombinant IL - 4 capture antibody was coated onto  
227 96 well plates overnight at 4°C. Samples were added in duplicate in 100µl volumes and  
228 incubated for 1.5 hours. IL - 4 detection antibody was added and incubated for a further  
229 hour. Streptavidin:HRP conjugate was added to the plate for 30 minutes, after which  
230 colour was developed by addition of substrate. The reaction was stopped and the plate

231 was read at 450nm with reference wavelength of 550nm. Results were quantified using a  
232 standard curve prepared from recombinant bovine IL - 4 and reported as pg/ml.

233

## 234 2.9 Statistical Analysis

235 SCITT and IFN -  $\gamma$  test results were expressed as percentages of animals in each  
236 group testing positive. Significant differences between groups were determined using the  
237 Chi - Squared test, as appropriate for categorical data. Measurement of arginase activity,  
238 IL - 4 levels, and antibody titers produced normal data, and differences between groups  
239 were accordingly tested using a one-tailed Students T - Test. All statistical analysis was  
240 carried out using MiniTab (Microsoft Inc.).

241

## 242 3. Results

243

### 244 3.1 *F. hepatica* burdens and specific immune response

245 Analysis of the serum antibody response of the 3 groups infected with *F. hepatica*  
246 showed that humoral immune responses specific for *F. hepatica* were unaffected by BCG  
247 immunisation in the two groups of co-infected animals (Figure 1). All infected groups  
248 seroconverted and continued to produce increasing levels of parasite-specific IgG1  
249 throughout infection. There were no significant differences between mean IgG1 titers  
250 amongst groups. Parasite-specific serum IgG2 did not increase above background during  
251 the course of the experiment (data not shown). Livers were collected post-mortem and  
252 examined to detect parasites present. The average parasite burden (+/- S.E.M.) in Groups  
253 1, 2, and 3 were  $26\pm 4$ ,  $15\pm 3$ , and  $8\pm 5$  respectively. These burdens were not statistically

254 different.

255

256 3.2 BTB test results

257 Thirteen weeks following immunisation with BCG, all animals were tested using  
258 the SCITT, and on the same day blood was collected for use in the whole blood IFN -  $\gamma$   
259 assay. The results of both tests are shown in Table 2. Group 3 contained one animal that  
260 responded to the IFN -  $\gamma$  assay, while the same animal was also a reactor on the SCITT  
261 along with a second animal from this group. No animals from Group 2 were classed as  
262 responders on either the IFN -  $\gamma$  assay or the SCITT. Group 2 animals had produced a  
263 patent infection at the time of BTB diagnosis (faecal egg positive- data not shown), while  
264 animals in Group 3 had a pre-patent infection at time of testing

265

266 3.3 Macrophage Phenotyping.

267 We sought to characterise the activation status (classically or alternatively  
268 activated) of blood-monocyte derived macrophages from animals in each treatment group  
269 We isolated CD14-positive cells using a microbead system from peripheral blood  
270 mononuclear cells of animals in each treatment group, cultured adherent cells *in vitro*,  
271 and measured their production of NO and arginase (Figure 2a). We found only trace  
272 amounts of NO, (data not shown) while we found selective upregulation of arginase  
273 production in the macrophages from animals infected with *F. hepatica*. These cells all  
274 showed significantly greater levels of arginase than those taken from animals which were  
275 inoculated with BCG only or from naïve control animals. We also examined the effect of  
276 *in vitro* stimulation with PPD-B or *F. hepatica* ES on blood monocyte-derived

277 macrophages from uninfected animals. (two calves obtained from the same source as the  
278 infected groups, and within the same age range). Mature macrophages were stimulated  
279 with PPD - B or *F. hepatica* Excretory-Secretory (ES) products. Following incubation,  
280 arginase and nitric oxide levels were measured and confirmed findings that ES induced  
281 alternatively activated macrophages, while PPD - B stimulation favoured NO generation  
282 indicative of classically-activated macrophages (Figure 2b & 2c).

### 283 3.4 Cytokine production in whole blood assays.

284 Levels of IL-4 and IFN- $\gamma$  produced following incubation of whole blood with  
285 PPD-B or *F. hepatica* ES are shown in Figures 3, 4 and 5 for Groups 2,3 and 4,  
286 respectively. We found that Group 4 animals produced a peak response of IL - 4 at week  
287 5. However Group 3 animals displayed a different pattern of IL - 4 expression. These  
288 animals produced a peak IL - 4 response at week 13 while levels were depressed at week  
289 5 (Figure 3). Group 2 animals in comparison to Group 4 animals had strong IL - 4  
290 responses directed at PPD - B one week following immunisation. This group also showed  
291 elevated IL - 4 responses at time of SCITT, however these levels were not significantly  
292 greater than those found in Group 4.

293 The IFN -  $\gamma$  response of animals to PPD - B in the whole blood assay also showed  
294 that some co-infected animals differed in respect of the kinetics and magnitude of this  
295 response as compared with BCG-infected animals (Figure 4). Animals in Group 4 that  
296 mounted an IFN -  $\gamma$  response, produced levels that were maximal between weeks 3 and 5  
297 post-infection; these animals were reactors on the SCITT. Two animals produced poor

298 responses but one of these was still a reactor on SCITT. In Group 3, one animal  
299 produced a strong and continuous IFN -  $\gamma$  response and was a reactor on SCITT. The  
300 others produced weak IFN -  $\gamma$  responses and failed to become reactors on SCITT. One  
301 animal produced an early IFN -  $\gamma$  response similar to Group 4 animals but this response  
302 collapsed following *F. hepatica* infection, and this animal subsequently failed to react on  
303 SCITT. IL - 4 is known to be inhibitory to IFN -  $\gamma$  production and this relationship was  
304 evident in the co-infected groups. Co-infected animals had peak IL - 4 responses at time  
305 of SCITT where IFN -  $\gamma$  is essential for the production of a positive response. Co-infected  
306 animals whose levels of IL - 4 elevated above those of BCG-immunised animals were  
307 those whose IFN -  $\gamma$  levels were suppressed and consequently responded poorly on  
308 SCITT.

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314 4. Discussion

315 The results presented here indicate that a secondary or concurrent helminth  
316 infection may influence the predictive capacity of tests used for diagnosis of BTB. The

317 finding that, following experimental infection, SCITT detection rates fell from 80% in  
318 Group 4 to 40% in Group 3, to 0% in Group 2 animals has grave implications for a  
319 control policy that relies on “test and slaughter”. There is also evidence for a potential  
320 effect of parasite burden visible within co-infected groups. Group 3 animals had a mean  
321 parasite burden less than those in Group 2, while this difference did not reach significant  
322 levels ( $P = 0.0662$ ), due to the relatively high variance within groups (commonly seen  
323 following experimental *F. hepatica* infection) it could indicate that higher parasite levels  
324 in Group 3 might have resulted in a further decrease in the number of animals that were  
325 reactors to SCITT and IFN -  $\gamma$  tests. It also raises the possibility that BCG immunisation  
326 had an “adjuvant” or non-specific effect on the ability of the animals to contain *F.*  
327 *hepatica* infection.

328  
329 Group 2 animals were 17 weeks post *F. hepatica* infection while Group 3 animals were 9  
330 weeks post *F. hepatica* infection at time of SCITT. This difference in numbers of reactors  
331 between these two groups of co-infected animals may be due to the difference in the  
332 relative timing of exposure to *F. hepatica* and BCG. This may indicate that the duration  
333 of helminth infection or the presence of helminth infection prior to BCG immunisation  
334 may have played some role in the influence on response to BTB diagnostic tests.

335  
336 Here we have shown that infection with a common helminth parasite can have a  
337 major effect on immune responses relevant to diagnosis of BTB. Positive responses in the  
338 SCITT and whole blood IFN -  $\gamma$  of BTB infected animals require sufficient cellular  
339 immune responses directed towards PPD - B (29). In general, dominance of humoral over

340 cellular immunity in cattle infected with BTB is correlated with a more progressive form  
341 of the disease (35). Even if the effects of natural infection with *F. hepatica* on the  
342 response to diagnostic tests for BTB are much less than those seen here following  
343 experimental *F. hepatica* infection with BCG immunisation, the potential implications for  
344 disease control programmes are grave. Only one previous study (6) has examined the  
345 effect of an intercurrent infection on the performance of SCITT and IFN -  $\gamma$  tests. Acute  
346 infection with bovine viral diarrhoea virus resulted in temporary suppression of  
347 lymphocyte and IFN -  $\gamma$  responses.

348 The ability of helminths to modulate their host's immune system has been  
349 extensively reviewed (22). A number of studies detailing the effects of co-infection with  
350 helminths highlight the detrimental effect this modulation can have on immune responses  
351 directed at a secondary or concurrent pathogen. In a murine model of *F. hepatica* and  
352 *Bordetella pertussis* infection, cellular immunity directed towards *B. pertussis* was  
353 abrogated resulting in delayed bacterial clearance of bacteria and poor *B. pertussis*  
354 specific IFN -  $\gamma$  production (4). This effect could also be produced by injection of the *F.*  
355 *hepatica* protease cathepsin L1 (28). This effect was also seen to be dependent on IL - 4,  
356 as IL - 4 knockout mice did not suffer the same effects when cathepsin L1 was  
357 administered (28). In our study we found IL - 4 levels to be elevated in co-infected  
358 animals, corresponding to downregulated IFN -  $\gamma$  production and in negative responses on  
359 SCITT. This suggests that IL - 4 plays a role in helminth immunomodulation in this  
360 model. *S. mansoni* infection is known to increase susceptibility to BCG infection in mice  
361 (16), with IFN -  $\gamma$  production in response to PPD - B downregulated in co-infected mice,  
362 while the Th2 cytokines IL - 4 & 5 were found to be unchanged in co-infected animals in



363 response to stimulation by *S. mansoni* antigen. Co-infected animals were also found to  
364 have higher CFUs of BCG in their lungs, liver, and spleen at three different time points  
365 post infection, when compared to singly infected animals. A study conducted in cattle  
366 infected with *F. hepatica* found that co-infection with *F. hepatica* and *Salmonella dublin*  
367 resulted in increased excretion of *S. dublin* and higher mortalities (2), although no  
368 specific mechanisms were identified. In summary, suppression of Th1 responses is  
369 known to occur in a variety of helminth infections. This is likely to be one of the factors  
370 resulting in increased susceptibility and to bacterial infections, and in altered performance  
371 of diagnostic tests.

372 The macrophage plays a vital role in the outcome of mycobacterial infection,  
373 acting as a host cell and providing anti - mycobacterial defenses. The effective  
374 recruitment of macrophages is needed for the induction of a DTH response, which is vital  
375 in the SCITT. Macrophage activation can follow two pathways, classical or alternative,  
376 depending on the metabolism of L - arginine *via* inducible nitric oxide synthase (iNOS)  
377 or arginase, respectively (19). Phenotyping of macrophages can therefore be carried out  
378 by monitoring the production of NO and the levels of arginase activity. Alternatively-  
379 activated macrophages are typically associated with helminth infections and have been  
380 highlighted as a possible cause of cellular anergy (33). In some cases this has been linked  
381 to the direct upregulation of programmed death ligand 2, (PD - L2), on the macrophage  
382 surface (33). PD - L2 has been shown to be responsible for decreasing cellular  
383 responsiveness by direct contact with T-cells. In this study we examined the possible role  
384 of macrophages in co-infection and found that animals infected with *F. hepatica* and  
385 those co-infected with *F. hepatica* and BCG produced macrophages which were

386 alternatively -activated. It has been shown that helminth-induced alternatively-activated  
387 macrophages have reduced IFN -  $\gamma$  and NO output (14). During the DTH response, Th1  
388 cells are responsible for recruiting and activating macrophages via IFN -  $\gamma$  secretion. We  
389 have shown in this study that cellular immune responses relevant to BTB diagnosis are  
390 compromised in animals following experimental *F. hepatica* infection. This lack of Th1  
391 effector mechanisms and presence of alternatively-activated macrophages may be  
392 responsible for the poor SCITT responses elicited from co-infected animals. The “cause  
393 and effect” relationship between alternatively-activated macrophages and failing Th1  
394 effector mechanisms is still to some extent unclear.

395 Our data demonstrates the continuing presence of IL - 4, a typical Th2 cytokine,  
396 at the time of SCITT in co-infected animals. Previous studies have shown that peak IL - 4  
397 expression in BTB occurs between weeks 5 -8 post-infection, with the response fading to  
398 background levels as infection proceeds (31). This is consistent with the results from  
399 Group 4 animals in our study, with IL - 4 production peaking at week 5, and levels  
400 declining to the week of SCITT. Levels of IL - 4 in Groups 2 and 3, however, indicate  
401 that cytokine expression patterns deviate from the norm in co-infected animals. In Group  
402 3, IL - 4 levels were significantly reduced at week 5 while being significantly higher  
403 when compared to Group 4 at time of SCITT. In Group 2, levels were also higher at time  
404 of SCITT but not significantly so. This suggests that a Th2 response was ongoing and  
405 being specifically directed at PPD - B.

406 The differences in IFN -  $\gamma$  responses seen between BCG-only and co-infected  
407 groups may also have been responsible for the poor SCITT test results. It appears that *F.*  
408 *hepatica* infection can cause the antigen specific IFN -  $\gamma$  response to shut down.

409 Notably, animal 3.3, the only one positive on both SCITT and  $\gamma$ -INF tests from either co-  
410 infected groups, was also the only one not to have a detectable IL-4 response and whose  
411  $\gamma$ -INF response remained elevated 13 weeks post-BTB immunization. The measurement  
412 of additional cytokines, such as IL-4, in helminth-infected animals in response to PPD-B  
413 restimulation of lymphocytes *in vitro*, may offer additional diagnostic potential.

414 The experiments described here deal with the possible influence of diagnosis of  
415 BTB in animals with *F. hepatica* infection. This experimental model, using *M. bovis*  
416 BCG, while useful, fails to take into consideration the complex interplay that may take  
417 place in active BTB where bacterial multiplication will have greater influence due to  
418 prolonged exposure to mycobacterial antigen. In view of the continuing spread of BTB,  
419 uncertainty in relation to transmission dynamics, and the impact of proposed control  
420 measures on wildlife populations, we consider it important to carry out further  
421 experimental and epidemiological studies on the effect of helminth parasitism on the  
422 diagnosis and control of this disease including a co-infection study involving an active  
423 BTB infection. Conclusions from this study may also have wider implications in the  
424 context of human health, where multiple co-infections are common and vaccination is  
425 seen as the most sensible route towards eradication of such diseases (3).

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574

## 575 **Figure Legends**

### 576 **Figure 1.**

577 *F. hepatica* specific IgG1 responses (+/- S.E.M.) in Groups 1, 2, 3, and 4 All animals in  
578 Group 1, 2, and 3 seroconverted while Group 4 did not. No significant difference in titers  
579 between Groups 1, 2, and 3 were found. Parasite-specific IgG2 levels did not rise above  
580 background during the course of the experiment.

581

### 582 **Figure 2**

583 (a) Levels of arginase production in blood-monocyte derived macrophages (+/- S.E.M.) ,  
584 24 hours post-isolation, from each group. Measurements were made 14 weeks post BCG-  
585 inoculation and 18 weeks post *F. hepatica* infection, except in the case of Group 3, when  
586 the sampling was 12 weeks post *F. hepatica* infection. Arginase production was  
587 significantly greater ( $P < 0.05$ ) by cells from Group 1, 2 and 3 than Group 4. The latter did  
588 not produce levels significantly greater than those from negative control animals. (b)

589 Arginase production in blood-monocyte derived macrophages from naïve (uninfected)  
590 calves stimulated with PPD - B and *F. hepatica* ES at 10µg/ml. Figure represents mean  
591 (+/- S.E.M.) values from three experiments using cells from two animals. (c) NO  
592 production by macrophages under conditions as described in (b).

593

594 **Figure 3.**

595 IL-4 (a) and  $\gamma$ -INF (b) production by individual animals (*F. hepatica*/BCG co-infected)  
596 animals in Group 2. Cytokine levels were measured in plasma recovered from cultures of  
597 whole blood taken at one week and thirteen weeks post-BCG immunisation and  
598 stimulated *in vitro* with PPD - B. Results are presented as mean of triplicate wells +/-  
599 S.E.M. The response of each animal to SCITT and whole blood  $\gamma$ -INF BTB test results is  
600 also indicated on each panel as follows; N:N negative on both assays, N:P SCITT  
601 negative whole blood positive, P:N SCITT positive whole-blood negative, and P:P  
602 positive on both assays.

603

604 **Figure 4.**

605 IL-4 (a) and  $\gamma$ -INF (b) production by individual animals (BCG/*F. hepatica* co-infected)  
606 animals in Group 3.). IL - 4 was measured in animals at week 1, 5, and 13 post-BCG  
607 immunization and IFN -  $\gamma$  at weeks 1, 3, 5, and 13 post-BCG immunization, as described  
608 for Figure 3. Response to BTB diagnostic tests is also indicated as for Figure 3.

609

610 **Figure 5.**

611 IL-4 (a) and  $\gamma$ -INF (b) production by individual animals (BCG-immunised) animals in

612 Group 4 Sampling times and other conditions were as described for Figure 4.

ACCEPTED

613

614

615

616 **Table 1.**

Treatment Group	Week 0	Week 4	Week 13	Week 17
1	<i>F. hepatica</i>	-	SCITT	-
2	<i>F. hepatica</i>	BCG	-	SCITT
3	BCG	<i>F. hepatica</i>	SCITT	-
4	BCG	-	SCITT	-

617

618 **Table 1. Experimental Design.** Calves were allocated to one of four treatment groups,

619 as shown. In the case of groups 2, 3 and 4 SCITT was carried out 13 weeks post-BCG

620 immunization. In addition to the groups shown above two additional calves, sourced

621 from the same farm as the other animals, and within the same age range, were used as

622 donors of blood monocytes for macrophage preparation and in vitro stimulation with

623 PPD-B and *F. hepatica* ES.

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630 **Table 2:**

631

Group	Treatment	Whole Blood IFN - $\gamma$ Assay	SCITT		
		Positive	Negative	Positive	Negative
1	<i>F. hepatica</i> (n = 4)	-	4*	-	4*

2	<i>F. hepatica</i> /BCG (n = 4)	-	4*	-	4*
3	BCG/ <i>F. hepatica</i> (n = 5)	1	4*	2	3*
4	BCG (n = 5)	4	1	4	1

632  
633

634 **Table 2: SCITT and IFN -  $\gamma$  assay results.**

635

636 Whole blood IFN -  $\gamma$  assay was carried out on the same day as injection of PPD antigens

637 for SCITT. Numbers of animals from each group that was classed as a positive or a

638 reactor was expressed as a percentage for statistical analysis.

639 \* Results were significantly different from those of the BCG-only group as tested by the

640  $\chi^2$  test,  $p \leq 0.05$ .

641

642

643

Figure 1

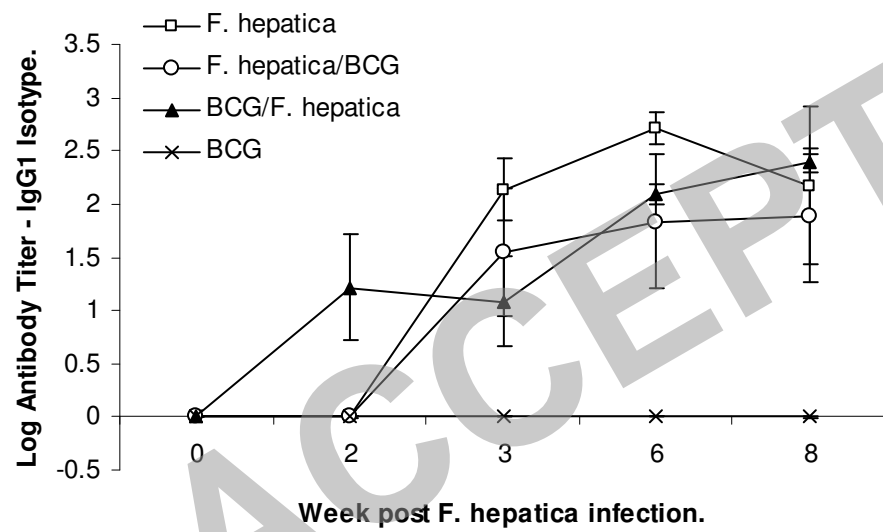
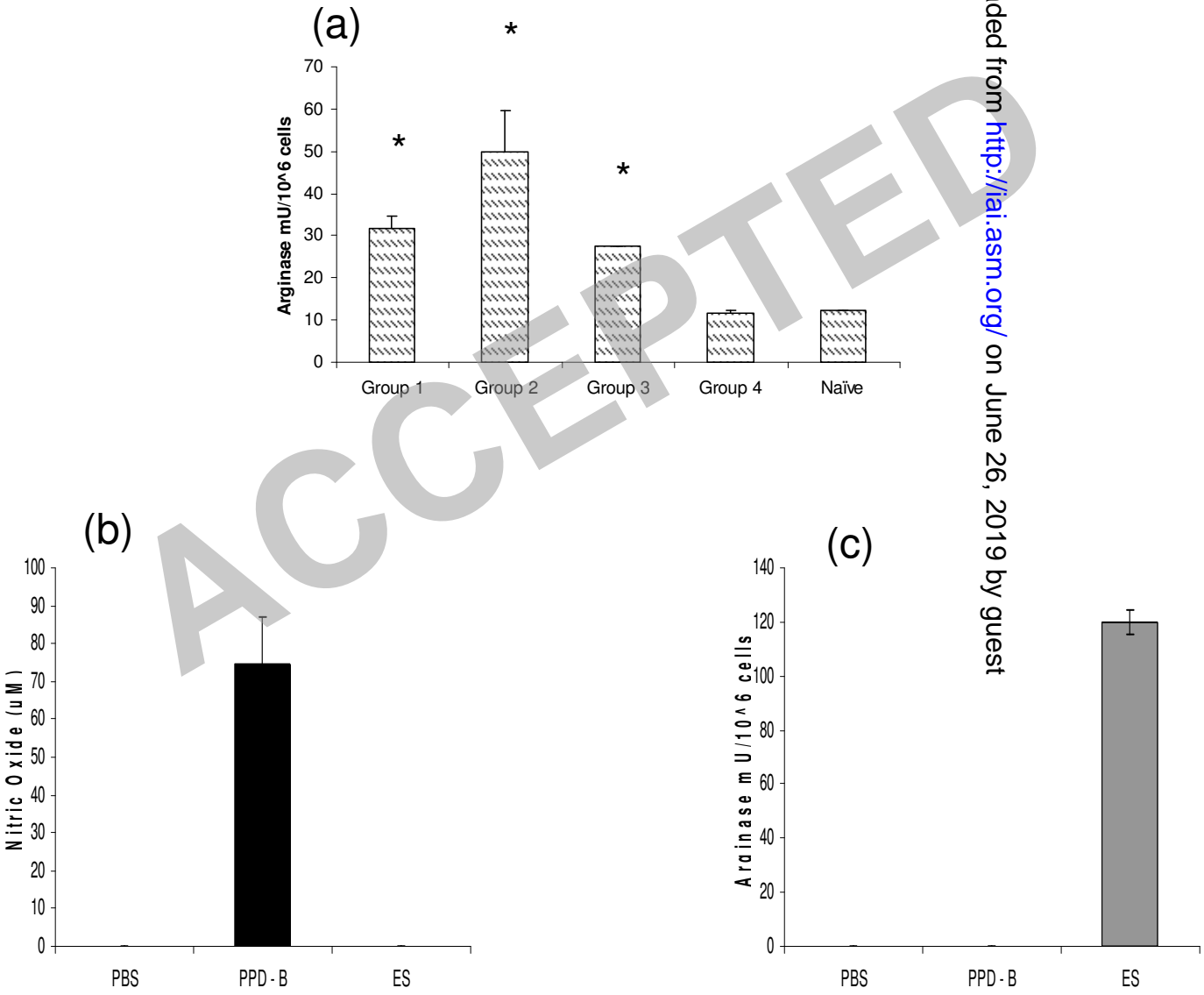


Figure 2

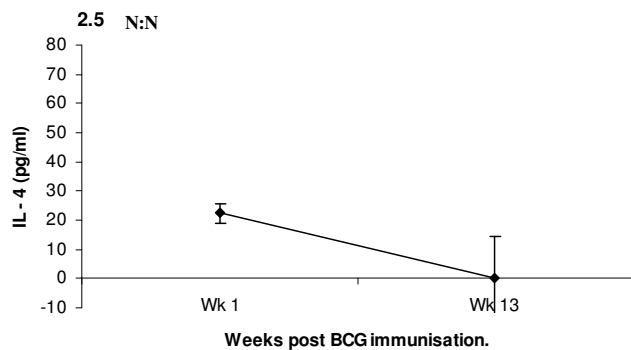
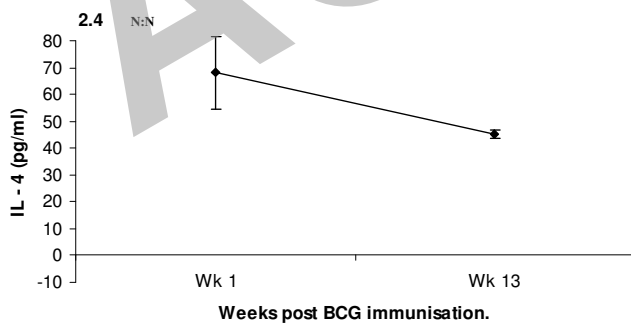
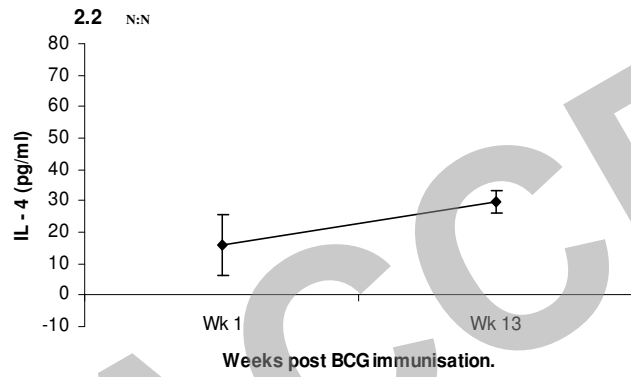
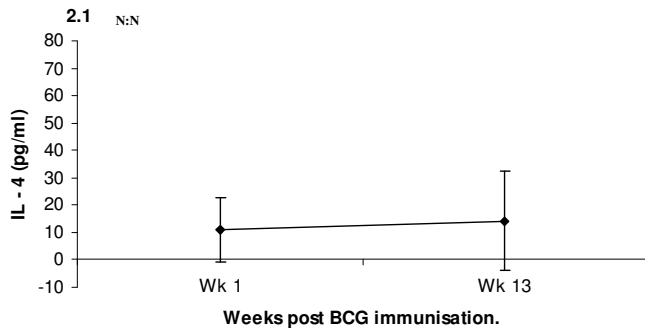


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# Figure 3

(a)



(b)

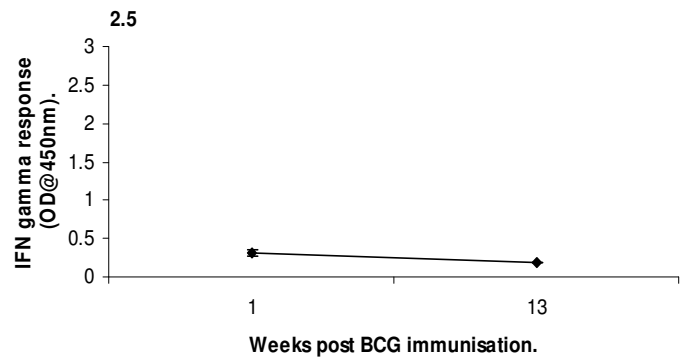
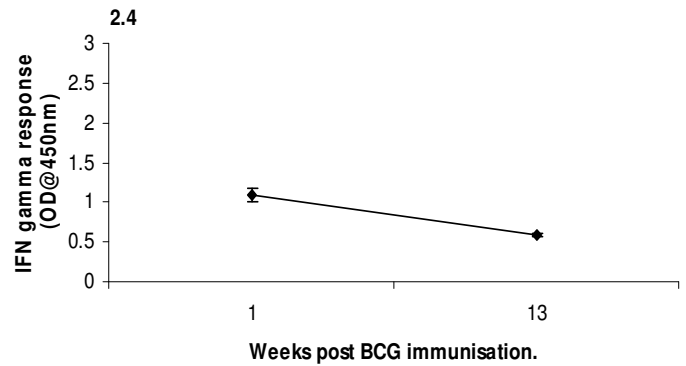
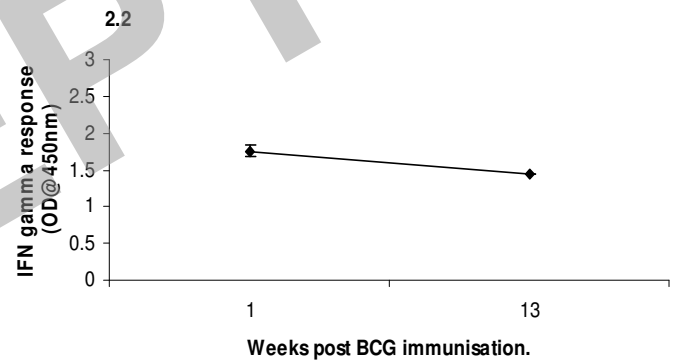
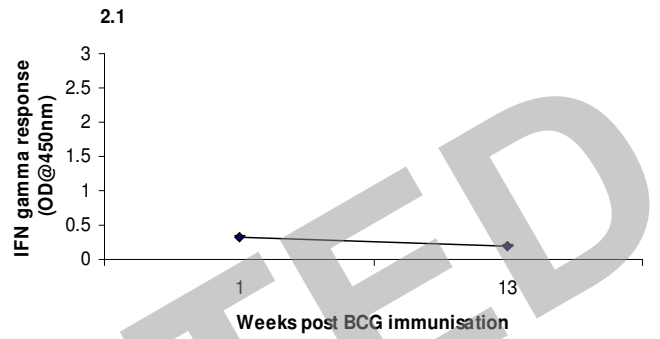
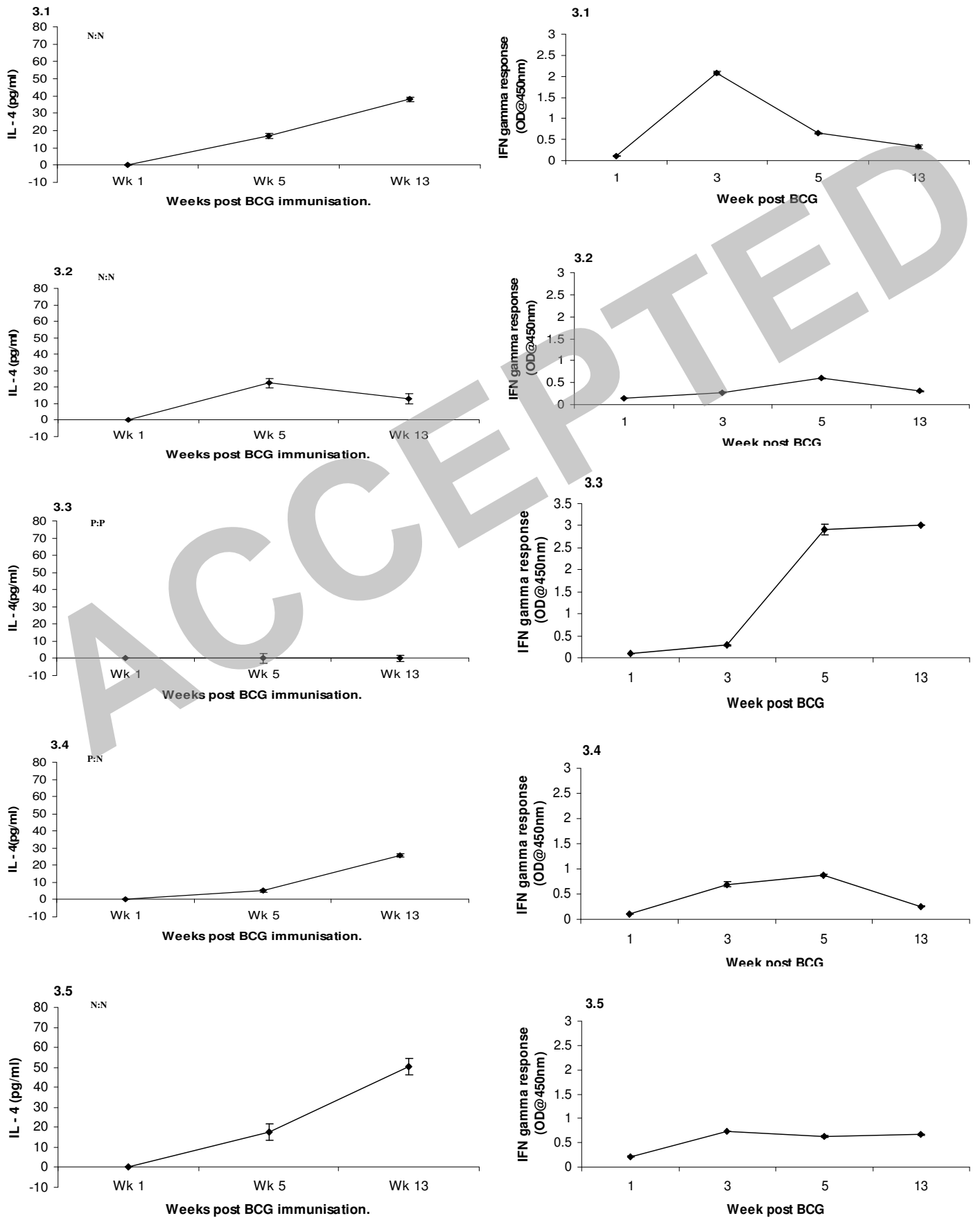
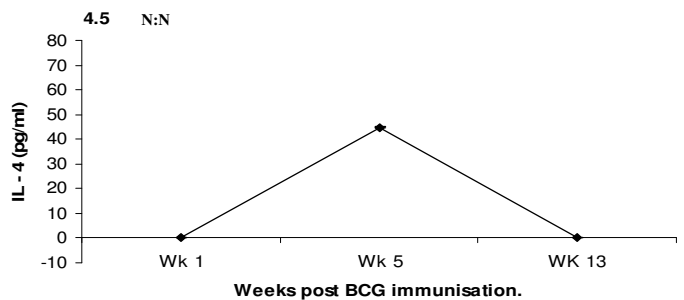
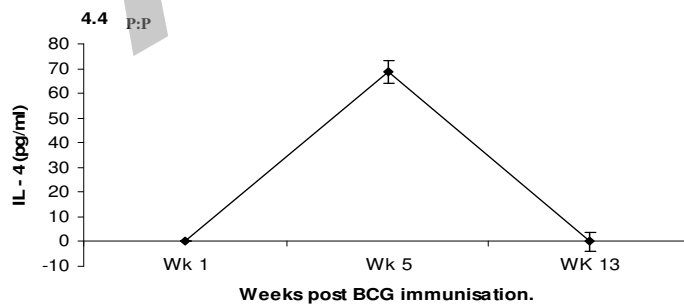
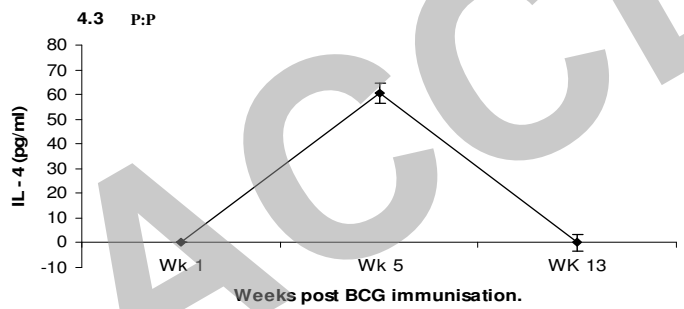
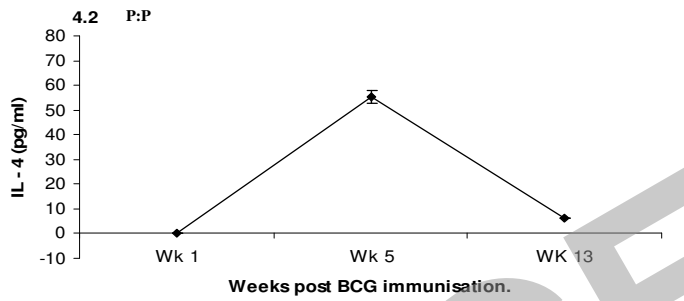
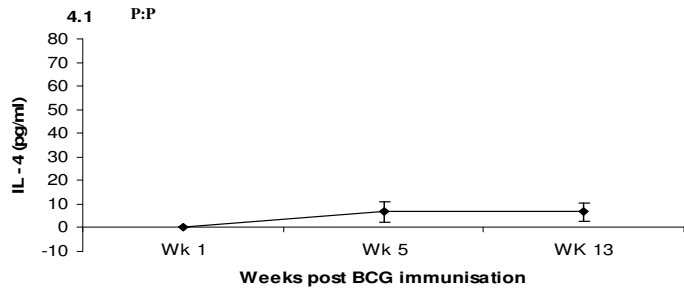


Figure 4:



**Figure 5:**

**(a)**



**(b)**

