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Co-infection with *Fasciola hepatica* may increase the risk of *Escherichia coli* O157 shedding in British cattle destined for the food chain

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Abstract

Escherichia coli O157 is a zoonotic bacterium that can cause haemorrhagic diarrhoea in humans and is of worldwide public health concern. Cattle are considered to be the main reservoir for human infection. *Fasciola hepatica* is a globally important parasite of ruminant

21 livestock that is known to modulate its host’s immune response and affect susceptibility to
22 bacterial pathogens such as *Salmonella* Dublin. Shedding of *E. coli* O157 is triggered by
23 unknown events, but the immune system is thought to play a part. We investigated the
24 hypothesis that shedding of *E. coli* O157 is associated with *F. hepatica* infection in cattle.
25 Three hundred and thirty four cattle destined for the food chain, from 14 British farms,
26 were tested between January and October 2015. *E. coli* O157 was detected by
27 immunomagnetic separation and bacterial load enumerated. *F. hepatica* infection status
28 was assessed by copro-antigen ELISA. A significant association (p = 0.01) was found
29 between the log percent positivity (PP) of the *F. hepatica* copro-antigen ELISA and *E. coli*
30 O157 shedding when the fixed effects of day of sampling and the age of the youngest
31 animal in the group, plus the random effect of farm were adjusted for. The results should
32 be interpreted cautiously due to the lower than predicted level of fluke infection in the
33 animals sampled. Nevertheless these results indicate that control of *F. hepatica* infection
34 may have an impact on the shedding of *E. coli* O157 in cattle destined for the human food
35 chain.

36 **Keywords: Escherichia coli O157, Fasciola hepatica, cattle, co-infection**

37 1 Introduction

38 *Fasciola hepatica,* or the common liver fluke, is a parasite of ruminant livestock, occurring
39 worldwide. Various studies have shown that *F. hepatica* can affect host immunity to other
40 pathogens (Moreau and Chauvin, 2010), by making the host more susceptible to infection
41 (Aitken et al., 1979, 1981; Brady et al., 1999); changing the pathogenesis of disease (Garza-
42 Cuartero et al., 2016); and interfering with diagnostic tests (DEFRA, 2005; Flynn et al.,
43 2007). This happens because infection with *F. hepatica* induces a mixed T helper type-2
44 (Th2) and T-regulatory response, with increased production of IL4, IL5, IL10, IL13 and TGFβ,

45 whilst T helper type-1 (Th1) responses are down regulated (Flynn et al., 2010; Graham-
46 Brown, 2016).

47 *Escherichia coli* O157 is a zoonotic bacterium that occurs worldwide and can cause
48 haemorrhagic diarrhoea in humans as a result of systemic Shiga toxin (Stx) activity. Cattle
49 are considered the main source of human infection, either through direct contact or
50 through contaminated food (Locking et al., 2001; Strachan et al., 2006; Gyles, 2007). An
51 estimated 20-40% of British cattle herds are reported to shed *E. coli* O157 (Paiba et al.,
52 2003; Ellis-Iversen et al., 2007; Gunn et al., 2007; Pearce et al., 2009). The annual reported
53 incidence of human *E. coli* O157 is 1.8 culture positive cases per 100,000 population in
54 England and Wales, and 4.5 cases per 100,000 in Scotland (Health Protection Network,
55 2013; Public Health England, 2013). In a proportion of cases, mainly in young children,
56 haemolytic uraemic syndrome may occur, and is potentially fatal (Chase-Topping et al.,
57 2008).

58 *Escherichia coli* O157 infections in cattle are usually asymptomatic as cattle lack vascular
59 receptors for Stx (Pruimboom-Brees et al., 2000), but both cellular and humoral immune
60 responses are induced and are required for immunity to *E. coli* O157 (Corbishley et al.,
61 2014, 2016). Furthermore, cellular responses to *E. coli* O157 are associated with Th1
62 responses (Corbishley et al., 2014). The relationship between the shedding of *E. coli* and
63 immunity is not fully understood, but shedding has been associated with stressful events
64 that could affect the immune response (Ellis-Iversen et al., 2007; Munns et al., 2015).

65 Recent estimates using bulk milk antibody detection ELISAs based on fluke excretory-
66 secretory antigens show 50 - 80% of UK dairy herds have been exposed to fluke (McCann et
67 al., 2010; Howell et al., 2015). Although the current status for the beef sector is unknown,
68 figures released by the Food Standards Agency report that 16.5% of cattle livers were
69 condemned due to liver fluke during 2015 (Ford and Hadley, 2015). Since liver fluke

infection down-regulates Th1 responses, which are associated with clearance of the bacteria from the bovine gut (Corbishley et al., 2014), we hypothesized that fluke infection could affect the propensity of cattle to shed *E. coli* O157. If so, the presence of co-infected cattle could increase the risk of zoonotic *E. coli* infections.

2 Methods

This study was designed to utilise samples collected for an existing larger study on *E. coli* O157 in cattle intended for human consumption, funded by Food Standards Scotland (FSS) and the Food Standards Agency (FSA; Project FS101055); referred to below as the FSS/FSA study. For the FSS/FSA study, sample size calculations showed that a minimum of 110 Scottish farms and 160 farms from England and Wales were required to estimate a prevalence of *E. coli* O157 of 20% and 35% respectively within a tolerance of 0.168 with 95% confidence (Henry et al., 2017).

A sample size calculation to determine the number of cattle that were required to investigate the association between *F. hepatica* infection and *E. coli* O157 shedding was performed by Hickey et al. (2015) using simulated datasets. The estimated prevalence of *E. coli* O157 was set at 4% of cattle and 20% of farms (Pearce et al., 2009) whilst the estimated prevalence of *F. hepatica* was set at 20% of cattle and 80% of farms (Salimi-Bejestani et al., 2005b; McCann et al., 2010). 100% sensitivity and specificity of both tests were assumed. The result of using these parameters was that the inclusion of 1645 individual samples, from 50 randomly selected farms, would give the study a power of 87% to detect a two-fold increase in the odds that an animal would shed *E. coli* O157 if it was also infected with *F. hepatica,* compared to cattle not infected with fluke.

2.1 Sample and data collection

Two hundred and seventy farms were sampled in the FSS/FSA study (Henry et al., 2017). These included a variety of types of enterprise and breeds of cattle. Of these, 110 were

95 Scottish farms, randomly selected from all Scottish farms that had participated in both of
96 two earlier studies. The inclusion criterion was that there was at least one male aged one
97 year or over, or female over two without calves on the farm, as these farms were most
98 likely to contain animals that would end up in the food chain. In addition, 160 farms for
99 England and Wales were recruited from a randomly selected subset with either a male of
100 any breed aged over 1 year, or a female of a non-dairy breed aged over 1 year. Farmers
101 were initially notified by letter and given the choice to opt out, and were then contacted by
102 phone in a randomised order to enrol them in the study.

103 Farms were visited once between September 2014 and November 2015. Individual fresh
104 faecal pat samples were taken from the floor or ground, for the group of cattle from each
105 farm that contained the animals closest to going off the farm for slaughter. The number of
106 samples collected from each group was determined by a protocol assuming that if 8% of
107 animals were positive, there would be a 0.9 probability of identifying groups containing at
108 least one positive animal (Chase-Topping et al., 2007). It was assumed that a pat sample is
109 equivalent to an animal level unit for analysis. These samples were then sent to the
110 Epidemiology Research Unit (ERU) microbiological facilities at Scotland's Rural College
111 (SRUC), Inverness, within 48 hours of collection, and tested for *E. coli* O157. The
112 recruitment and visits were done by members of SRUC project team in Scotland, and the
113 ADAS project team in England and Wales.

114 Farms for which samples were submitted to SRUC's ERU laboratory on or after 5th January
115 2015, and which consented to further use of their samples and data for research purposes,
116 were included in the study. Delays due to funding and contractual issues meant that
117 samples received prior to this date were not retained.

118 Information on animal characteristics and farm management was collected from the
119 livestock keeper or farm manager on each farm, via a questionnaire administered by the

120 survey staff. The information was collected in an electronic format and was a shortened
121 version of a questionnaire used in a previous study (Chase-Topping et al., 2007). The
122 questionnaire was piloted with several farmers before use. The finalised questionnaire was
123 approved by the FSS Survey Control team (Henry et al., 2017). The questionnaire was
124 conducted in Welsh for Welsh-speaking respondents.

125 The information obtained was at the farm level, for example the age of animals was given
126 as a range for the group, and all animals in a group were treated as having been managed
127 the same in terms of housing, feeding and treatments given. The information relevant for
128 the current study was identified and extracted. As the aim was to develop a model to
129 determine the presence of an association between fluke and *E. coli* O157, rather than a
130 predictive model, only management information relevant to fluke was taken for use in the
131 model, to control for possible confounders which may be linked to both fluke and *E. coli*
132 O157. A summary of these is shown in Table 1.

133 ## 2.2 *E. coli* testing

134 One gram of faeces was added to 20 ml of buffered peptone water (BPW, Thermo
135 Scientific, UK). The BPW was incubated for six hours at 37°C (±1°) then subjected to
136 immunomagnetic separation (IMS). Briefly, a 1 ml aliquot from each 20 ml BPW sample was
137 added to 20 µl paramagnetic beads coated with polyclonal antibody for *E. coli* O157
138 lipopolysaccharide (Lab M Ltd., UK). The aliquots were mixed on a rotary mixer for 30
139 minutes before being washed three times in PBS with 0.05% Tween 20 (PBST, Sigma-Aldrich
140 Co. Ltd.). After the third wash, the beads were re-suspended in 100 µl PBST and cultured
141 onto MacConkey agar containing sorbitol, cefixime (0.05 mg/l) and tellurite (2.5 mg/l) (CT-
142 SMac, Thermo Scientific, UK)(Jenkins et al., 2003).

143 Following overnight incubation at 37°C (±1°) plates were examined for non-sorbitol-
144 fermenting colonies and any suspect colonies were subcultured onto Chromocult coliform

145 agar (Merck KGaA., Germany). After a further overnight incubation at 37°C (±1°) any
146 resulting red colonies were tested with anti-*E. coli* O157 latex (Thermo Scientific, UK) for
147 agglutination. Colonies that agglutinated were identified as presumptively positive and
148 enumerated by limiting dilution.

149 Polymerase Chain Reaction (PCR) was used to confirm the serogroup of the isolates as
150 *E. coli* O157 (ISO/TS, 2012). For all positive samples, the number of *E. coli* O157 were
151 enumerated by culturing 10-fold dilutions of faeces in minimum recovery diluent, starting
152 from 1:10, on duplicate CT-SMac plates. Typical colonies were counted after overnight
153 incubation at 37°C (±1°) and counts expressed as colony forming units (cfu) per gram of
154 faeces.

155 IMS is considered to be a highly sensitive and specific method of identifying *E. coli* O157,
156 and has a lower limit of detection of 50 cfu/g (Aydin et al., 2014; Wright et al., 1994). Lower
157 cfu counts can be detected with decreased sensitivity. IMS has a specificity of 99% (Ekong
158 et al., 2017), and all positive isolates were confirmed as such by the Scottish *E. coli*
159 Reference Laboratory. For the positive/negative analysis, an *E. coli* O157 positive cow was
160 defined as one that tested positive by IMS. The limit of accurate enumeration was 100 cfu/g
161 of faeces (Pearce et al., 2004), and samples from which too few *E. coli* were cultured to be
162 enumerated were assigned a cfu/g of 10.

163 2.3 *F. hepatica* testing

164 Extraneous faecal material (2g), from each faecal sample was weighed into polypropylene
165 tubes and frozen (-20°C). When the *E. coli* O157 status of the farms was known (as defined
166 in Henry et al., 2017), all the samples from eligible *E. coli* O157 positive farms were
167 transported to Moredun Research Institute (MRI) in batches. Here they were tested using a
168 copro-antigen ELISA according to the manufacturer's instructions (Bio-X Diagnostics,
169 Jemelle, Belgium). MRI staff members were blinded to the *E. coli* O157 status of the

170 individual samples. Freezing the samples prior to performing the copro-antigen ELISA is
171 reported to make no difference to the sensitivity or specificity of the test (Brockwell et al.,
172 2013; Flanagan et al., 2011), and this was also confirmed before this study commenced
173 (Personal communication, Dr Philip Skuce).

174 The result was determined by calculating the percentage positivity (PP) of each sample
175 relative to the optical density (OD) of the positive control, after subtracting the OD of the
176 negative control (provided in the kit). The positive/negative cut off was determined by the
177 quality control insert supplied with the kit, and was either 7 or 8 for all the kits used for this
178 study. This test has a reported sensitivity of 0.77 and a specificity of 0.99 (Mazeri et al.,
179 2016).

180 2.4 Statistical analysis

181 The epidemiological unit of interest was the individual animal. For each animal for which a
182 sample was tested, the following results were obtained: *E. coli* O157 positive/negative, *E.*
183 *coli* O157 cfu/g, *F. hepatica* positive/negative derived by applying the cut off to the copro-
184 antigen ELISA results, and *F. hepatica* PP result (on a continuous scale). Farms without a
185 single fluke positive animal were excluded from further analysis, to ensure that cattle at
186 least had a possibility to be infected by fluke, which would not necessarily be the case if
187 there was no fluke on the farm. R (R Core Team, 2011) was used, with the lme4 (Bates et
188 al., 2015) and ggplot2 (Wickham, 2009) packages. Due to confidentiality agreements
189 relating to FSS/FSA project FS101055 which funded the faecal sample collection, figures or
190 data relating to groups of fewer than five farms cannot be shown.

191 2.5 Multilevel model

192 Correlations between the numerical explanatory variables were checked to ensure highly
193 correlated variables were not entered simultaneously into the model. All models were
194 fitted using maximum likelihood. Linear and logistic regression models were built with $\log_{10}$
195 *E. coli* cfu/g and a positive *E. coli* result respectively as the outcome variable. Either log

196 fluke ELISA PP or a positive fluke result was used as the only level 1 explanatory variable,
197 and all other animal and farm management information were level 2 variables. One and 2
198 respectively were added to the *E. coli* O157 count and fluke ELISA PP results before logging
199 to deal with zero and negative values.

200 The starting point was a variable intercept model including a positive fluke result as a level
201 1 explanatory variable and farm as a level 2 random effect. Management variables which
202 met the inclusion criteria were then added one at a time. A seasonal pattern was expected
203 for *E. coli (Ferens and Hovde, 2011)*, so day was modelled as a sinusoidal function to allow
204 for this. The same process was repeated with fluke PP as the explanatory variable. The
205 process was then repeated again with $\log_{10}$ *E. coli* cfu/g as a continuous outcome variable.
206 Variable slopes were also tested. The Akaike information criterion (AIC) was used to
207 compare models, with a lower AIC considered better than a higher one.

208 # 3 Results

209 Between 13th January and 19th October 2015, of 39 farms sampled with one or more cattle
210 testing positive for *E. coli* O157, two declined to take part in further research and samples
211 from two farms were delayed in transit and were therefore not suitable for fluke testing.
212 There was insufficient sample for testing from a further five cows. Therefore, samples from
213 810 cattle from 35 herds were tested using the *F. hepatica* copro-antigen test. Of these, 14
214 farms had at least one cow testing positive for *F. hepatica.* Between 7 and 40 cattle were
215 sampled from each of these farms (median = 22, total = 334) and are included in the
216 following analysis.

217 ## 3.1 Descriptive statistics

218 The characteristics of the farms are shown in Table 1. The data were examined to find out
219 whether groups of cattle were housed or grazing, how long they had been housed or
220 grazing for, and whether they had received a worming or flukicide treatment within the

221 past 3 months. However, even in groups for which flukicide use was recorded, fluke copro-
222 antigen ELISA positive cattle were still present, and similarly some groups of cattle which
223 had been housed for several months still had significant numbers of fluke positive animals.
224 Therefore treatment history was not used to exclude farms and all animals that came from
225 groups with at least one fluke case were included, on the basis that they would all have had
226 the chance to become fluke infected.

227 ### 3.1.1 Animal level

228 Overall, 50.9% of cattle tested positive for *E. coli* O157 and 13.2% tested positive for *F.*
229 *hepatica.* The distributions are shown in figure 1.

230 ### 3.1.2 Farm level

231 Within farms, between 4 and 100% of cattle tested positive for *E. coli* (mean = 43.5%,
232 median = 43.1%) whilst for *F. hepatica* the range was 2.1 to 100% (mean = 14.7%, median =
233 6.5%). The distribution of log *E. coli* O157 cfu/g varied between farms, but in general it was
234 right skewed in ten farms whilst four farms showed a more symmetrical platykurtic
235 distribution. For fluke PP, all except one farm had a right skewed distribution.

236 The farms were spread throughout Great Britain with six from Scotland, four from England
237 and four from Wales. North Wales, South Wales, the Welsh borders, Northern England and
238 a variety of Scottish locations were represented.

239 ## 3.2 Associations between fluke and *E. coli* O157

240 Inspection of scatterplots revealed no visible association between the fluke PP and log *E.*
241 *coli* O157 cfu/g, either at individual or farm level (data not shown). More detailed
242 inspection of three farms with more than 10% of cattle testing positive for fluke revealed
243 no consistent pattern with regard to which individuals were positive for which pathogen. In
244 one farm all of the fluke positive animals were also *E. coli* positive, in a second farm all of
245 the fluke positives were *E. coli* negative, and in a third farm all animals had fluke and the PP
246 values were evenly spread between the *E. coli* positive and negative animals.

247 ## 3.3 Multi-level models

248 The plotting of management variables against log_{10} *E. coli* O157 cfu/g did not reveal any
249 non- linear relationships. No correlations of r >0.7 were seen between any of the
250 explanatory variables, except between numbers of animals of different ages/types.

251 Four different combinations of output and input variables were tested, to include all
252 combinations of the *F. hepatica* and *E. coli* data. The inclusion of random effects improved
253 the model fit in every case, indicating that there were important differences between
254 farms. The explanatory management variables shown in Table 1 were each added to the
255 model as level 2 variables, but it was not possible to add more than two variables at once
256 because of the relatively small number of fluke cases, which led to non-convergence of the
257 model due to perfect partitioning.

258 The best models for each combination of *E. coli* O157 positive and log_{10} *E. coli* O157 cfu/g,
259 and log fluke PP and fluke positive are shown (Table 2). The fluke result did not explain any
260 additional variation in three out of four models, however, log fluke PP was significant when
261 modelled against positive *E. coli* O157 result. Day of sampling and the age of the youngest
262 animal in the group were included in all of the models at level 2 and were highly significant
263 in all models ($p < 0.0001$). The higher the age of the youngest animal in the group, the
264 lower the odds of infection with *E. coli* O157. The model fitted better with day of sampling
265 as a linear variable, and the odds of *E. coli* O157 was found to decrease throughout the
266 year, from January until October. The introduction of random slopes worsened the model
267 fit in each case so this was not pursued.

268 # 4 Discussion

269 This study aimed to use samples available from the FSS/FSA study to investigate whether
270 shedding of *E. coli* O157 is associated with *F. hepatica* infection in cattle (Hickey et al.,
271 2015). *E. coli* O157 is the serogroup most commonly detected in humans in the UK, Europe

272 and the US, and is associated severe clinical outcomes in humans (Anon, 2015; Browning et
273 al., 2016). The advantage of using the samples from a pre-existing study was efficiency in
274 terms of reducing resources needed for planning, recruitment of farmers, visiting farms and
275 testing samples for *E. coli* O157. However, the biggest disadvantage of using the samples
276 gathered for the FSS/FSA study was that the sampling method was designed to treat the
277 group of cattle as the unit of interest; specifically, to identify groups where at least one
278 animal was shedding *E. coli* O157 (Gunn et al., 2007; Pearce et al., 2009). *E. coli* O157
279 shedding varies widely from day to day (Robinson et al., 2009), and the effective sensitivity
280 may be as low as 40% for a one-off faecal sample (Echeverry et al., 2005). Whilst this was
281 not a problem for the FSS/FSA study, where the group was treated as the epidemiological
282 unit, it may have affected the current study because individuals that were shedding *E. coli*
283 O157 may have been missed.

284 Reaching the required sample size depended on the initial assumptions about prevalence of
285 the two pathogens being reasonably accurate, particularly as the collection of additional
286 samples was not possible given the constraints of the study. However, the levels of fluke
287 infection seen in this study, both at the farm and the animal level, were much lower than
288 had been assumed for the sample size calculations (Hickey et al., 2015), and *F. hepatica*
289 only occurred in 43% of farms compared to the predicted 70-80% (Salimi-Bejestani et al.,
290 2005a; McCann et al., 2010; Hickey et al., 2015). This lower fluke prevalence may be partly
291 explained by differences between the cattle populations that were the subject of the
292 FSS/FSA study and previous studies. Data on herd level prevalence of infection is from
293 lactating dairy cows, whereas the FSS/FSA study sampled mostly beef breed or cross bred
294 store or finishing animals. Differences in management exist between these groups that are
295 likely to affect their risk of fluke infection. For example, treatment for fluke is more difficult
296 in dairy animals due to the long milk withhold times of flukicides. Also, of the 35 groups of
297 cattle tested for fluke, only nine were currently grazing, and of those, three had been

298 turned out onto pasture within the three weeks prior to sample collection. This could have
299 been due to the time of year when the samples were collected but also the nature of the
300 farming units tested. It is possible that some of the groups were permanently housed,
301 which would put them at low risk of fluke exposure, although this information was not
302 available from the questionnaire.

303 The lower than expected prevalence of fluke could also be due to the relatively low
304 sensitivity of the *F. hepatica* copro-antigen test, which in naturally infected cattle has been
305 estimated to be below 50%-60% (Duscher et al., 2011), whereas the bulk milk tank antibody
306 ELISA used to estimate prevalence in previous studies has a sensitivity of 96% (Salimi-
307 Bejestani et al., 2005a). The difference in sensitivity between the two types of test was not
308 taken into consideration in the feasibility study (Hickey et al., 2015). The relatively low
309 sensitivity of the diagnostic tests used for both fluke and *E. coli* could have led to non-
310 differential misclassification. This is expected to bias the observed effect size towards zero,
311 although sometimes, by chance, the effect size can be over-estimated (Jurek et al., 2005).

312 Delays in implementation of this study led to the loss of samples from 17 farms enrolled in
313 the FSS/FSA study that were sampled between September 2014 and January 2015 and
314 which had agreed to take part. This contributed to the failure to reach the required sample
315 size. The missed samples were taken during the season when the within-herd prevalence of
316 fluke might have been expected to be at its highest (Bloemhoff et al., 2015).

317 In spite of these challenges, one of our models showed a significant association between *F.*
318 *hepatica* and *E. coli* O157. This would be consistent with our initial hypothesis that *F.*
319 *hepatica* mediated down-regulation of Th-1 immunity may limit the ability of cattle to clear
320 *E. coli* O157 from the intestinal tract: indeed, this would be similar to the previous
321 observation that *F. hepatica* infections in cattle result in increased susceptibility to

322 *Salmonella dublin* which is associated with reduced cellular immune responses against the

323 bacteria (Aitken et al., 1979).

324 The cut-off of the fluke copro antigen ELISA has been the subject of debate, with some

325 studies setting their own cut-off to increase sensitivity (Brockwell et al., 2014). A

326 continuous measure of PP avoids this problem and PP is a biologically meaningful measure

327 as antigen level is correlated with fluke burden (Kamaludeen, 2016).

328 The addition of more than two additional explanatory variables was prevented by

329 insufficient variability within the data. This could partly explain the observed large random

330 effect of farm, which indicates that there were large differences between farms. Another

331 interesting question is whether the inter farm differences could be partially explained by

332 differences between strains of *E. coli* O157 at the molecular level that might be related to

333 shedding events and immune status. Indeed it is known that different strains of *E. coil* O157

334 induce different types of immune response (Corbishley et al., 2014) and different genetic

335 traits of *E. coli* O157, such as phage type (Chase-Topping et al., 2007), presence of *stx2a*

336 and *stx2c* genes and polymorphisms in the *tir* gene (Arthur et al., 2013), are associated with

337 either high or low shedding from infected individuals. Although it was important to control

338 for day of sampling, as season is associated with observed prevalence of both fluke and *E.*

339 *coli* O157 (Bloemhoff et al., 2015; Ferens and Hovde, 2011; Smith et al., 2016; Synge et al.,

340 2003), the strong effect seen here is more likely to be due to all animals from a single farm

341 being sampled on the same day than a genuine seasonal effect. Therefore caution should

342 be used when interpreting the direction and size of the seasonal effect. There may also be

343 other explanatory or confounding variables that are not included in these models. The

344 result should therefore be interpreted with caution, particularly as the effect size is small

345 and it is only seen in one of the model combinations.

346 The results of our study hint at an association between *E. coli* O157 shedding and *F.*
347 *hepatica* infection that merits further investigation. Based on our experiences, use of pre-
348 collected samples represents a cost–effective way of obtaining data, however, care needs
349 to be taken to avoid certain pit falls. In the planning stage of future studies, worst case
350 scenarios for prevalence should be considered, taking into account diagnostic test accuracy
351 and differences between populations which may affect apparent prevalence. Even more
352 importantly, efforts should be concentrated on ensuring that the true infection or shedding
353 status of each individual can be ascertained, and that the type, number and size of sample
354 are suitable for this. For *E. coli* shedding this is likely to include longitudinal sampling to
355 address the issues of intermittent shedding and uneven distribution of bacteria within the
356 faeces.

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549

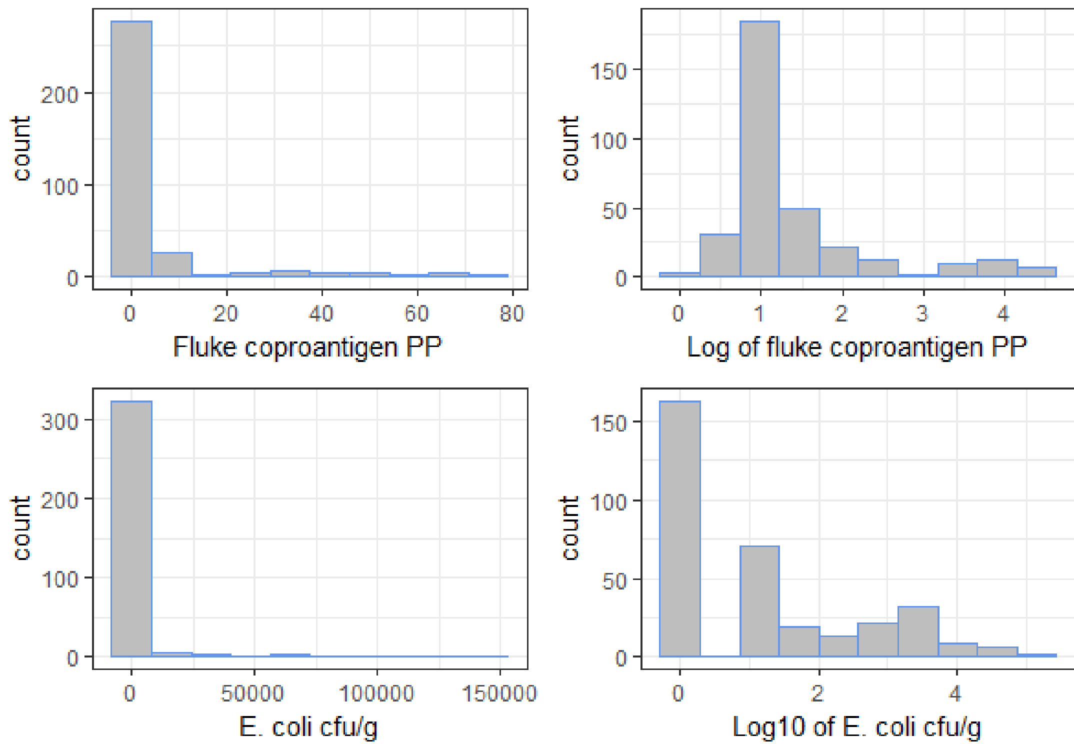
550 **Table 1. Characteristics of the animals and farms in the fluke and *E. coli* O157 study**

	Farm-level (n=14)	Individual animals (n=334)
Day of sample collection (Day 1=1st Jan 2014)	Range = 20-293 Median = 126	
Grazing Housed	3 (21.43 %) 11 (78.57 %)	
Dairy Suckler beef Finisher Other	2 (14.29 %) 8 (57.14 %) 2 (14.29 %) 2 (14.29 %)	
Youngest in group (months) Oldest in group	Range = 6-26 Median = 14.5 Range = 11-48 Median = 20	
Total number of cattle on farm	Range = 41-516 Median = 117	
Total number of cows on farm (Females that have had a calf)	Range = 0-208 Median = 33	
Total number of heifers on farm	Range = 0-65 Median = 6	
Total number of cattle under 1 year on farm	Range = 0-215 Median = 30	
Total number of ewes on farm	Range = 0-700 Median = 0	
Total number of sheep overwintering on farm	Range = 0-433 Median = 0	
Water supply from mains Water supply from spring or well Water supply from natural source	10 (71.43 %) 6 (42.86 %) 11 (78.57 %)	
Median percentage of fluke positive cows[1] Range of positive cows Fluke positive Median PP Range PP	6.55% 2.13-100%	 44 (13.17%) 0.82 -1.07-73.74
Median percentage of *E. coli* O157 positive cows[2] Range of positive cows *E. coli* positive Median cfu/g[3] Range cfu/g[3]	43.10% 4.00-100%	 170 (50.9%) 10 0-1.45 x 10^5

551 [1]'Fluke positive' refers to an animal which tested positive on the copro-antigen ELISA result

552 [2]'E. *coli* O157 positive' refers to an animal with a positive IMS *E. coli* test

553 [3]Samples from which *E. coli* numbers fell below the limit of enumeration were assigned cfu/g = 10



554

555 **Figure 1. Distribution of *F. hepatica* coproantigen PP values and *E. coli* O157 cfu/g for**
556 **animals across all farms.**

557

558 **Table 2. Summary of the multi-level models. Farm was included as a random intercept.**
559 **Day of sampling and age of the youngest animal in the group were controlled for in all**
560 **models.**

Outcome variable	Input variable	Co-efficient	*p* value
E. coli O157 positive	*F. hepatica* positive	0.50	0.34
E. coli O157 positive	Log *F. hepatica* ELISA PP	0.48	0.010
$\log_{10}$ *E. coli* O157	*F. hepatica* positive	-0.02	0.90
$\log_{10}$ *E. coli* O157	Log *F. hepatica* ELISA PP	0.09	0.26

561 *E. coli* O157 positivity was determined using immune-magnetic separation. $\log_{10}$ *E. coli* O157 refers
562 $\log_{10}$ of the *E. coli* O157 count (cfu/g). *F. hepatica* positivity was determined using a copro-antigen
563 ELISA. ELISA PP is the percentage positivity compared to a known positive sample.

564