The use of Cathepsin L1 (FhCL1) serological ELISA in sentinel screening for liver fluke on sheep farms

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Abstract

Fasciola hepatica is a parasitic helminth (worm) that poses a significant economic threat to the ruminant livestock industry worldwide. The disease, fasciolosis, can result in a range of clinical signs including anaemia, weight loss and death, with the most severe symptoms attributed to early acute infection when the parasite is migrating through the liver. Early diagnosis and intervention are essential for the control and management of the disease to prevent productivity losses. The traditional gold standard method of diagnosis uses faecal egg counts (FEC) that is limited to

detecting patent infections from 10-12 weeks post infection (WPI). In contrast, serological assays can detect pre-patent infections as we have shown that enzyme-linked immunosorbent assays (ELISA) using the *F. hepatica* cysteine peptidase cathepsin L1 (FhCL1) can detect liver fluke infections from 3 to 4 WPI. Here, we used FEC and ELISA to monitor liver fluke infections in sentinel lambs from three commercial farms in Ireland from September 2021 to March 2022. All three farms showed a significant increase in FhCL1 antibody levels and FEC over this time, with a substantial rise in positive infection detection between late November and January. However, ELISA screening detected infection at least two months prior to FEC (September). This suggests that the regular screening of sentinel lambs for *F. hepatica* seroconversion in a "test and treat" approach could mitigate the negative damaging impact of early fasciolosis on flock health, welfare and productivity and inform management strategies. In addition, we show that whole blood samples taken on Whatman® protein saver cards could replace conventional serum blood tubes for blood collection. Cards can be stored at room temperature for long periods of time and samples revisited at any time for re-analysis. The adoption of these cards on farm together with the FhCL1 ELISA would provide a simpler, cost-effective, and eco-friendly method for testing sentinel lambs for liver fluke disease.

Keywords: *Fasciola hepatica,* Diagnostics, ELISA, Cathepsin peptidase L1, Whatman® protein saver cards, Sentinels

Introduction

Fasciolosis is a worm infection by the liver fluke, *Fasciola hepatica,* that causes extensive liver damage and loss of productivity in ruminants. The disease has three clinical forms (acute, subacute and chronic), which can cause symptoms ranging from weight loss and anaemia to sudden death (Sustainable Control of Parasites, 2022; Andrews et al., 2021). It is estimated that fasciolosis is

47 responsible for ~€2.5 billion losses in productivity (milk, meat, wool etc.) to the global livestock and
48 food sectors, with €90 million of this being apportioned to the Irish economy alone (Animal Health
49 Ireland, 2021b).

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51 In Ireland and the UK, the mud snail *Galba truncatula* is the intermediate snail host of *F. hepatica*
52 (Jones et al., 2017). Infected snails shed cercariae that encyst as the infective form on pasture and in
53 water, which is then eaten/drank by a grazing animal. The metacercariae excyst in the intestine and
54 the newly excysted juveniles (NEJs) penetrate through the intestinal wall and migrate to the liver.
55 The closely related paramphistome or rumen fluke, *Calicophoron daubneyi,* shares *G. truncatula* as
56 an intermediate host (Jones et al., 2015), and in *G. truncatula*-associated habitats livestock are often
57 co-infected with the two flukes.

58

59 The effective and sustainable management of fasciolosis in agriculture can be facilitated by a "test
60 and treat" approach. The current gold standard diagnostic for fasciolosis is the faecal egg count
61 (FEC). While a positive FEC is indicative of an active infection, *F. hepatica* do not start to shed eggs
62 until the parasites have reached maturity in the bile ducts, typically around 10-12 weeks post-
63 infection (WPI), so a negative result does not discriminate between a non-infected animal or one
64 that may have parasites less than 10-12 weeks old. This is a major drawback of the FEC since it is the
65 immature migratory parasites that cause the extensive liver damage (tunnelling and haemorrhaging)
66 during the acute stages.

67

68 Recently, we have shown that a serological test, an enzyme-linked immunosorbent assay (ELISA) that
69 detects antibodies against the *F. hepatica* cathepsin peptidase L1 (FhCL1) can identify infected sheep
70 as early as 3 WPI (López Corrales et al., 2021). Recent guidelines recommend the regular monitoring

of sentinel animals (7 – 10 per herd/flock), ideally first grazing season lambs or calves, using tests for serum antibodies to give an indication as to the infection status of the flock or herd (Sustainable Control of Parasites, 2022). This method of regular infection monitoring would enable farmers to accurately and timely detect when an infection occurred in these animals, and therefore choose the most suitable time and drug for treating the flock/herd; this strategy would avoid misuse or overuse of chemicals that could encourage the emergence of drug-resistant parasites (Fairweather et al., 2020). It is also useful for identifying infected pastures, which farmers should avoid using in autumn/winter to prevent further infections and thereby reduce the need for anthelmintic use (Animal Health Ireland, 2021a; Opsal et al., 2021).

In the current study, we conducted a 6-month sentinel surveillance across three sheep farms in Ireland, monitoring *F. hepatica* infection using the FhCL1 ELISA compared with the gold standard FEC. Furthermore, with the view to improving the ease and utility of our test, including energy-saving measures, we evaluated the use of Whatman® protein saver cards for blood sampling compared to traditional polypropylene blood tubes. Finally, we demonstrate absolute specificity and sensitivity for the FhCL1 ELISA for monitoring liver fluke as animals solely infected with rumen fluke do not express antibodies that cross react with FhCL1. Therefore, our studies fully support the use of our FhCL1 ELISA for serologically screening sentinel sheep to predict on-farm sheep fasciolosis.

Methods

Ethical Statement

All animal procedures were carried out on commercial farms under license from the Health Products Regulatory Authority (HPRA) in accordance with EU Directive 2010/63/EU (License No.

AE19132/P115) following ethical approval from the Teagasc Animal Ethics Committee (TAEC2021-298).

Sample Collection

Three commercial farms in Ireland located in Co. Galway (Farm A), Co. Sligo (Farm B) and Co. Cork (Farm C), with previous history of *Fasciola hepatica* infections were selected for this study. To assess the parasite burden at farm level, seven spring-born lambs from each farm were randomly selected to act as flock sentinels. This represented 5-7% of the total flock as each farm has 100-150 animals. These animals were monitored regularly for *F. hepatica* and *C. daubneyi* infections over a six-month period from September 2021 to March 2022. Faecal and blood samples were obtained from each animal at five separate time points (in September, early November, late November, January, and March), although in some cases samples were not obtained. In total, 103 blood samples and 94 faecal samples were collected throughout the study. Blood samples taken for the recovery of sera were collected in BD Vacutainer™ SST™ II Advance Tubes (FisherScientific) and on Whatman® protein saver cards (Cytiva) via jugular venepuncture. Droplets of blood were collected on the Whatman® protein saver cards until a sample collection circle marked on the card was saturated via the jugular venepuncture.

Elution of samples from Whatman® protein saver cards

A 5 mm diameter circle was cut from the Whatman® protein saver card using a steel paper punch. The card piece was transferred to 1 mL PBS supplemented with 0.15% (v/v) Tween 20 and incubated overnight at 4°C on a rocker. The 1 mL suspension was used in place of a serum sample in the card ELISA.

ELISA

The ELISA was carried out as previously described by López Corrales *et al.* (2021). Flat-bottom 96-well microtitre plates (Nunc™ Maxisorp ™, ThermoFisher Scientific) were coated in triplicate with 100 µL of 1 µg/mL of the recombinant antigen (rFhCL1) in 0.05 M carbonate buffer, pH 9.6, and incubated overnight at 4°C. After three washes with 100 µL of PBS-0.05% Tween 20 (PBST), 100 µL/well of blocking buffer (2% bovine serum albumin diluted in PBST) was added and incubated for 1 h at 37°C. After washing three times with PBST, 100 µL of serum or 100 µL eluted card samples from sheep diluted 1:100 in serum dilution buffer (PBS, 0.5% Tween 80, 0.5 M NaCl) were added and the plates were incubated for 1 h at 37° C. After washing five times, 100 µL/well of HRP-conjugated donkey anti-sheep IgG (ThermoFisher Scientific), diluted 1:50,000 in blocking buffer, was added and the plates were incubated for 1 h at 37°C. Following five washes, 100 µL/well of 3,3',5,5'-Tetramethylbenzidine (TMB; Sigma-Aldrich) was added and the plates incubated at room temperature for 4 min. The reaction was stopped by the addition of 100 µL/well of 1 M sulphuric acid. The optical density was determined at a wavelength of 450 nm (OD450) in a PolarStar Omega spectrophotometer (BMG LabTech).

Faecal Egg Counts (FEC)

FECs were completed for each animal to monitor patent *F. hepatica* and *C. daubneyi* infections. The samples (5 g) were processed as described by López Corrales *et al.* (2021) and results were presented as eggs per gram of faeces.

Statistical analysis

All statistical analysis was carried out in NCSS2022. Determination of the significance of the association between seropositivity and month, and seropositivity and farm was determined by the

Chi-squared test. The optimal OD cut-off for determination of seropositive and seronegative samples by the ELISA assays was derived from a receiver operating characteristic (ROC) curve of data values as the OD value which optimised the accuracy of diagnosis of FEC positive and FEC negative samples. The overall efficiency of the ELISA assays was determined from the area under the curve (AUC) Analysis (empirical estimation). Determination of the significance of the difference of two ROC curves was determined by test comparing two AUCs (empirical estimation). The significance of the slope of the regression of serum immunoassay absorbance and of card immunoassay absorbance against fluke egg number was determined by linear regression analysis. Fluke egg numbers were first logged to promote linearity. Normality of the residuals was assessed by the D'Agostino omnibus test and linearity by the Lack of Linear Fit Test. The correlation was assessed by correlation analysis. Determination of the significance of the association between seropositivity and egg positivity was determined by the McNemar test.

Results

Infection rates are highest in January and March on all three farms.

We used our cathepsin peptidase ELISA (López Corrales et al., 2021) based on FhCL1 to determine at what point the animals displayed *F. hepatica* antibodies, in comparison to the FEC data (Figure 1; Table 1). Infection status was determined by *F. hepatica* FEC for the 94 faecal samples collected across the three farms over the course of the study. When infection was compared over time by Pearson's Chi-square, there was significantly higher infection in January and March compared to September, early November and late November ($p < 0.0001$). Comparable numbers of samples were collected from each farm allowing comparative analyses to be carried out (Farm A: 33; Farm B: 29; Farm C: 32). Farm A and C had similar numbers of positive faecal samples (Farm A: 14, 42.4%; Farm C: 15, 46.9%), whereas a slightly lower number of samples on Farm B were identified as being

positive for liver fluke infection (Farm B: 9, 31%) (Figure 1). However, no significant difference was observed between the farms based on the Pearson's Chi-squared test ($p > 0.05$).

As would be expected, there was a significant difference in the infection levels depending on the time of year the samples were collected (Table 1; Pearson's Chi-squared test, $p < 0.0001$). In September, at the beginning of our study, we observed lower levels of infection, with only two animals positive for liver fluke infection out of the 20 faecal samples collected. The infection level rose slightly over November, with three and five animals positive for liver fluke eggs in early and late November, respectively. The highest number of liver fluke egg positive animals were observed in early 2022, with 12 animals in January and 16 animals in March. At the end of our study, two animals were still negative by *F. hepatica* FEC.

There is no significant difference in the sensitivity and accuracy of the FhCL1 ELISA using sera samples compared to samples eluted from Whatman® protein saver cards

To improve the utility of our cathepsin peptidase ELISA, we assessed the sensitivity and specificity of the FhCL1 ELISA using serum collected via the traditional method via polypropylene blood tubes (serum ELISA) compared with the eluate from Whatman® protein saver cards (card ELISA). Both protocols could significantly detect liver fluke infection based on the data from the FEC, which gave an estimated prevalence of infection of 0.40 across the three farms. No significant difference was detected between the two tests using Test to Compare Two AUCs (Empirical Estimation; $p > 0.05$). Based on this data, the optimal cut-off for the serum ELISA was 0.26A, giving an accuracy of 0.85, and for the card ELISA was 0.31A, giving an accuracy of 0.87 (Figure 2).

190 The similar life cycle of *F. hepatica* and *C. daubneyi,* which utilise the same intermediate host *(G.*
191 *truncatula)*, often results in animals being co-infected with these two fluke species. We assessed the
192 impact of *C. daubneyi* infections on our FhCL1 ELISA to determine if any cross reactivity was
193 observed (Figure 2). Seventy-nine of the 94 faecal samples collected across the three farms over the
194 course of the study were positive by *C. daubneyi* FEC (estimated prevalence of 0.84). No cross
195 reactivity was observed for the serum ELISA ($p > 0.05$; AUC = 0.63 ± 0.08), although a significant
196 impact of rumen fluke infection was observed for the card ELISA ($p = 0.01$; AUC = 0.66± 0.08). To
197 assess whether this was because of the presence of both *F. hepatica* and *C. daubneyi* eggs, we re-
198 analysed the data focusing only on the samples negative for liver fluke eggs, representing 56
199 samples, of which 44 were positive for *C. daubneyi* eggs. No significant cross reactivity was observed
200 for these samples by either ELISA protocol ($p > 0.05$).

201

202 *There is no significant difference in the accuracy of FEC compared to either of the FhCL1 ELISA tests.*

203 Comparative analysis of the *F. hepatica* positive FEC data with our serological data showed a
204 significant correlation between these two diagnostic assays (serum ELISA: $p = 0.0001$; card ELISA: $p =$
205 0.0008), indicating that our ELISA could accurately detect *F. hepatica* infections earlier than the FEC.
206 The only exception was the misdiagnosis of six samples by the serological assays that were positive
207 by FEC (Figure 3A and B, blue circles). However, all six samples had a *F. hepatica* FEC of <3, indicating
208 a possible limit of sensitivity to the ELISA methods. Three of these samples were from Farm B that
209 displayed a lower level of liver fluke infection, particularly at the early stages of our study (Animal 2
210 in early November and Animal 5 in September and March). Similarly, a sample from Farm C collected
211 in September (Animal 3) was positive by *F. hepatica* FEC but negative by the serological assays. Two
212 further samples were not consistent across the serological assays. Animal 1 of Farm A was negative
213 by serum ELISA but positive by both the card ELISA and *F. hepatica* FEC in late November, and animal

3 of Farm C was negative by the card ELISA but positive by both serum ELISA and *F. hepatica* FEC in March.

The accuracy of the serological assays to detect liver fluke infection compared with the FEC was further supported by comparing these two datasets using the McNemar test. The probability that the serological assays would not result in the same diagnosis as the *F. hepatica* FEC was insignificant (serum ELISA and *F. hepatica* FEC $p > 0.05$; card ELISA and *F. hepatica* FEC $p = > 0.05$). Comparing the data with the *C. daubneyi* FEC data, resulted in a significant probability that the *F. hepatica* serological data did not correlate with the rumen fluke egg data ($p < 0.0001$), further highlighting that the *F. hepatica* serological assays can be used for animals co-infected with both rumen and liver fluke due to the lack of cross-reactivity (Table 2).

Discussion

Typically, in Ireland, animals pick up *F. hepatica* from the pasture in the late summer and early autumn, with the infection becoming chronic over the winter and into spring (Control of Cattle Parasites Sustainably, 2022). Since 1959, the amount of rainfall and evapotranspiration have been used for the Ollerenshaw forecasting model to predict the risk of liver fluke infection within specific geographical areas (Ollerenshaw and Rowlands, 1959). The liver fluke forecast based on weather data from the summer of 2021 predicted high risk of disease in Co. Sligo and medium levels of risk in Co. Cork and Co. Galway (Department of Agriculture, Food and the Marine, 2021). The Animal Health Ireland Beef Health Check programme reported low levels of live liver fluke identified in post-mortem inspection of livers in abattoirs across Ireland (1.6% of 144,000 animals) in spring 2022, with highest number of reported cases in the northwest of Ireland (Co. Sligo, Co. Roscommon, Co. Leitrim, Co. Longford, and Co. Mayo). By the summer of 2022, the percentage of herds in which at

least one animal had a live liver fluke infection at slaughter was up to 40% in Co. Sligo, up to 20% in Co. Galway and up to 10% in Co. Cork (Animal Health Ireland, 2022a, b), indicating that infection levels, albeit high in some counties, did not fully align with predictions. In the present study, there was no significant difference in infection levels between farms, despite Farm B being located in a high risk area and Farms A and C being located in a medium risk area (Teagasc, 2021). This indicates that, while geography and weather are major determinants of infection rates, their accuracy is compromised by generalised predictions that cannot take into consideration the level of pasture contamination on individual farms, nor individual farming practices that may influence infection rates. However, in agreement with the seasonal lifecycle of *F. hepatica*, the present study identified a significant increase in infection rates across all farms in January and March compared to September and November. Therefore, infections begin in September to November consistent with the weather data predictions but were not detectable by *F. hepatica* FEC until at least 10 WPI when eggs were produced by mature flukes in the bile duct and were shed in the faeces (Craig, 2009). This delay in detecting infection using FEC highlights the need for a diagnostic that can detect infection prior to egg production and liver damage.

There is currently no system in place to monitor the impact of fasciolosis in sheep across Ireland. Recently the Irish Regional Veterinary Laboratory (RVL) carried out an ELISA based survey to assess liver fluke infections in sheep using blood samples collected by the Irish Department of Agriculture, Food and the Marine at time of slaughter, indicating that there is a potential mechanism for a surveillance program (Regional Veterinary Laboratory, 2021). In our previous study, we showed that antibodies in naturally infected lambs against FhCL1 could be measured to determine infection status, and accurately distinguish between infected and non-infected lambs, including animals infected with low liver fluke burdens (López Corrales et al., 2021). In the present study, using the same FhCL1 ELISA we showed that antibodies against FhCL1 were found to significantly increase

over the course of infection and detected infection at least two months earlier than FEC, consistent with our previous study.

An aim of this study was to develop an ELISA that used serum samples eluted from Whatman® protein saver cards that could detect *F. hepatica* infections as well as the current gold standard FEC and was consistent with our established FhCL1 ELISA. Whatman® protein saver cards offer several environmental and logistical advantages over conventional blood tubes, including that they do not require low-temperature storage. Laboratories are huge users of energy and single use plastics, consuming up to six times more energy than an equal sized office building (Lopez et al., 2017). It is estimated that research labs used approximately 5.5 million tonnes of single-use plastics in 2014 (Urbina et al., 2015). A large proportion of the energy consumed by laboratories is a result of requirements for cold storage, with a new ultra-low temperature freezer running at -70°C to -80°C consuming approximately 22 kWh per day (Henderson, 2014), compared to the 0.84 kWh that a standalone kitchen freezer consumes per day (Energy Use Calculator, 2023). Whatman® protein saver cards also facilitate collection of blood samples by the farmer, e.g. capillary blood sampling with a lancet, rather than requiring a vet to collect the blood samples, which could lead to more end-user uptake of the test.

The card ELISA in the present study had 87% specificity compared to 85% specificity for the serum ELISA, indicating that the Whatman® protein saver cards offer a comparable alternative for sample collection compared to polypropylene tubes. While some studies indicate that there may be variability in antibody elution from Whatman® protein saver cards (Dauner et al., 2015; Barin et al., 2005), further optimisation of our antibody elution SOP could be made following further studies that include farmers to collect the sample to investigate the effect of user handling. Nevertheless, no

287 statistical difference was observed between the card-elution method compared to serum isolation
288 from blood for the present study.

289

290 In addition, both the serum and card ELISA diagnoses showed a significant correlation with the
291 results of the *F. hepatica* FEC, with IgG levels increasing in congruence with the number of eggs per
292 gram of faeces. This finding agrees with that of the study by Valero *et al.* (2020), who identified a
293 positive correlation between egg shedding and IgG levels in rats that were experiencing their first *F.*
294 *hepatica* infection, albeit not in re-infected rats which suggested that egg shedding is influenced by
295 the immune status of the individual. However, this finding disagrees with the previous study by
296 Brockwell *et al.,* (2013) who did not find a correlation between IgG levels and FEC and found that
297 FEC levels varied day by day. The correlation seen in the current study could be due to the different
298 ELISA assay used, CL1 as opposed to the assay available from Bio-X Diagnstics, or because the
299 current study used samples from naturally infected lambs as opposed to experimentally infected
300 cattle. It would be important to further evaluate the serum and card ELISA's across a larger number
301 of farms, and to include a larger sample size at each farm, as the current study only availed of seven
302 animals across three farms.

303

304 Of the 38 samples positive by *F. hepatica* FEC, six samples were misdiagnosed by our ELISA assays.
305 The exact cause for these discrepancies is unknown but could be related to technical aspects of
306 sampling out on farm, including mislabelling of either the blood tube, Whatman® protein saver card
307 or faecal sample or contamination of a faecal sample. However, all the animals misdiagnosed by
308 either of the ELISAs had a FEC of <3, indicating a possible cut-off for sensitivity of the methods.

309

The paramphistome *C. daubneyi* is a closely related species of *F. hepatica,* sharing a similar excretory-secretory (ES) proteome including a range of cysteine proteases, including cathepsins, (Huson et al., 2018). The *C. daubneyi* NEJ ES proteome overlaps with that of *F. hepatica* by 24%, while the adult ES proteomes overlap by 20% (Huson et al., 2021). The finding that neither the serum nor the card ELISA significantly detect animals that were infected with *C. daubneyi* but not *F. hepatica* indicates that the FhCL1 antigen is specific to *F. hepatica.* This is of particular importance due to the shared intermediate host, *G. truncatula,* of the two species and also the finding that infection with either species is associated with an increased risk of becoming infected with the other (Naranjo-Lucena et al., 2018). Most importantly, our study shows that both the serum and card ELISAs provide diagnostic tests that are capable of specifically diagnosing *F. hepatica* infection independently of *C. daubneyi* infection.

Conclusions

Previous studies using ELISA assays for *F. hepatica* utilise either serum collected in traditional blood tubes (Carnevalea et al., 2001; López Corrales et al., 2021; Mokhtarian et al., 2018) or milk samples (Bloemhoff et al., 2015; Munita et al., 2016; Munita et al., 2019; Selemetas et al., 2014) to test for anti-FhCL1 antibodies. This study is the first study to demonstrate that blood samples collected on Whatman® protein saver cards can be used to determine an animal's *F. hepatica* infection status by FhCL1 ELISA. Not only do the Whatman® protein saver cards provide environmental benefits by reducing plastic use and the need for cold storage, but they also allow for a sample to be reanalysed multiple times without any freeze thawing. Secondly, this study has proven the suitability of both the serum and card ELISA for diagnosing infection in naturally infected animals, which contrasts with the study by Walsh et al. (2021) that found naturally infected animals have a much weaker antibody response to recombinant FhCL1 compared to experimentally infected animals. Both ELISA assays in the current study were able to detect naturally acquired *F. hepatica* infections with 85-87%

accuracy, showing strong levels of correlation with *F. hepatica* FEC but remained unaffected by *C. daubneyi* infections. Finally, this study provides evidence that using new season lambs as sentinels provides an accurate method of monitoring infection within sheep flocks, consistent with the study by the Irish RVL and as recommended by SCOPS (Regional Veterinary Laboratory, 2021; Sustainable Control of Parasites, 2023). This offers both a time and cost-saving incentive for farmers to implement a "test then treat" approach, helping to combat anthelmintic resistance.

Declaration on competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by Science Foundation Ireland (SFI) Professorship grant (17/RP/5368) awarded to J.P. Dalton.

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Figure legends

Figure 1. Infection rates do not differ between farms, but infection is higher in January and March compared to September and November. Infection status was determined by *F. hepatica* FEC for the 94 samples collected across the three farms (Farm A, B and C) over the course of the study. Optical density values for total IgG against FhCL1 were plotted for each sample, and samples were coded according to comparative *F. hepatica* FEC analysis (red circles: positive FEC; blue circles: negative FEC). The cut-off for seropositivity of the ELISA was 0.26, indicated by the dashed line. The degree of association between farms was measured by Pearson's Chi-square analysis. There was no evidence that farms had different rates of infection ($p > 0.05$).

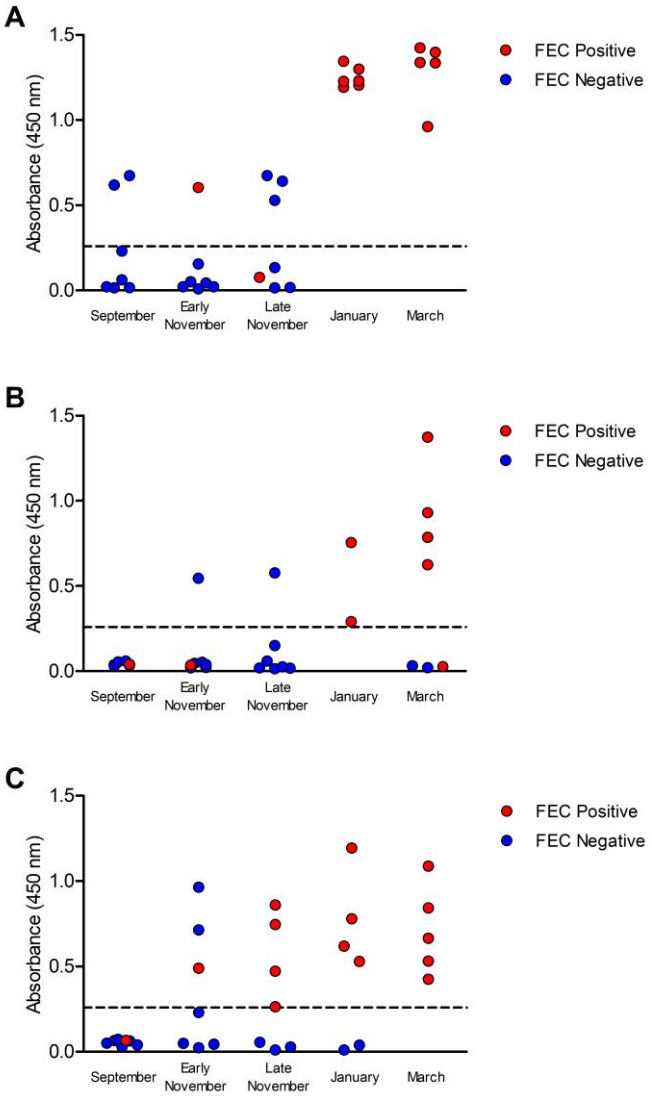

Figure 2. Comparable levels of sensitivity and specificity are observed for the FhCL1 ELISA using serum samples or blood samples eluted from Whatman® protein saver cards. Ninety-four sera samples collected from the three farms over the course of the study with corresponding *F. hepatica* and *C. daubneyi* FEC were used to calculate the sensitivity and specificity of the serum and card ELISAs. The optimal cut-off for the serum ELISA was 0.26A with a specificity of 85% (red line). The optimal cut-off for the card ELISA was 0.31A with a specificity of 87% (blue line). AUC analysis found a highly significant ($p < 0.0001$) selective detection of *F. hepatica* FEC negative and positive samples by both serum and card ELISA. Forty-four of the 94 sera samples were negative by *F. hepatica* FEC and positive by *C. daubneyi* FEC; however, no significant cross reactivity was observed with the ELISA data (orange and purple lines).

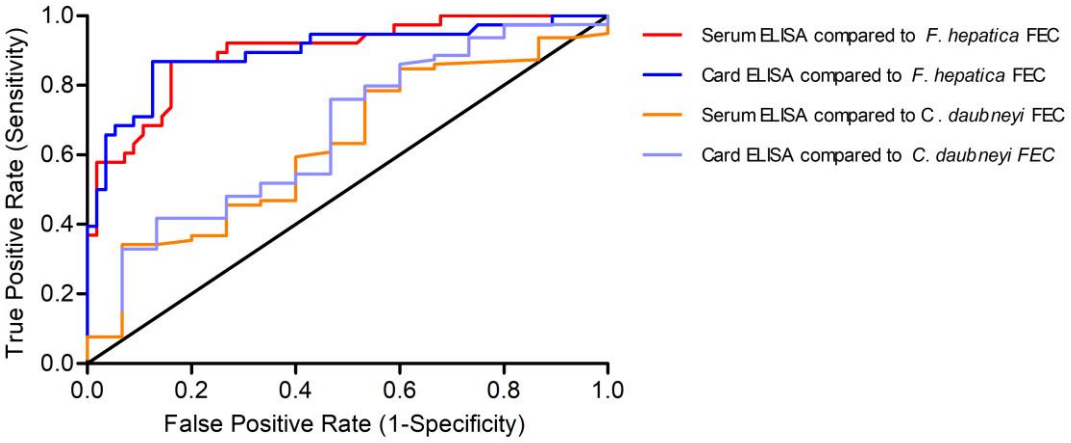

Figure 3. There is a significant linear relationship between *F. hepatica* FEC results and serum and card FhCL1 ELISA absorbance values. Thirty-eight faecal samples collected across the three farms over the course of the study were positive by *F. hepatica* FEC. The results of the FEC were plotted against absorbance values of the serum (A) and card (B) ELISAs. The serum ELISA has a y-intercept of 0.5759 with a SE of 0.0779 and a significant (p = 0.0001) slope of 0.3613 with a SE of 0.0789. The R^2 is 0.3683 and the correlation between the serum ELISA and the *F. hepatica* FEC is 0.6069. The card ELISA has a y-intercept of 0.5017 with a SE of 0.0692, and a significant (p = 0.0008) slope of 0.2560 with a SE of 0.07. The R^2 is 0.2709 and the correlation between the card ELISA and the *F. hepatica* FEC is 0.5204. The slope is significant (p = 0.0008). Both the serum and card ELISAs each misdiagnosed five individuals that were positive by *F. hepatica* FEC (blue circles).

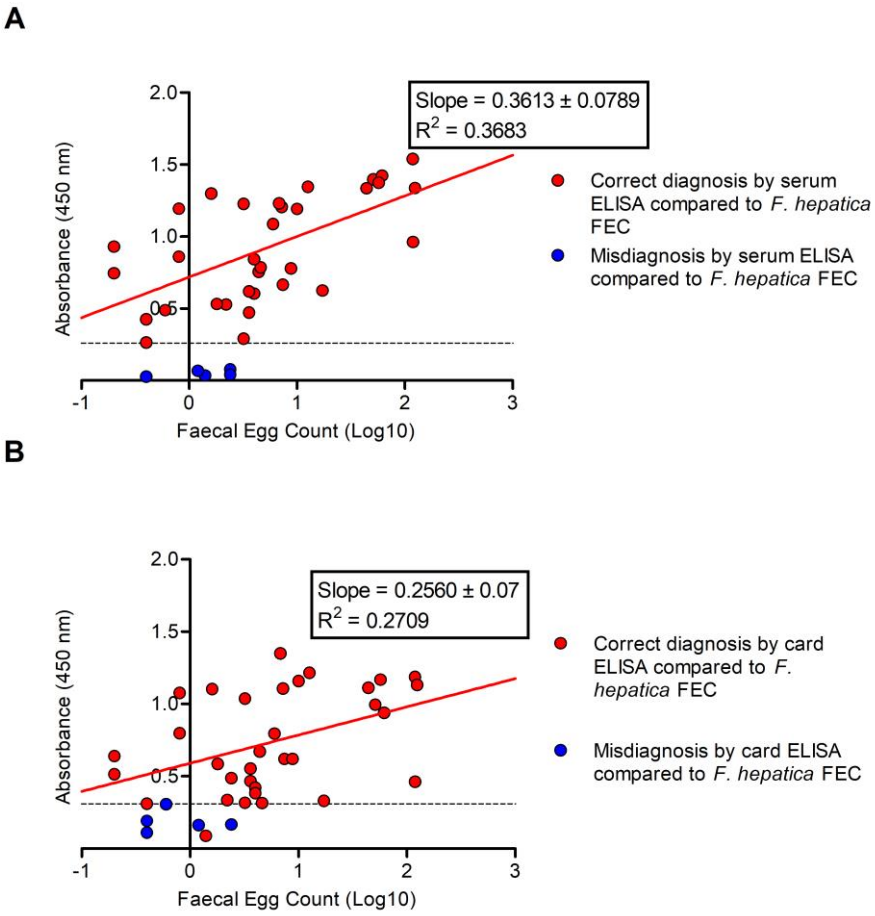

Figure 4. There is a strong association between the diagnosis obtained for the serum and card ELISAs and the *F. hepatica* FEC, but not the *C. daubneyi* FEC. Ninety-four serum and card samples with corresponding faecal samples were collected across the three farms over the course of the study. Absorbance values for the serum and card ELISA's are plotted and colour coded for positive (red circles) or negative (blue circles) *F. hepatica* (A) or *C. daubneyi* (B) FEC. There is no evidence that the serum and card ELISAs are not giving the same result as the *F. hepatica* FEC (p = 0.2850 and 0.7815, respectively). There is evidence that the serum ELISA and the *C. daubneyi* FEC are not giving the same diagnosis (p <0.0001 for both tests). Cut-off values for the serum ELISA (x = 0.26) and card ELISA (y = 0.31) are indicated by the dashed lines.

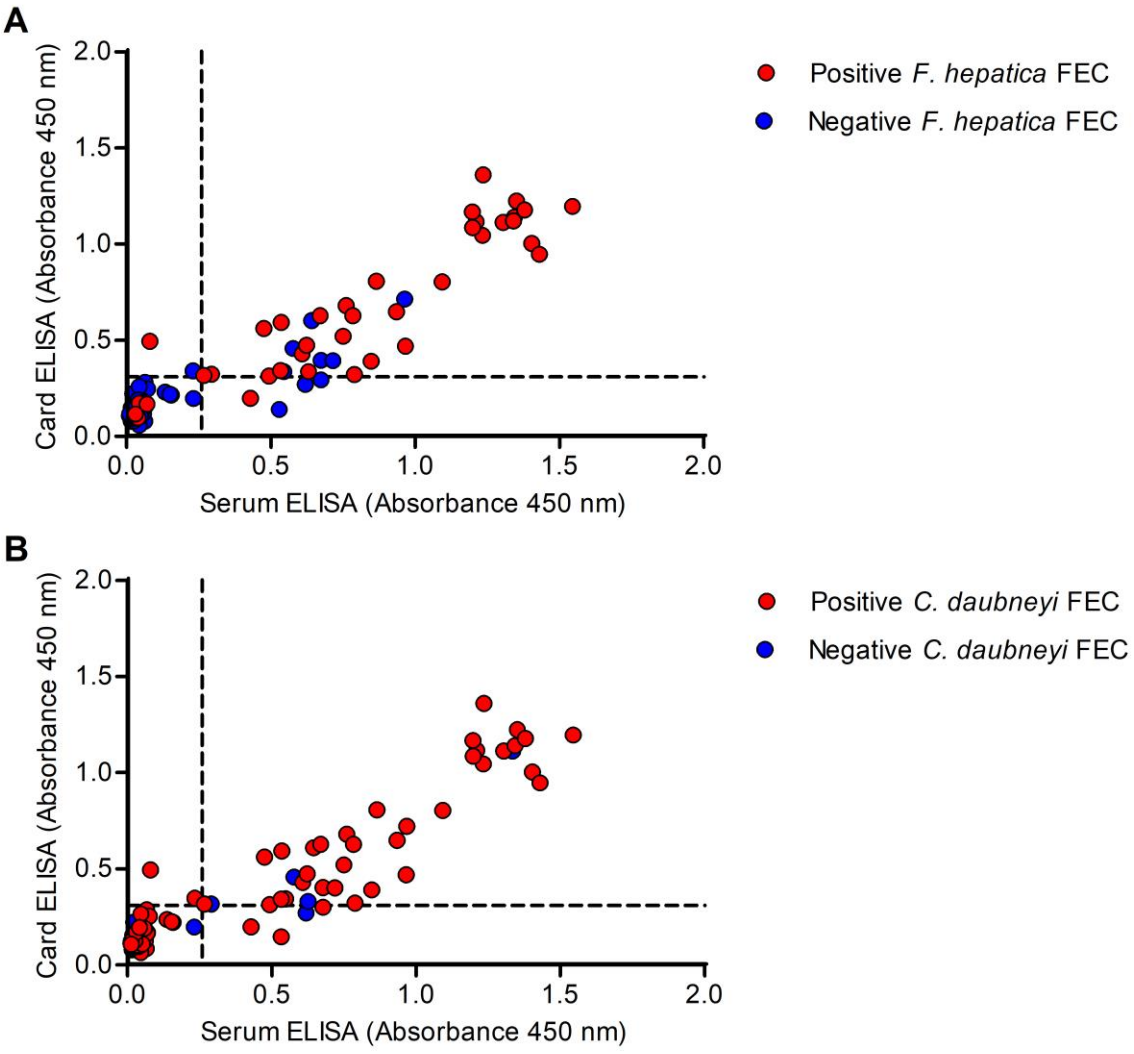

Table 1. *F. hepatica* FEC data

Month	Number of Positive *F. hepatica* FEC	Number of Negative *F. hepatica* FEC
September	2	18
Early November	3	18
Late November	5	16
January	12	2
March	16	2

Table 2. Summary of the results from each of the diagnostic assays (FEC and ELISA) used over the course of the study to determine positive and negative *F. hepatica* infections compared with *C. daubneyi* FEC data.

Diagnostic Test	Number of Positive Results	Number of Negative Results
Serum ELISA	42	52
Card ELISA	39	55
***F. hepatica* FEC**	38	56
***C. daubneyi* FEC**	79	15

Table S1. Raw data for the serum ELISA, card ELISA, *F. hepatica* FEC and *C. daubneyi* FEC used in the study. Cells highlighted in green indicate a negative result and cells highlighted in red indicate a positive result. N/A indicates that the sample was unavailable.

Serum-ELISA		Month				
		Sept	Early Nov	Late Nov	Jan	March
Farm A	Animal 1	0.619	0.604	0.077	1.466	1.542
	Animal 2	0.016	0.010	0.530	1.346	1.539
	Animal 3	0.017	0.022	0.016	1.228	1.398
	Animal 4	0.022	0.022	0.134	1.206	1.338
	Animal 5	0.674	0.044	0.674	1.300	1.335
	Animal 6	0.232	0.155	0.642	1.231	1.425
	Animal 7	0.063	0.052	0.018	1.193	0.962
Farm B	Animal 1	0.038	0.022	0.014	0.012	0.032
	Animal 2	0.035	0.036	0.151	0.756	0.785
	Animal 3	0.031	0.047	0.059	0.055	1.374
	Animal 4	0.055	0.545	0.577	0.291	0.626
	Animal 5	0.041	0.020	0.018	0.032	0.027
	Animal 6	0.059	0.053	0.020	0.055	0.930
	Animal 7	0.043	0.040	0.025	0.030	0.021
Farm C	Animal 1	0.051	0.230	0.056	0.039	N/A
	Animal 2	0.029	0.049	0.011	0.011	N/A
	Animal 3	0.067	0.489	0.264	0.530	0.425
	Animal 4	0.066	0.715	0.861	0.780	0.666
	Animal 5	0.064	0.044	0.028	0.922	0.843
	Animal 6	0.073	0.024	0.472	1.194	1.088
	Animal 7	0.041	0.964	0.746	0.619	0.532

Card-ELISA 1 ml extraction		Month				
		Sept	Early Nov	Late Nov	Jan	March
Farm A	Animal 1	0.270	0.423	0.487	1.224	1.161
	Animal 2	0.079	0.108	0.141	1.215	1.188
	Animal 3	0.085	0.095	0.149	1.037	0.996
	Animal 4	0.135	0.222	0.231	1.107	1.132
	Animal 5	0.294	0.060	0.396	1.104	1.113
	Animal 6	0.197	0.216	0.602	1.351	0.939
	Animal 7	0.079	0.184	0.142	1.160	0.463
Farm B	Animal 1	0.129	0.124	0.121	0.199	0.151
	Animal 2	0.107	0.090	0.219	0.673	0.316
	Animal 3	0.141	0.148	0.121	0.098	1.170
	Animal 4	0.192	0.337	0.457	0.317	0.331
	Animal 5	0.168	0.126	0.099	0.136	0.111
	Animal 6	0.125	0.111	0.086	0.135	0.641
	Animal 7	0.176	0.132	0.081	0.115	0.095
Farm C	Animal 1	0.176	0.340	0.184	0.189	N/A
	Animal 2	0.098	0.105	0.118	0.103	N/A
	Animal 3	0.163	0.307	0.311	0.335	0.193
	Animal 4	0.159	0.394	0.799	0.621	0.621
	Animal 5	0.281	0.259	0.165	0.873	0.385
	Animal 6	0.248	0.121	0.554	1.077	0.796
	Animal 7	0.183	0.715	0.515	0.467	0.586

Liver fluke FEC		Month				
		Sept	Early Nov	Late Nov	Jan	March
Farm A	Animal 1	0.0	4.0	2.4	N/A	N/A
	Animal 2	0.0	0.0	0.0	12.6	118.8
	Animal 3	0.0	0.0	0.0	3.2	51.0
	Animal 4	0.0	0.0	0.0	7.2	124.4
	Animal 5	0.0	0.0	0.0	1.6	44.2
	Animal 6	0.0	0.0	0.0	6.8	61.6
	Animal 7	0.0	0.0	0.0	10.0	119.2
Farm B	Animal 1	0.0	0.0	0.0	N/A	0.0
	Animal 2	0.0	1.4	0.0	4.4	4.6
	Animal 3	0.0	0.0	0.0	N/A	57.2
	Animal 4	0.0	0.0	0.0	3.2	17.2
	Animal 5	2.4	0.0	0.0	N/A	0.4
	Animal 6	0.0	0.0	0.0	N/A	0.2
	Animal 7	N/A	0.0	0.0	N/A	0.0
Farm C	Animal 1	0.0	0.0	0.0	0.0	N/A
	Animal 2	0.0	0.0	0.0	0.0	N/A
	Animal 3	1.2	0.6	0.4	2.2	0.4
	Animal 4	0.0	0.0	0.8	8.8	7.4
	Animal 5	0.0	0.0	0.0	N/A	4.0
	Animal 6	0.0	0.0	3.6	0.8	6.0
	Animal 7	0.0	0.0	0.2	3.6	1.8

Rumen fluke FEC		Month				
		Sept	Early Nov	Late Nov	Jan	March
Farm A	Animal 1	0.0	0.8	4.2	N/A	N/A
	Animal 2	0.0	36.8	50.0	158.7	5.6
	Animal 3	0.0	0.0	0.8	8.4	0.2
	Animal 4	0.0	0.0	0.4	2.1	1.2
	Animal 5	13.0	133.4	>200	184.4	0.0
	Animal 6	0.0	3.4	8.4	57.4	1.8
	Animal 7	4.8	75.2	172.8	>200	95.8
Farm B	Animal 1	43.4	137.6	89.0	N/A	> 200
	Animal 2	0.0	16.2	0.4	43.6	134.8
	Animal 3	6.6	0.4	0.4	N/A	>200
	Animal 4	5.4	63.2	0.0	0.0	0.0
	Animal 5	10.0	41.4	36.4	N/A	> 200
	Animal 6	0.0	0.0	0.6	N/A	> 200
	Animal 7	N/A	6.0	0.0	N/A	6.6
Farm C	Animal 1	1.8	12.6	7.8	0.4	N/A
	Animal 2	30.4	44.6	>200	>200	N/A
	Animal 3	36.2	50.2	40.2	134.4	>200
	Animal 4	14.2	171.2	17.4	28.0	41.2
	Animal 5	37.6	76.4	18.4	N/A	35.6
	Animal 6	22.8	41.4	126.0	>200	>200
	Animal 7	28.2	24.2	>200	173.4	>200