

Original Article

Assessment of accuracy of liver fluke diagnostic tests using the gold standard of total worm counts

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ABSTRACT

In many regions of New Zealand liver fluke is endemic, infecting most grazing ruminants, including cattle, sheep, and deer. Restricting the economic losses and welfare costs associated with liver fluke relies on accurately identifying those animals with a production limiting infection. This has proven a difficult goal and although several antemortem quantitative tests are available, including faecal egg counts (FEC), serum ELISA and copro-antigen ELISA, none can be considered a gold standard test of liver fluke infection. The accepted gold standard test for fascioliasis is the total fluke count, which is both laborious and can only be completed at post-mortem. This study aimed to compare the performance of four liver fluke diagnostic tests, against the results of a gold standard total fluke count test. Two groups of cattle were selected, 29 culled mixed age beef cows (MAC) and ten 30-month-old steers. The cattle were blood sampled and faecal sampled prior to slaughter and their whole livers recovered post slaughter at the abattoir. Liveweight was also recorded at slaughter. After collection, each liver was weighed, scored for gross pathology, then serum, faeces and livers were frozen at -20°C for later analysis. Faecal egg counts and *F. hepatica* copro-antigen ELISA tests were completed on the faecal samples and total fluke counts were completed on the livers. *Fasciola hepatica* antibody concentration in serum samples were quantified using a commercial ELISA test. Poisson regression models were built to model the association between each diagnostic test and the total fluke count, and a linear regression model was built to examine the relationship between each diagnostic test and live weight at slaughter.

The median fluke count was significantly higher in MAC than steers ($p = 0.01$), and *F. hepatica* eggs were present in 100% steers and 66% MAC. There was a significant effect of copro-antigen ELISA value on total fluke count ($p < 0.0001$), with a coproantigen ELISA value = 20.1 predicting 10 flukes and a value = 44.8 predicting 30 flukes. There was also a significant effect of FEC on total fluke count ($p = 0.002$) but the R-squared value for this model was lower. There was no association between liver fibrosis score or antibody ELISA test and total fluke count ($p = 0.95$, $p = 0.73$, respectively). There was a significant effect of total fluke count ($p = 0.03$) on liveweight at slaughter, with liveweight falling 20.4 kg for each unit increase in \log_e (total fluke count). There was no effect of FEC ($p = 0.11$), antibody ELISA ($p = 0.55$) or copro-antigen ELISA value ($p = 0.16$) on liveweight at slaughter. Taken together, these results show that the coproantigen ELISA test is the better test for estimating the true liver fluke burden and that the number of flukes in the liver has a negative effect on cattle live weights at slaughter.

1. Introduction

Liver fluke is endemic in some regions of New Zealand (Charleston, 1990, Harris, 1980), being reported in all grazing ruminants, however despite this, the ante mortem diagnosis of liver fluke infection in farmed livestock is still problematic. There are several tests currently available

to diagnose liver fluke infection in cattle and these broadly fall into four categories: gross pathology, conventional microscopy, antibody detection and antigen detection. Gross pathology includes liver scoring and detection of flukes in the bile ducts of the liver, conventional microscopy includes total fluke count and faecal egg counts, with antibody and antigen detection tests using enzyme linked immunosorbent assays

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(ELISA) which are performed on either serum, milk, or faecal samples.

Total fluke count (TFC) is considered the gold standard test (Mazeri et al., 2016)) but is not practical in the field since it can only be completed at post mortem and is also very time consuming. Gross pathological inspection of livers for damage consistent with liver fluke, as part of abattoir post-mortem inspections, is a common surveillance practice in many countries. However, the purpose of liver inspection is to determine the suitability of the organ for human consumption, not diagnosis of the cause of the pathology. Consequently, the resulting sensitivity of this technique can be quite low (63–68%), although when detecting the presence of liver flukes the specificity can be higher (88–100%) (Rapsch et al., 2006; Mazeri et al., 2016). It should be noted that the fungal disease pithomycototoxicosis (facial eczema), a common cause of pathological change to the livers of farmed ruminants in New Zealand, resulting in fibrosis and sclerosis (Lawrence et al., 2022) is a confounding factor when determining the cause of liver pathology in New Zealand. In the United Kingdom, it was found that the presence of liver pathology was a good indicator of either past or present liver fluke infection, with the extent of liver pathology strongly associated with the number of flukes present (Salimi-Bejestani et al., 2008). However, the United Kingdom does not suffer from facial eczema so it is unlikely that the accuracy of these results would be reproducible for New Zealand cattle. For this reason, it is important that other methods of diagnosis of liver fluke infection are thoroughly researched and validated for New Zealand conditions.

Faecal egg counts (FEC) have historically been used as a relatively simple method of investigating and monitoring liver fluke infection, However, the correlation between adult fluke burdens and FEC is low and variable (Charlier et al., 2008; Mezo et al., 2010a). FEC has a low sensitivity, ranging from 30% to 88% (Boray et al., 1969; Rapsch et al., 2006; Charlier et al., 2014; Kelley et al., 2021) and the poor accuracy which is further confounded by the lack of a universally accepted protocol for conducting the test. A consequence of the latter means that results can even vary between laboratories in the same country. For example, the specificity of the FEC was found to be higher using 4 g of faeces (100%) than 10 g of faeces (96%), due to less faecal debris obscuring the eggs (Charlier et al., 2008).

Several ELISA methods have been developed to detect and quantify liver fluke specific antibodies in the host either individually or as pooled samples and have subsequently been released as commercial products. These tests are marketed as both qualitative and quantitative, whereby a qualitative ELISA result can only determine a positive and negative diagnosis, whilst a quantitative ELISA can estimate the concentration of antibodies present, from which the burden of infection can be inferred. However, a common problem with the antibody based ELISA tests is that after removal of adult flukes, e.g., with anthelmintic treatment, the ELISA will continue to test positive for an indeterminate period, with the time taken for a titre to become negative being dependant on both the initial titre and the assay used (Munita et al., 2019).

The IDEXX Fasciolosis Verification test (IDEXX, Hoofdorp, Netherlands) is a commercial ELISA kit which utilises the f2 fraction of the ESA to detect host antibodies and is both highly immunogenic and specific for *F. hepatica* with sensitivity ranging from 82 to 99% and specificity 80–99% (Hutchinson and Macarthur, 2003; Molloy et al., 2005; Rapsch et al., 2006; Charlier et al., 2008).

More recently the ELISA protocol has also been modified to detect the presence of ESA rather than antibodies in either serum or faeces, using a highly specific monoclonal antibody, indicating current or very recent infection. To date, only one of these antigen detection assays has been commercialised, the coproantigen ELISA, BIO K 201 – Monoscreen AgELISA *Fasciola hepatica* test (Bio-X Diagnostics Rochefort, Belgium) which detects and quantifies the presence of parasite ESA in cattle faeces (Mezo et al., 2004; Brockwell et al., 2013; Mazeri et al., 2017). Since the test kit was launched, there have been two recent modifications to the manufacturer's instructions. The addition of an overnight incubation of the diluted faeces was found to increase the reactivity of the sample by

25%, without compromising the background OD reactivity, and the use of a lower cut-off value was found to increase the sensitivity of the test (Brockwell et al., 2013). Using the overnight incubation and lower cut-off value of 1.4 the test had a reported sensitivity of 87% and specificity >99% (Palmer et al., 2014), although results can be variable in chronic infections and where fluke numbers are low (Kelley et al., 2021).

The aims of this present study are to investigate and compare the performance of four diagnostic tests against a gold standard test, to detect and quantify liver fluke infection in two groups of naturally infected cattle from an endemically infected property in the North Island of New Zealand. The evaluated diagnostic tests included gross pathological assessment of livers, conventional microscopic FEC, a commercial antibody serum ELISA test and a commercial antigen faecal ELISA test. The ability of each of these diagnostic tests to accurately estimate the true burden of infection was assessed by comparing their results to the gold standard of the total liver fluke count performed on livers obtained post-mortem.

2. Materials and methods

2.1. Animal selection

The study population were Angus cattle from a beef and sheep farm in Taranaki, New Zealand (Latitude –38.925° South, Longitude 174.782° East). This farm was selected based on having a history of endemic liver fluke infection and the farmer's willingness to be part of the study. The same farm had previously been identified in 2011 as having triclabendazole-resistant *F. hepatica* in sheep (Hassell and Chapman, 2012) and liver fluke is commonly detected at necropsies of cull sheep, or at slaughterhouse post-mortem inspections of cattle and sheep from this property. As a result, faecal egg counts are regularly used to monitor infections, with anthelmintic treatments administered up to twice a year to sheep and young cattle (<3 years old), with older cattle (>3 years old) only receiving annual treatments. The cattle and sheep co-graze pastures on the farm, with limited use of forage crops for yearling cattle during the winter, which are grazed in-situ. Since identification of the triclabendazole resistance, the farmer has almost exclusively used an oral drench containing 1 mg/ml abamectin and 50 mg/ml closantel in sheep (Clomax Oral, Nexan Corporation Limited, Auckland, New Zealand) dosed at 1 ml/5 kg body weight, providing 200 micrograms/kg abamectin and 10 mg/kg of closantel and an injectable treatment containing 10 mg/ml ivermectin and 100 mg/ml clorsulon in cattle (Animec Super Solution for Injection, AHD Ltd., Hastings, New Zealand) dosed at 1 ml/50 kg, providing 200 micrograms/kg ivermectin and 2 mg/kg of clorsulon.

Two groups of cattle naturally exposed to liver fluke infection and with unknown infection status were selected for the study in autumn 2017, comprising all the 29 culled mixed age breeding beef cows (MAC) aged from four to ten years, identified for culling in the herd and the ten remaining 30-month-old finished steers (steers) that were grazed together and being sent for processing before winter. Cattle were not housed at any stage. All animals were born and raised on the farm and were identified with individual ear tags. The steers had been treated with a flukicidal anthelmintic nine months prior to slaughter and the cows had received no treatment in the previous 12 months.

2.2. Preslaughter sample collection

All animals in both groups of cattle were blood sampled at the farm of origin on the same day (1 May 2017) from the coccygeal vein using an 18 gauge 1" needle and 10 ml plain serum) vacutainer. Animals were also faecal sampled by gently inserting a gloved hand into the rectum with collected faeces placed in a 70 ml plastic pottle, with the rectal glove changed between animals. Each blood and faecal sample was labelled with the animals' herd ID tag. Blood and faecal sampling occurred on farm either five days (MAC) or 28 days (steers) prior to

transport to the abattoir. Faecal and serum samples were transported for four and a half hours in an insulated bag containing ice blocks to the Massey University, Palmerston North. On arrival at the laboratory, whole blood samples were centrifuged at 1100g for 15 min with duplicate serum samples pipetted from each tube and frozen at -20°C for up to 800 days for serological analysis. Faecal samples were split with 10 g being used for faecal egg counts and the remainder frozen for up to 800 days at -20°C for coproantigen analysis.

2.3. Slaughter sample collection

On the day of slaughter, the cattle were transported three hours to a commercial abattoir in Eltham, Taranaki and killed within six hours of arrival. Both groups of cattle, MAC and steers, were processed sequentially, together with lines of cattle from other farms, and their pre slaughter live weight, and post slaughter trimmed carcasses weight were recorded.

Printed unique sequential identifier number, with multiple tear-off replicates, were used to track the carcasses and respective offal through the abattoir. The tear-off portions adhered well to the tissues when wet, with only a slight risk of becoming dislodged. Each liver and gall bladder, identified by the unique identifier number, was removed from the processing line by abattoir staff, and placed in a plastic bag. The bagged livers were then packed into a box, containing up to three livers. The livers and gall bladders were then transported two and a half hours by car from the abattoir to Massey University, Palmerston North.

2.4. Gross pathological inspection of livers

The livers and gall bladders were initially examined either within three hours of collection (steers) if collected during the day or after chilling overnight (MAC) if collected overnight. Each liver was weighed, photographed and scored for gross pathology using a scoring system developed by Sargent et al. (2009). After the initial examination of the livers, they were then frozen at -20°C and stored for up to seven weeks to prevent putrefaction and until such time that they fluke counts could be completed.

2.5. Total fluke counts

The frozen livers were removed from the freezer, placed in high sided plastic trays (to hold the liver and fluids) and left to thaw over two to four days in a chiller, before being held at room temperature for dissection. Each liver was cut into segments with sides no longer than 20 cm to facilitate handling, then sliced into 1-2 cm wide strips. These strips were then massaged and flushed with warm water over a 250 μm aperture sieve to remove any flukes with all expressed material caught by the sieve. The free tissue caught in the sieve was then examined using an illuminated lens with 2 \times magnification and any suspected partial or complete fluke bodies removed, to be identified and counted using a stereoscopic dissecting microscope at 4 \times magnification. Total fluke counts were determined by summing all the entire flukes, all flukes that were $\geq 75\%$ intact and all segments containing oral suckers. No attempt was made to differentiate between adult and immature flukes.

2.6. Fluke faecal egg counts

Faecal egg counts (FEC) were performed using a Flukefinder® (Soda Springs, Idaho, USA) faecal egg detection kit following the manufacturers' instructions with the sedimentation modification described by Reigate et al. (2021). In brief, a tea sieve (mesh size 500 μm , 65 mm diameter) containing 10 g of faeces was placed in a stainless-steel bowl with tap water added to cover the faeces to facilitate mixing using a plastic spoon. After mixing, the liquid was collected in the stainless-steel bowl which was transferred into the Flukefinder®. Tap water was passed through the Flukefinder® flushing eggs through onto the lower section

of the device. Eggs and residual faecal matter from the lower section of the Flukefinder® were flushed into a bowl and then transferred into a 100 ml vertical cylinder. Tap water was added to the 100 ml mark, allowed to stand for 5 min then siphoned down to 5 ml before rinsing with an additional 95 ml of tap water. This process was repeated three to four times until minimal flocculant remained after siphoning to 5 ml. After agitation, the remaining liquid was transferred into a circular counting chamber supplied with the kit. One drop of methylene blue was added, and eggs were identified at 4 \times magnification. The total number of eggs counted was divided by 10 to calculate eggs per gram and a count greater than zero was classed as a positive FEC.

2.7. Antibody ELISA test

The concentration of *F. hepatica* antibody in serum samples were quantified using a commercial ELISA test (IDEXX) following the manufacturer's instructions. The IDEXX kit measures the antibody present to the f2 antigen and contains a positive control (PC) and negative control (NC). The colour reaction is read as an optical density (OD) at 450 nm on the microplate spectrophotometer and the sample-to-positive percentage ratio (SP%) is calculated with the following formula:

$$SP\%_{\text{sample}} = 100 \times \left(\frac{OD_{\text{sample}} - OD_{\text{NC}}}{OD_{\text{PC}} - OD_{\text{NC}}} \right)$$

Test interpretation categorises the individual serum samples as; *negative* (SP% ≤ 30), *mild positive* (30 < SP% ≤ 80), *positive* (80 < SP% < 150) and *strong positive* (SP% ≥ 150) for the presence of *F. hepatica* antibodies. The IDEXX ELISA testing was completed on all samples on the same day within 18 months of collection.

2.8. Antigen ELISA test

The concentration of *F. hepatica* antigen in faecal samples was quantified using a commercial *F. hepatica* copro-antigen ELISA test kit (Bio K 201, Bio-X Diagnostics, S.A. Rochefort, Belgium), following the manufacturer's instructions. Briefly two grams of faeces from each animal was diluted in 2mls of the buffer, with the following modification: all diluted samples were stored overnight at room temperature before centrifuging at 1000g for 10 min and collection of the supernatant. The kit comes with two 96 (8 \times 12) well microplates: Rows A, C, E and G of the 96 well microplate are coated in a polyclonal antibody specific to *F. hepatica* and rows B, D, F and H are coated in a polyclonal antibody not specific to *F. hepatica*. Thus, rows B, D, F and H act as the corresponding negative controls (NC) for rows A, C, E and G, with rows A to F reserved for testing faecal samples and rows G and H reserved for testing the positive control reference antigen, provided with the kit. The optical density (OD) was read at 450 nm using a microplate spectrophotometer (BioTek Epoch 2, Vermont, USA) and the Value (Val) for each sample calculated using the following formula.

$$Val_{(ue)} = 100 \times \left(\frac{OD_{\text{sample}} - OD_{\text{NC}}}{OD_{\text{PC}} - OD_{\text{NC}}} \right)$$

The positive cut-off value was ≥ 1.4 and all coproantigen ELISA testing was completed on all samples on the same day within 26 months of collection.

2.9. Statistical analysis

The raw data was summarised by the mean or median (if skewed) and range, for all cattle and categorised by age of cattle, i.e., MAC or steers. The distribution of the continuous data was checked for normality using histograms and an appropriate transformation was applied if the distribution was found to be skewed. Initial exploratory data analysis included constructing scatter plots to assess the relationship between two continuous variables and boxplots to assess the

relationship between continuous and categorical variables.

The *t*-test was used to test whether there was a significant difference in the mean liveweight, mean IDEXX ELISA SP% results, and mean coproantigen value between MAC and steers. However, the non-parametric Wilcoxin signed rank sum test was used instead for faecal egg counts (eggs/g), number of flukes and liver score data, which were all not normally distributed. For categorical data, Fisher's Exact Test was used to test whether there was independence between MAC and steers for IDEXX categories, for being coproantigen positive or being total fluke count positive animals.

Poisson regression models were built to examine the association between each diagnostic test and the total fluke count, and a linear regression model was built to examine the relationship between each diagnostic test and live weight at slaughter. For all models, only two variables, in addition to the diagnostic test, were tested in the model, group (MAC or steer) and an interaction between group and the diagnostic test, with variables retained at $p < 0.05$. Model fit for the Poisson model was checked by calculating Nagelkerke's pseudo- R^2 and assessing the distribution of the model residuals using the DHARMA package (Hartig, 2020) and for the linear model, was checked by assessing the distribution of the residuals.

Two by two tables were constructed to estimate the sensitivity and specificity of each diagnostic test against the gold standard total fluke count using the epiR package developed by Stevenson et al. (2022).

All analysis was performed using R studio (RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL <http://www.rstudio.com/>)

3. Results

Exploratory data analysis showed that there were two steers with high coproantigen values (value = 54 and 28 respectively), positive faecal egg counts but zero total fluke counts. The blood and faecal samples were collected at the same time, on-farm, by the first author, but the livers were collected by abattoir staff, without oversight by the first author. Since a zero-fluke count is incompatible with a high coproantigen result and a positive FEC and given that the steers had not been treated with an anthelmintic active against liver fluke for 9 months, the results for these two steers were removed from those analyses where gross liver score, liver weight or the total fluke count was a variable. However, the results from these two steers were retained for analyses of results from diagnostic tests completed on blood and faeces.

3.1. Summary data

The results for each test are summarised in Table 1 for the mixed age cows (MAC), steers and combined.

3.2. Liveweight

The mean liveweight of the ten steers was 100.8 kg (95% CI, 55.3–146.3) more than the mean liveweight of the 29 MAC ($p = 0.0004$), Table 1.

3.3. Liver examination and total fluke counts

The median liver fibrosis score was significantly higher for MAC than for the steers ($p < 0.0001$), but there was no difference in the mean liver weight between the two groups ($p = 0.92$). Flukes were recovered from the livers of 6/8 (75%) steers, with a median count of 3.5 and a maximum count of 10 flukes per animal, and in 28/29 (97%) MAC livers, with a median count of 8 and a maximum count of 74 flukes per animal. There was no evidence of a difference in the proportion of MAC with flukes compared to the steers ($p = 0.12$), although the median fluke count was significantly higher in MAC than steers, ($p = 0.01$), Table 1.

Table 1

Summary of results of the diagnostics tests performed on samples from 29 mixed age cows (MAC), and 10 steers, with the results combined over both groups.

Sample	Parameter	MAC	Steers	All animals
Liveweight	Mean Liveweight kg (range)	520 (416–606) ^a	620 (517–686) ^b	545 (416–686)
	Liver flukes present (%)	28/29 (97%) ^a	6/8 (75%) ^a	34/37 (92%)
Liver*	Median number of flukes (range)	8 (0–74) ^a	3.5 (0–10) ^b	7 (0–74)
	Mean Liver weight kg (range)	7.6 (6.5–8.9) ^a	7.5 (6.4–9.1) ^a	7.6 (6.4–9.1)
	Median Liver Score (range)	5 (4–5) ^a	4 (2–5) ^b	5 (2–5)
	Mean IDEXX	235	282	247
Serum	Elisa SP% (range)	(129–343) ^a	(251–305) ^b	(129–343)
	IDEXX ELISA Category			
	Strong Positive (%)	28/29 (97%)	10/10 (100%)	38/39 (97%)
	Positive (%)	1/29 (3%)	0/10 (0%)	1/29 (3%)
	Mild Positive (%)	0/29(0%)	0/10(0%)	0/39 (0%)
	Negative (%)	0/29(0%)	0/10(0%)	0/39 (0%)
	Fluke faecal eggs present (%)	20/29 (69%) ^a	10/10 (100%) ^a	30/39 (77%)
Faeces	Median Faecal Egg Count epq (range)	0.1 (0–3.5) ^a	0.5 (0.1–5.2) ^a	0.3 (0–5.2)
	Coproantigen positive (%)	24/29 (83%) ^a	5/10 (50%) ^a	29/39 (74%)
	Mean Coproantigen Value (range)	19.2 (–2.1–58.7) ^a	10.8 (–0.1–54.55) ^a	17 (–2.1–58.7)

^a ^b different suffixes in same line denote statistical significance at $p < 0.05$, contrasting MAC and steers.

* The results of all liver related tests are shown for only 8 steers

3.4. Serum antibody ELISA testing

All cattle in both the MAC and steer groups tested positive in the IDEXX ELISA with one MAC being in the *positive* diagnostic category and all other cattle being in the *strong positive* category (Table 1). The IDEXX ELISA SP% results for the steers were on average 50 (95% CI, 25.8–70.2) SP% higher than the MAC ($p < 0.0001$), but there was no evidence that the distribution of the IDEXX ELISA category results for MAC was different to steers ($p = 1$). Since all ELISA results were positive no meaningful sensitivity and specificity could be calculated.

3.5. Faecal analysis

Fasciola hepatica eggs were present in the faecal samples collected from 10/10 (100%) steers and 21/29 (66%) MAC. There was no evidence for a difference in the proportion of animals with a positive FEC ($p = 0.08$) or the median FEC ($p = 0.13$) between MAC and steers (Table 1).

Furthermore, there was no evidence for a difference in the proportion of animals that were antigen ELISA test positive ($p = 0.09$) or for the mean antigen ELISA test value ($p = 0.22$), between MAC and steers.

The coproantigen test had a sensitivity of 0.76 (95% CI, 0.59–0.89) and a specificity of 0.67 (95% CI, 0.09–0.99) and the FEC had a sensitivity of 0.76 (95% CI, 0.59–0.89) and a specificity of 0.33 (95% CI, 0.01–0.91), using the total fluke count as a gold standard.

3.6. Multi-variable analysis

There was no evidence for an association between liver fibrosis score and total fluke count ($p = 0.95$) adjusting for the effect of group. Nor was there a significant association between the antibody-based ELISA test

and total fluke count ($p = 0.73$).

A negative binomial regression model was fitted to the data, after a Poisson model previously fitted to the same data was found to be over-dispersed. The final model showed a significant effect for coproantigen ELISA value ($p < 0.0001$). Group was not significant ($p = 0.08$), nor was the interaction between group and coproantigen ELISA value ($p = 0.21$). The final predictive model was:

$$\text{Total fluke count} = 1.407 + 0.044 \times \text{coproantigen ELISA value}$$

Inverting the regression equation, an infection of 10 adult fluke would be predicted by a coproantigen ELISA value of 20.1 while a burden of 30 flukes would be predicted by a coproantigen ELISA value of 44.8. Overall, an increase of 5 for the coproantigen ELISA value, e.g., from 20 to 25 or from 5 to 10, predicts a 25% increase in the adult fluke count. The residuals from the final model showed no evidence of non-linearity, KS test $p = 0.83$, with a Nagelkerke pseudo- $R^2 = 0.63$. The fitted regression line from the model shows a curvilinear relationship between coproantigen values and total fluke count, Fig. 1.

The final negative binomial model showed significant support for an effect of FEC ($p = 0.002$) and an interaction between FEC and group ($p = 0.02$). The final predictive model was:

$$\text{Total fluke count} = 2.165 + 0.607 \times \text{FEC} - 0.743 \times \text{steer} - 0.668 \times \text{FEC} \times \text{steer}$$

At the overall mean FEC of 0.7 epg, the model predicts that MAC have 13.0 (95% CI, 9.0–13.0) adult flukes and that steers have 4.0 (95% CI, 1.3–6.7). The residuals from the final model showed no evidence of non-linearity, KS test $p = 0.41$, with a Nagelkerke pseudo- $R^2 = 0.41$.

The model found support for a significant effect of total fluke count ($p = 0.03$) and group ($p = 0.001$) on liveweight at slaughter, with no significant interaction between total fluke count and group found ($p = 0.2$).

$$\text{liveweight (kg)} = 565.6 - 20.4 \times \log_e(\text{total fluke count} + 1) + 79.1 \times \text{steer}$$

The model showed that the liveweight fell by 20.4 kg for each unit increase in \log_e (total fluke count) and that steers were on average 79.1 kg (95% CI, 38.4–120) kg heavier than MAC at slaughter. The $R^2 = 0.48$ and the residuals were normally distributed ($p = 0.62$ Shapiro-Wilk test) (Fig. 2).

Adjusting for group, there was no support for an effect of FEC ($p = 0.11$), antibody ELISA SP% ($p = 0.55$) or antigen ELISA value ($p = 0.16$), on liveweight at slaughter.

4. Discussion

The use of the total fluke count as a gold standard test in this study confirms the contradictory nature of liver fluke diagnostic tests in naturally infected animals and that their interpretation is likely to lead to biased conclusions about *Fasciola hepatica* infection in cattle. Although the total fluke count is considered a gold standard diagnostic test for liver fluke (Mazeri et al., 2016), the test itself requires meticulous dissection of the whole liver and even for just 39 livers was a formidable undertaking, such that the challenges of performing this diagnostic technique on a larger cohort of animals cannot be underestimated. Thus, having reliable laboratory serological or faecal tests would be advantageous for the diagnosis and management of *F. hepatica* infections in cattle. Taken together, the results of this study provide the

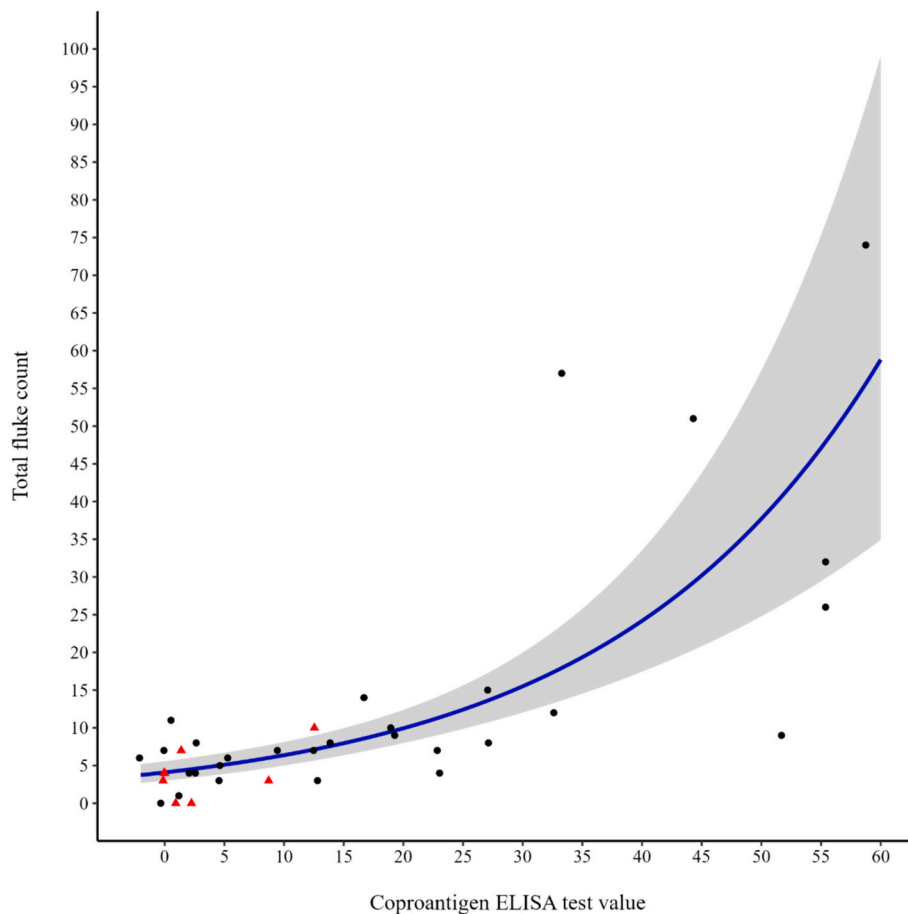


Fig. 1. Scatterplot of total fluke count against coproantigen ELISA test value, categorised by group, with fitted negative binomial regression line, blue line, and 95% confidence interval, grey area. Black dots are mixed age cows and red triangles are steers.

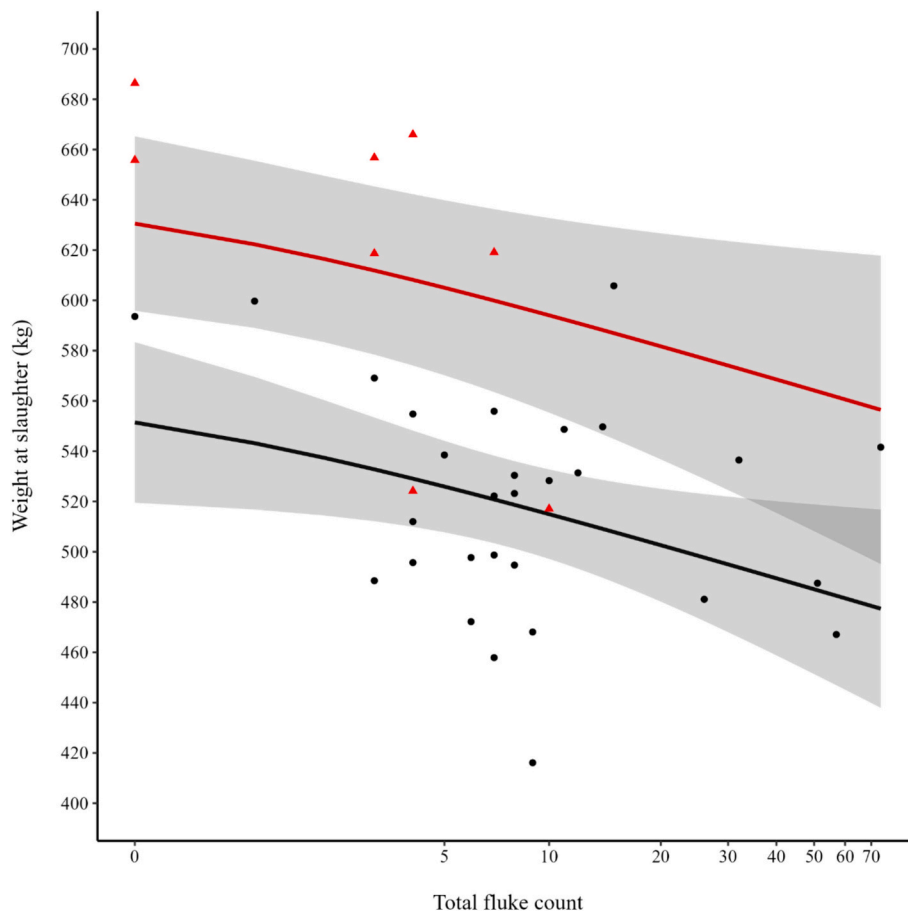


Fig. 2. Scatterplot of weight at slaughter against total fluke count, categorised by group, with fitted linear regression line, and 95% confidence interval. The black line and dots are mixed age cows, and the red line and triangles are steers. Please note the X-axis is on the natural log scale.

first indication that the coproantigen ELISA test can estimate the number of adult flukes in the liver with reasonable accuracy. In addition, this study is the first to suggest that the number of adult flukes in the liver has a negative effect on cattle live weights at slaughter in New Zealand beef cattle.

The reduction in weight described in this study was similar to that reported by Malone (1986) and is possibly the first time this association has been observed in New Zealand. Overseas studies have found substantial reductions in live weight gain for individual cattle with artificial liver fluke infections, of 8% with 40 flukes, 8–9% with 54 flukes and 28% with 140 flukes (Cawdery et al., 1977; Malone 1986). Others have suggested that production losses in individual cattle can occur at even lower fluke numbers, at 1–10 adult flukes (Mazeri et al., 2017), at >10 adult flukes (Charlier et al., 2008) or at ≥ 30 adult flukes (Vercruyse and Claerebout, 2001). In the current study, the animals were managed as comparably as possible within a farm system and sent to slaughter based on the need to reduce farm numbers, before the winter period, thus not selected on weight, but rather age and other management factors. Had selection been based on reaching a minimum target weight then it is likely that the more heavily infected animals would have taken a lot longer to finish, which supports the findings of a Scottish study where naturally infected cattle with as few as 1–10 flukes took 10 days longer to reach slaughter weight compared to cattle with no evidence of fluke (Mazeri et al., 2017).

Extrapolating the impact of fluke infection at the individual to the herd level is, however, confounded by several factors including herd prevalence of infection and the different stages of infection within the herd at any one time point. Significant production losses were noted in herds with infection prevalence >25% (Koopman, 1968, Malone 1986),

which has led to the recommendation that a herd infection prevalence of >25% is the threshold for treatment (Vercruyse and Claerebout, 2001). In this study the herd prevalence of infection was 92% based on the gold standard so it is not surprising that an effect on weight was seen.

An association between coproantigen ELISA value and fluke count was determined in naïve, artificially infected cattle in Australia with $R^2 = 0.87$ (Brockwell et al., 2013) and in a different Australian study of naturally infected dairy cows the $R^2 = 0.64$ (Kelley et al., 2021). In this study we determined a significant association ($p < 0.001$) between the antigen-detection coproantigen test and total fluke count with an adjusted $r^2 = 0.61$. Using our sample of cattle, a coproantigen ELISA test value >20.1 and > 44.8 are consistent with an infection of 10 or more and 30 or more adult flukes respectively.

The coproantigen ELISA detects fluke ESA once the flukes are in the biliary system, so the assay is reliant on the ESA protein being present in the faeces and not on the host response to the presence of the flukes. Daily variation of the coproantigen ELISA value of 2.6 to 8.9-fold have been recorded in cattle (Kelley et al., 2021) due to episodic ESA release from the gut of the flukes, changes in faecal consistency (Brockwell et al., 2013), ELISA kit variability (Kelley et al., 2021) and the volume of faeces passed each day. Despite these confounding factors, this study confirms the value of the antigen ELISA test to estimate the total adult flukes and potentially detect production limiting infections. It should be noted that the resulting model from this study does have difficulties in estimating low numbers of adult flukes in the liver. The cut point value used for a positive animal was 1.4, which would give a predicted liver fluke burden 4.3 (95% CI, 3.2–5.9). This may explain why there was no effect of coproantigen ELISA value on live weight at slaughter, since even low numbers of adult fluke had a significant effect on live weight,

which the coproantigen ELISA test cannot accurately estimate.

The study had two potential limitations, the first was the issue of two steers with high coproantigen ELISA values (54 and 28) and positive FEC that had zero total fluke counts, which suggests that mismatched samples or tissues had been collected, and the second was the long storage of faeces before testing (795 days). The slaughterhouse operates as a continuous chain with identification relying on the animals being allocated the correct unique identifier. Many hundreds of animals from different farms are processed in a day, with animals of similar carcass dimensions being processed as a group to improve efficiency of the processing and packaging of the meat and other products, creating the risk of animals being misidentified. It is possible that mislabelling may have also occurred between the time of faecal sampling and slaughter although this risk is low as the individual animals were identified using their unique animal identification tag. These two data points were dropped whenever an analysis involved the total fluke count, gross liver score or liver weight. The faeces were frozen at -20°C for 795 days and in this time the antigens may have degraded reducing the test reactivity. Although no literature was found on the effect of freezing on the performance of the test in cattle, faecal samples were frozen for 12 weeks in sheep, 17 weeks in humans and 12 months in red deer without impacting the optical density (Ubiera et al., 2009; Flanagan et al., 2011; French et al., 2016). Given that the animals in this study returned high coproantigen values at the time the samples were tested, it seems unlikely that the antigens degraded during storage.

Liver fluke prevalence of cattle of 20–50% (Charlier et al., 2008; Salimi-Bejestani et al., 2008; Mazeri et al., 2016; Zalizar et al., 2021) and 78% (Anderson et al., 1999) are less than the very high overall prevalence found in this study (92%). The prevalence in the subset of steers (75%) was similar to other studies for cattle of a similar age (Salimi-Bejestani et al., 2008; Mazeri et al., 2016). Based on published suggestions for liver fluke burdens, the total fluke counts in this study (median 7, maximum 74) and the number infected (34/37, 92%) are considered high enough to impact animal performance at both the individual (Charlier et al., 2008) and herd level (Vercruysse and Claerebout, 2001).

The *F. hepatica* faecal egg counts observed in this study were very low and considerably less than previous studies ((Anderson et al., 1999; Phiri et al., 2005; Elitok et al., 2006; Novobilsky and Hoglund, 2015; Kelley et al., 2021), with only the study by May et al. (2019) having similar FEC. Experimental studies using artificial infections, have shown that egg output reaches a peak 18–20 weeks post-infection (WPI) and then decreases to very low levels 30–40 WPI (Dixon, 1964; Doyle, 1971; Kendall et al., 1978; de León et al., 1981). This shedding dynamic is possibly due to calcification of the bile ducts trapping eggs as the host appears to tolerate the chronic infection and focus on tissue repair (Donnelly et al., 2021), with adult cattle usually having low egg counts (Boray, 1969). Although it is not known when the cattle used in this study became infected, the farm has a history of chronic infection in both sheep and cattle (Hassell and Chapman, 2012). Thus, it is plausible that the infection had become chronic in nature at the time of slaughter despite annual anthelmintic treatment. The delay between faecal sampling and slaughter (5 days MAC and 28 days steers) was not considered to impact the FEC results, as the cattle had been exposed to metacercariae throughout the previous 12 months, since last treatment in the cows and nine months in the steers. The farm has a temperate climate, so intermediate host snails are likely to be active from at least spring to autumn ensuring a continuous population of metacercariae over the summer and autumn, but this should be investigated further.

Internationally, an egg count of $\geq 5\text{epg}$ is considered to indicate a production limiting fluke infection (Malone and Craig, 1990; Vercruysse and Claerebout, 2001). However, this study showed that reliance on FECs alone could lead to a contradictory result. For example, in this study 28% of cattle had a fluke burden ≥ 10 , indicative of a production limiting infection, while the FEC were very low (0.3epg (range 0–5.2)) which would not indicate a production limiting infection. Although an association between FEC and total liver flukes was shown, there was a

significant interaction between FEC and group showing that the relationship in this group of cattle is different depending on whether the animal was a MAC or steer, with MAC having much higher total fluke counts at similar FEC. Charlier et al. (2008) reported that the presence of any eggs in 10 g of faeces is 2.5 times more likely to indicate an animal with a production limiting infection (> 10 flukes) as compared to an animal with a lower level of infection or not being infected at all, while the presence of any eggs in 4 g of faeces was 10.7 times more likely to detect an animal with a production limiting infection. Therefore, as this current study used 10 g of faecal material the interpretive value for the assay could be improved by using only 4 g of faeces. Moreover, for sampling cattle in a population where fluke is endemic, the use of 4 g of faeces could have more diagnostic value.

All the animals tested in this study were positive using the serum antibody ELISA test, with 38/39 (97%) IDEXX results being *strong positive*. Moreover, many of the positive sample readings were beyond the linear range of the assay. Antibody concentration in the host is dependent on both parasite and host factors and has been shown to be a complex interaction in naturally infected animals with repeated or chronic liver fluke infection (Gorman et al., 1997; Ortiz et al., 2013; Walsh et al., 2021). The very high antibody ELISA results made test analysis almost impossible and as a result, further studies are required in populations with a greater range of antibody concentrations to determine if there is any association between ESA antibody titre and current fluke burdens.

As these antibody assays detect the host response to the presence of the liver flukes, they reflect both current and past exposure. Thus, the detection of antibodies in serum or milk could be due to new infection, the continued development of the fluke life cycle within the host, fluctuations of the host's immune response or antibody decay from an initial high concentration (Mazeri et al., 2016), with the difficulty for the veterinarian being that none of these potential reasons are easily identified. In addition, seasonal variation of sensitivity and specificity has also been reported for the antibody and antigen ELISA with the test characteristics for summer and autumn samples being lower than for winter and spring samples (Charlier et al., 2008; Mazeri et al., 2016). It is not known if a similar effect occurs in New Zealand but since these aforementioned studies were in winter housed cattle it possibly doesn't occur. However, it potentially adds an additional level of complexity when interpreting ELISA results.

The host antibody response is greatest 3–12 weeks post infection (Mezo et al., 2010b) and the IDEXX SP% can double over 90 days as the fluke infection matures (Mazeri et al., 2016). The inability of the antibody ELISA to quantify the current fluke burden found in this study is a frustration identified in other studies conducted in Australia and Belgium (Hutchinson and Macarthur, 2003; Charlier et al., 2008).

Taken together, the results from this study revealed that the coproantigen ELISA was superior to other diagnostic tests at quantifying the fluke burden in naturally infected cattle. The presence of one egg in a faecal sample confirms infection while the antibody ELISA tests reflect the immune response of the host, but in this study the coproantigen ELISA gives the veterinarian the ability to estimate the number of flukes present and the production cost of that burden as indicated by the lower slaughter weight of animals with higher fluke counts.

In conclusion the coproantigen ELISA test appears the best diagnostic test for estimating the true liver fluke burden, which means that practitioners can now advise their clients whether anthelmintic treatment will result in a production response.

Ethical approval

This experiment was performed under the approval of Massey University Animal Ethics Committee, Protocol 15/92

CRedit authorship contribution statement

A. Dowling: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **K.E. Lawrence:** Writing – review & editing, Supervision, Methodology, Formal analysis. **L. Howe:** Writing – review & editing, Supervision, Methodology. **I. Scott:** Writing – review & editing, Supervision. **W.E. Pomroy:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology.

Declaration of competing interest

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.

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