Horses are susceptible to natural, but resistant to experimental, infection with the liver fluke, *Fasciola hepatica*.

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Highlights:

- Infection of horses with the liver fluke, *Fasciola hepatica*, is relatively common
- There is little information available on the epidemiology and pathogenesis of fasciolosis in this host
- Experimental challenge did not establish either infection or seroconversion in this study
- The performance of antibody-detection and coproantigen tests established for diagnosis of fasciolosis in ruminants is not optimal in horses
- Many questions remain to be answered in understanding the importance of liver fluke infection for equine health and welfare

Abstract

Fasciola hepatica is a common parasite of livestock in Ireland, causing significant economic losses and affecting animal welfare. A previous abattoir study of 200 horses led to an estimated 9.5% prevalence of infection in horses slaughtered in Ireland. However, the epidemiology and pathogenic significance of this infection in this species is not well-described.

The objectives of this study were to determine the susceptibility of horses to oral challenge infection with *F. hepatica* metacercariae, and to document the course of the infection along with serological and biochemical response.

We attempted an experimental infection of horses (n=10; 9 geldings and 1 mare) with *F. hepatica*. Four were given 1000 metacercariae, four 500 metacercariae and two were sham-infected. Blood and faecal samples were taken at intervals up to 18 weeks post-infection (wpi). ELISA assays were used to assess sero-conversion in the experimental horses and also in a panel of sera from horses of known fluke status.

No flukes were recovered from any of the livers, and neither were any lesions that could be attributed to *F. hepatica* infection observed. Coproantigen ELISA was negative throughout for all horses. Three antibody detection ELISAs, useful in diagnosing fasciolosis in other species, had limitations as diagnostic aids as determined using a panel of sera from horses of known *F. hepatica* infection status.

This study is limited by the relatively small number of animals included, and the relatively short duration of the study period.

Failure to establish infection after oral challenge raises fundamental questions on the pathophysiology and epidemiology of equine fasciolosis.

Keywords: Liver fluke, *Fasciola hepatica*, horses, fasciolosis, susceptibility, serology.

1. Introduction

Infection with *Fasciola hepatica* or the common liver fluke is widespread in livestock in Ireland, due to the favourable environmental conditions for the liver fluke and its intermediate host, most commonly the mud snail G*alba truncatula*. In dairy herds, bulk milk ELISA surveys have led to estimates of liver fluke exposure in 82% (Selemetas et al., 2015). Other mammalian species including goats, deer and horses which graze on fluke-infected pastures can also become infected (Taylor et al., 2007).

F. hepatica is found in equine livers in Europe as documented in anecdotal reports, peer-reviewed clinical cases and surveys [Howell et. al., 2019; Williams and Hodgkinson, 2017). The prevalence of liver fluke infection in the horse can be high, for example with 60% seroprevalence reported in Spain (Arias et al., 2012) but tends to be less than in ruminants (Quigley et al., 2015).

Despite these reports, experimental infections have not been successful, and relatively few investigations documenting the equine response to experimental infection are found in the literature (Nansen et al., 1975; Alves et al., 1988;. Soulé et al., 1989). The reasons for this dichotomy are not well understood, but could be due to method of infection, strain of parasite, or factors related to individual hosts. We therefore undertook an experimental challenge to further understand the equine response to *F. hepatica* challenge.

We also used a sub-group of 82 equine sera of known fluke status to compare the sensitivity and specificity of three antibody-detection ELISAs in the horse; an in-house CL1 ELISA (Collins et al., 2004), an ELISA using a recombinant surface protein, Paz-Silva et al., (2012) and an ELISA based on purified ES proteins from the parasite. (Howell et al., 2019).

2. Materials and Methods

2.1 Animals

Ten horses destined for the food chain were procured from a commercial source and maintained on pasture at UCD Lyons Research Farm. The horses were of mixed breeds, and between 2 and 20 years of age, as described in Table 1. Horses were randomly assigned to either infection or control groups, and none had *F. hepatica* eggs in faeces. Prior to the start of the experiment all horses were treated with 0.4mg /kg moxidectin and 2.5mg/kg praziquantel (Equest Pramox, Zoetis), and 12mg/kg triclabendazole (Fasinex 10% oral solution, Elanco).

2.2 Experimental infection

F. hepatica metacercariae (n=10,000), Italian strain, were obtained from Ridgeway Research, Gloucester, UK). Fluke viability was assayed by *in vitro* excystation followed by observation of metacercarial mobility. A suspension of 150 metacercariae per ml of distilled water was prepared. The fluid was swirled to ensure even distribution of the metacercariae prior to loading syringes, and doses of 500 (horses 1-4, Group A) and 1000 (horses 5-8, Group B) individual viable metacercariae were administered by syringe. The 20 ml syringe was inserted through the side of the mouth, and the contents expelled onto the back of the tongue. The same syringe was then re-loaded with water and the horses dosed again to ensure any metacercariae remaining in the syringe were administered. Two of the 10 horses (Group C) were sham infected using water only.

2.3 Sampling

Blood was taken on day of infection and at four weekly intervals thereafter. Blood samples were obtained by jugular venepuncture. The vein was raised using digital pressure, and 9 ml of blood collected in a plain vacutainer using a 19-gauge needle. The jugular region was examined post-

sampling for any signs of haematoma, swelling or bleeding. The side of the neck used was alternated at every venepuncture. After the blood had clotted, vacutainers were centrifuged at 5000 g for 10 min and supernatants aliquoted and stored at -80°C until assay. Faecal samples were taken per rectum at 14 and 18 wpi.

2.4 Serological analysis

Sera from the 10 experimental horses were examined for antibodies specific for *F. hepatica* using an in-house ELISA based on a recombinant mutant cathepsin L1 antigen (rmFhCL1, Collins et al., 2004) at day of infection, and at 4, 8, 12, 16, and 18 weeks post-infection (wpi). Eighty-two serum samples collected as part of a previous abattoir survey of horses (6) were also examined using this assay, plus two other antibody detection assays, one based on purified *F. hepatica* ES antigens (Howell et al., 2019) and the second on FhrAPS, a 2.9 kDa recombinant protein derived from the fluke tegument (Paz-Silva et al., 2012).

2.4.1 Recombinant mutant Cathepsin L1 ELISA

ELISA plates were coated with recombinant mutant CL1 (Collins et al., 2004) at a concentration of 1 µg/ml in 50 mmol/l carbonate/bicarbonate coating buffer and incubated for 1 h at 37°C. Columns were alternately coated with antigen or with buffer only to provide a background control. Plates were washed 3 times with phosphate-buffered saline with Tween 20 (PBST), and this wash protocol repeated after each incubation. Plates were blocked with 5% skimmed milk powder in PBST at 100 µl per well, and incubated for 1 h at 37°C. Sera were diluted 1:100 in 2% skimmed milk powder in PBST, and 100 µl per well, added (in duplicate), and incubated for 1 h at 37°C. HRPO-conjugated goat anti-horse immunoglobulin IgGT (Biorad) was diluted 1:20,000 in the same buffer, added at 100 µl per well, and incubated for 1 h at 37°C. 3,3',5,5'- Tetramethylbenzidine (TMB) substrate was added, at 100 µl per well, colour was allowed to develop for 10 min and then stopped with 1 mol/l H_2SO_4, at 100 µl per well. Plates were read on a Dynamica LEDetect plate reader at 450nm and corrected optical densities (ODs) were calculated by subtracting the background OD for each serum sample

incubated on non-antigen coated wells. The cut-off value for the test was determined to be 0.15 at OD 450 nm.

2.4.2 FhrAPS Indirect ELISA

ELISAs using the *F. hepatica* FhrAPS, a 2.9 kDa recombinant protein were performed on serum samples as previously described (Paz-Silva et al., 2005). The protein concentration used to coat the wells of the polystyrene plates was 3 µg/ml, sera were diluted (tested in duplicate) at 1:200 in PBS-0.3% Tween 20 and 10% skimmed milk, and horseradish peroxidase conjugated protein G (Nordic Immunology Laboratories) at 1:1000. Substrate consisting of 10 mg of ortho-phenylenediamine, 12 ml citrate buffer and 10 µl of 30% hydrogen peroxide was then added to each well. The plates were incubated in the dark for 10 min at room temperature. The enzymatic reaction was stopped with 100 µl per well of 3N sulphuric acid, and absorbances were read using a spectrophotometer (Titertek Multiskan) at 492 nm.

2.4.3 *F. hepatica* Excretory-Secretory (ES) ELISA

An ES antibody detection ELISA validated in cattle was used, with minor modifications as described by Howell et al., 2019. Briefly, the modified protocol involved the use of 2% BSA as a blocking buffer, dilution of equine serum samples to 1:200, and use of a goat anti-horse HRPO conjugate (Biorad) as secondary antibody.

2.5 Biochemical Analysis

Serum glutamate dehydrogenase([GLDH) and g-glutamyltransferase (GGT) were measured using a Randox RX Imola analyser. Samples were also tested for bile acid levels using the same analyser

2.6 Faecal Analysis

Faecal samples were collected at 14 and 18 wpi and were assayed by sedimentation for *F. hepatica* eggs and by *F. hepatica* coproantigen ELISA (Bio-X Diagnostics).

The horses went to abattoir at 20 wpi. On the day of collection, livers were examined for signs of pathology or overt signs of fluke infection. They were then kept frozen at -20°C until determination of fluke burden. Livers were thawed for 24h minimum, and each liver was cut into approximately 8 cm slices and placed into warm water. Each slice was then cut into approximately 1 cm cubes and further examined for flukes. Liver cubes (approximately 15 at a time) were collected in muslin gauze and thoroughly squeezed to release any parasites, following which the liquid was filtered twice, first in a sieve and then in a 0.35 micron mesh filter, and any particulate matter retained for examination.

3.0 Results

3.1 Viability of metacercariae.

The viability of the metacercariae used for this protocol was 70%, in line with other batches received by our laboratory. The same batch of metacarcariae were used for experimental infection of a group of young cattle, in a separate study, three months after the challenge in this study. Each animal in this study was orally dosed with 150 metacercariae, and the establishment rates were between 42-63 flukes at post-mortem examination.

3.2 Gross morphology of livers and fluke burden

Observations were made on each liver post-mortem. There were no overt signs of liver fluke infection. Bile ducts were not calcified or enlarged and no liver flukes or tracts were observed. Evidence of prior *Echinococcus granulosis* infection (large hydatid cyst) was present in liver number seven. Experimental challenge with *F. hepatica* failed to establish patent infection.

3.3 Faecal analysis

160 Faecal samples collected at both time points were negative for fluke eggs, and also for the *F.*
161 *hepatica* coproantigen test.

162 3.4 Serum biochemistry and antibodies

163 Three of the horses in Group B, which were challenged with 1000 *F. hepatica* metacercariae, had
164 serum levels of GGT above the reference range at various timepoints (Figure 1a). However, in the
165 case of two of these animals, numbers 5 and 8, elevated values were already present at the day of
166 infection. Values in horse 5 returned to the normal range by 8 wpi, but generally remained elevated
167 in horse 8. In horse 7, a transient elevation was measured at 8 wpi only. Horse 5 also had elevated
168 GLDH levels at the day of infection (Figure 1b). For all other samples GLDH was within the reference
169 range. No elevations in bile acids above the reference range were recorded (Figure 1c).

170 Of the ten horses assayed from the three experimental groups, four were positive for antibodies
171 specific to rmFhCL1 (Figure 2). Two horses had moderately high levels of antibodies, namely, #3
172 and #7, from Group A and Group B, respectively. Horses #6 (Group B) and #10 (control, Group C)
173 both had slightly elevated levels of antibodies. No *F. hepatica* parasites were found in any of the
174 livers.

175 **3.5 Comparison of Cathepsin L1, 2.9 kDa recombinant surface protein (FhrAPS) and ES ELISAs for**
176 **detection of antibodies against *F. hepatica* in horses.**

177 When used to compare performance on 82 serum samples from horses of known fluke infection
178 status collected as part of a previous abattoir survey [6)] the FhrAPS ELISA gave the highest
179 sensitivity, 72% Sn (95% C.I. 46.5 to 90.3%), but also the lowest specificity, 30% Sp (95% C.I. 18.9 to
180 42.4%). The ES ELISA showed a sensitivity of 67% (95% C.I. 40%-87%) and a specificity of 97% (95%
181 C.I. 89%-100%) and the rmFhCL1 ELISA had the lowest sensitivity, 50% Sn (95% C.I. 26%-74%) and
182 the highest specificity of 100% Sp (95%C.I. 94%-100%), as shown in Table 2.

Kappa values were determined to measure agreement between the tests, (Table 3). The rmFhCL1 ELISA and the ES ELISA have a kappa value of 0.57, which indicates moderate agreement. The kappa value for the CL1 ELISA and the FhrAPS ELISA is 0.067, which indicates there is slight agreement (Dohoo et al., 2003).

4.0 Discussion

Our experimental challenge study supports previous observations on the difficulty in establishing experimental infection of horses with *F. hepatica* (Nansen et al., 1975; Alves et al., 1988;. Soulé et al., 1989), in spite of a prevalence of 9.5 % of infection in horses in a relatively recent abattoir study in Ireland (Quigley et al., 2017). Neither did our experimental challenge protocol provide convincing evidence of seroconversion, or of pathology within the liver in the experimental time frame. Coproantigen results were negative and ELISA results on the sera were mixed. In two horses, #3 and 7, high levels of antibodies specific for rmFhCL1 were detected, however as the antibody level was elevated on the day of infection, we considered that this positive result was more likely due to previous exposure or to poor specificity, rather than experimental infection. Negative coproantigen results in horses have previously been reported (Palmer et al., 2017) and may be due to the extensive hindgut fermentation in equids.

Previous studies on the experimental infection of horses with *Fasciola hepatica* led to the conclusion that horses are largely resistant to liver fluke infection. Nansen et al., (1975), infected horses both orally and by intraperitoneal implantation and found that only one of ten horses dosed orally became infected. Both of the horses that were implanted with metacercariae developed a patent infection. Hence, the authors concluded that given by the oral route, the majority of parasites were eliminated or immobilised at an early stage of the infection. Alves et al., (1988) dosed horses with both *F. hepatica* and *F. gigantica* and found that the horses were resistant to infection with oral doses of metacercariae ranging from n= 500 up to 9,500. Boulard et al., (1989) conducted an experimental infection in which a patent infection was seen in only two of eight horses infected. The

question remains, therefore, why natural infection with *F. hepatica* is reasonably common (Howell et al., 2019; Quigley et al. 2017).

In our hands, experimental challenge of both cattle and sheep with *F. hepatica* metacercariae, including with the Italian strain, invariably produces infection, at lower dose rates than those used in this study (150 metacercariae for sheep and 200-400 for cattle). Furthermore, we used the same batch of metacercariae to infect cattle, in a separate study. It is unlikely, therefore, that failure to infect horses with this protocol was due to technical factors.

It is possible that the pre-patent period in horses is considerably longer than in ruminants, and this may be one reason why this and previous experimental challenges have not demonstrated patent infection (Alves et al., 1988). It is possible that horses are only susceptible to infection within a certain age range, perhaps as foals, and that the prevalence can be explained by long-lived infection. Alternatively, the possibility of strain-specific infection of horses has been mooted, although this is a relatively unlikely possibility given the high genetic diversity of *F. hepatica* populations (Beesley et al., 2017). Furthermore, Howell et al. (2019) recently demonstrated no differences in the genetic diversity of flukes recovered from horses and from ruminants in the UK, and a high level of gene flow between these populations, showing that at least in this geographical region equine-specific strains do not occur. Cross-infection between horses and ruminants is also suggested by the work of this group by showing an increased risk of *F. hepatica* infection in horses co-grazing with ruminants. The ability of horses to mount an effective protective immune response following a primary infection can also not be excluded as a possibility, and we acknowledge that the horses in this study may have been exposed to prior infection. There may also be individual animal risk factors/susceptibilities within equine populations, that are as yet undefined. In any event, it is clear that there are significant gaps in our knowledge of equine fasciolosis that cannot be extrapolated directly from the established picture of ruminant infection. Further understanding of the epidemiology of equine fasciolosis could be achieved, for example, by studying the age-prevalence, or by prospective natural

challenge studies on horses co-grazed with infected ruminants. These studies will be useful not only in determining optimal control programmes for equine fasciolosis, but also potentially in illuminating the more basic aspects of host-parasite relationships pertaining to *F. hepatica*.

Another issue hindering elucidation of the epidemiology of equine fasciolosis is the relative lack of reliable diagnostic tests. In our hands, a serum antibody-detection ELISA assay based on a recombinant form of the major adult fluke ES protein, cathepsin L1, provided a relatively low sensitivity, although a high specificity. An assay based on purified ES protein from adult flukes provided a higher sensitivity and a relatively high specificity. We expected that the performance of these two assays would be comparable as FhCL1 is a major component of the ES fraction (Jeffries et al. 2001). The difference in sensitivity is likely to be due to the presence of additional antigens in the ES ELISA that are recognised during liver fluke infection of the horse. Although a third ELISA based on a 2.9kDa tegumental protein expressed in *E. coli* identified all horses with confirmed active infection, it had a low specificity, and hence did not have a high level of concordance with the other two assays. The optimisation of serological diagnosis for equine fasciolosis will also be an important factor in understanding its pathophysiology and epidemiology, while also perhaps shedding light on the factors underlying susceptibility of different species as definitive hosts. An intriguing phenomenon which is deserving of future study also is the apparent failure of some horses at least to mount a strong antibody response to the FhCL1 antigen, which is immunodominant in ruminants (Garza-Cuartero et al., 2018) and humans (O'Neill et al., 1999) infected with *F. hepatica*. Understanding this aspect of the equine immune response to *F. hepatica* may be useful in the broader context of understanding host-parasite relationships in fasciolosis.

5.0 Conclusions

In summary, this and previous studies confirm that while *F. hepatica* infection is relatively common in horses, there are many unanswered questions relating to its epidemiology, pathophysiology and diagnosis. These gaps, together with the absence of any licensed treatment, limit the advice that

258 can be provided in relation to equine fasciolosis. Co-grazing or rotational grazing of horses with
259 ruminants on pastures where fluke infection is known to occur should prompt vigilance for potential
260 related clinical signs in horses. Improved diagnostics will be required to improve our knowledge of
261 the epidemiology and clinical importance of *F. hepatica* infection in the horse.

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Horse no.	Group	Age (yrs)	Breed	Sex	Dose (metacercariae)
1	A	8	TB	Gelding	500
2	A	14	TB	Gelding	500
3	A	9	ISH	Mare	500
4	A	20	ISH	Gelding	500
5	B	8	ISH	Gelding	1000
6	B	11	ISH	Gelding	1000
7	B	11	ISH	Gelding	1000
8	B	17	TB	Gelding	1000
9	C	12	TB	Gelding	nil
10	C	17	TB	Gelding	nil

Table **1. Horses included in the study**. These were horses destined for slaughter, purchased from a variety of locations throughout Ireland. They were maintained on fluke-free grazing for the duration of the study. TB= Thoroughbred, ISH= Irish Sport Horse. Four horses were challenged orally with 500 *F. hepatica* metacercariae (Italian strain), four with 1000 metacercariae, and two were given a sham challenge.

Assay	Sensitivity (%)	Specificity (%)	Indicative sero-prevalence(%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy(%)
rmFhCL1	50 (26-74)	100 (94-100)	22 (14-33)	100	88 (82-92)	89

ES ELISA	67 (40-87)	97 (89-100)	22 (13-32)	85.7 (85-95)	91 (85- 95)	90 (82-96)
FhrAPS	72(47-9)	30(19-42)	22(14-33)	23 (18-29)	78 (61-89)	39(28-50)

346 **Table 2. Performance of ELISAs.** Sensitivity, specificity, indicative sero-prevalence, positive and
347 negative predictive values, and accuracy determined for each of the three antibody-detection ELISA
348 assays using a sub-group of 82 equine sera collected from horses in a previous abbatoir survey.
349 Each sample was from a horse of known status with respect to current liver fluke infection, and of
350 this population, 22% had evidence of infection. All values are given as percentages with the upper
351 and lower limit of the 95% confidence interval in brackets.

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	CL1	ES ELISA	Fhr APS
CL1	n/a	0.57	0.067
ES ELISA		n/a	0.179
FhrAPS			n/a

353 **Table 3 Agreement between assays.** Kappa values, indicating moderate agreement between the
354 CL1 and ES ELISAs, and slight agreement between the FhrAPS and each of the other two assays.

355

356 **Figure Legends**

357 Figure 1. Serum GLDH (a) GGT (b) and bile acid (c) levels for each horse from day of infection through
358 18 weeks post-infection. The horizontal line indicates the upper level of the reference range, in each
359 case. Horses 1-4 (Group A) were challenged with 500 metacercariae, 5-8 (Group B) with 1000, and 9
360 and 10 (Group C) were unchallenged. Serum bile acids did not rise above the reference range in any
361 animal. Two horses from Group B, numbers 5 and 8, had elevated serum GLDH on the day of
362 infection, but these levels subsequently declined. These two horses also had serum GGT levels
363 above the reference range at this timepoint, and in the case of horse 8 elevated levels persisted
364 throughout the study period. Horse 7 had transiently elevated GGT at 4 wpi.

365 Figure 2. ELISA results as corrected O.D. 450 nm values of horse sera were determined by rmFhCLq
366 ELISA. Results from individual horses numbered 1-10 are displayed on X-axis. Horses 1-4 were
367 dosed with 500 metacercariae (Group A) 5-8 were dosed with 1000 metacercariae (Group B) and 9
368 and 10 were uninfected controls (Group C).

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