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Title	Diagnosis of sheep fasciolosis caused by Fasciola hepatica using cathepsin L enzyme-linked immunosorbent assays (ELISA)
Author(s)	López Corrales, Jesús; Cwiklinski, Krystyna; De Marco Verissimo, Carolina; Dorey, Amber; Lalor, Richard; Jewhurst, Heather; McEvoy, Amanda; Diskin, Michael; Duffy, Catherine; Cosby, S Louise; Keane, Orla M; Dalton, John Pius
Publication Date	2021-07-13
Publication Information	López Corrales, Jesús, Cwiklinski, Krystyna, De Marco Verissimo, Carolina, Dorey, Amber, Lalor, Richard, Jewhurst, Heather, McEvoy, Amanda, Diskin, Michael, Duffy, Catherine, Cosby, S. Louise, Keane, Orla M., Dalton, John Pius. (2021). Diagnosis of sheep fasciolosis caused by Fasciola hepatica using cathepsin L enzyme-linked immunosorbent assays (ELISA). Veterinary Parasitology, 298, 109517. doi: https://doi.org/10.1016/j.vetpar.2021.109517
Publisher	Elsevier
Link to publisher's version	https://doi.org/10.1016/j.vetpar.2021.109517
Item record	http://hdl.handle.net/10379/18038
DOI	http://dx.doi.org/10.1016/j.vetpar.2021.109517

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Veterinary Parasitology 298 (2021) 109517

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Diagnosis of sheep fasciolosis caused by *Fasciola hepatica* using cathepsin L enzyme-linked immunosorbent assays (ELISA)

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A R T I C L E I N F O

Keywords:
Fasciola hepatica
Diagnostics
ELISA
Coproantigen
Cathepsin L peptidases

A B S T R A C T

Fasciolosis, a global parasitic disease of agricultural livestock, is caused by the liver fluke *Fasciola hepatica*. Management and strategic control of fasciolosis on farms depends on early assessment of the extent of disease so that control measures can be implemented quickly. Traditionally, this has relied on the detection of eggs in the faeces of animals, a laborious method that lacks sensitivity, especially for sub-clinical infections, and identifies chronic infections only. Enzyme linked immunosorbent assays (ELISA) offer a quicker and more sensitive serological means of diagnosis that could detect early acute infection before significant liver damage occurs. The performance of three functionally-active recombinant forms of the major *F. hepatica* secreted cathepsins L, rFhCL1, rFhCL2, rFhCL3, and a cathepsin B, rFhCB3, were evaluated as antigens in an indirect ELISA to serologically diagnose liver fluke infection in experimentally and naturally infected sheep. rFhCL1 and rFhCL3 were the most effective of the four antigens detecting fasciolosis in sheep as early as three weeks after experimental infection, at least five weeks earlier than both coproantigen and faecal egg tests. In addition, the rFhCL1 and rFhCL3 ELISAs had a very low detection limit for liver fluke in lambs exposed to natural infection on pastures and thus could play a major role in the surveillance of farms and a 'test and treat' approach to disease management. Finally, antibodies to all three cathepsin L proteases remain high throughout chronic infection but decline rapidly after drug treatment with the flukicide, triclabendazole, implying that the test may be adapted to trace the effectiveness of drug treatment.

1. Introduction

Fasciolosis, also known as liver fluke disease, is caused by trematodes of the genus *Fasciola*. *F. hepatica* has one of the greatest geographical distributions for parasites infecting livestock and causes enormous losses to the global agricultural sector (Charlier et al., 2020). Infection is acquired when livestock ingest infective encysted metacercariae that have emerged from the intermediate host, the mud snail *Galba truncatula*, and settled on grass. After excystment in the intestine, the parasite penetrates the intestinal wall and migrates to the liver, after which the disease progresses in two clinical phases. Acute fasciolosis begins within approximately two weeks of infection and results from tissue damage and haemorrhaging caused by the parasites' tunnelling and feeding activity in the liver tissue, and as a result of the immunopathology associated with the host's immune responses. Then, after 8–12 weeks the chronic phase begins when the flukes migrate into the bile ducts, where they mature and produce eggs. The chronic phase can last for months and even years if untreated.

The extent of clinical pathology depends on the host, specifically their breed, condition, nutritional status and, most importantly, the burden of infection (Chauvin et al., 2001). In general, infections in sheep tend to be more severe than in cattle due to their apparent lack of resistance to the parasite. While acute infections with large numbers of parasites that penetrate the liver within a short period of time can cause extensive damage to the liver and sudden death in sheep, trickle infections over weeks or months are considered more typical and,

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https://doi.org/10.1016/j.vetpar.2021.109517
Received 7 May 2021; Received in revised form 29 June 2021; Accepted 3 July 2021
Available online 6 July 2021
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therefore, acute and chronic infection can occur simultaneously. The most common form of fasciolosis on sheep farms is sub-clinical with low burdens of liver fluke; nevertheless, these can reduce weight gain, wool quality (Hayward et al., 2021) and increase the susceptibility of sheep to other infections (Munita et al., 2019b).

Management of fasciolosis on sheep farms depends on predicting the extent of infection early so that the appropriate anthelminthic interventions can be applied to prevent losses without the risk of over-treatment and the possibility of abetting the development of drug resistance in parasites (Kelley et al., 2016; Kamaludeen et al., 2019; Fairweather et al., 2020). Therefore, a diagnostic that can accurately detect the early onset of infection is imperative so that a targeted treatment programme can be employed. Traditionally, diagnosis relies on the identification of liver fluke eggs in faecal samples (faecal egg count, FEC), but this method is not only time-consuming and laborious, it is also insensitive, especially in the case of low parasite burdens. In addition, the method detects infection only after the parasites have matured and produced eggs within the bile ducts, well after liver damage has ensued. Accordingly, recent approaches have employed immunological techniques to improve the sensitivity and accuracy of fluke detection in sheep and cattle, for example, the use of capture enzyme linked immunosorbent assays (ELISA) for the detection of liver fluke coproantigens in faeces (Martínez-Sernández et al., 2016) and serological ELISA for the detection of anti-fluke antibodies in blood samples (Cornelissen et al., 2001; Salimi-Bejestani et al., 2005; Mezo et al., 2007; reviewed by Alvarez Rojas et al., 2014).

Research in our laboratory has shown that as the liver flukes migrate through the tissues of its host they release a variety of proteolytic enzymes which assist in the breakdown of host tissue, the formation of the migratory path, blood feeding and immunomodulation (Cwiklinski et al., 2019). These cathepsin-like cysteine peptidases are produced by the gastrodermal cells of the parasite gut and are strictly developmentally-regulated (Cwiklinski et al., 2019); thus, the predominant proteases released by adult flukes in the bile ducts are the cathepsin L peptidases termed FhCL1 and FhCL2 while those released by the juvenile flukes that penetrate the intestine are the cathepsin L, FhCL3, and three cathepsins B, FhCB1, 2 and 3. FhCL1 is the best characterised of these enzymes, biochemically and structurally (Smith et al., 1993; Dowd et al., 1994; Collins et al., 2004; Stack et al., 2008), and has been shown by us and several other laboratories to be highly immunogenic and, therefore, useful in the serological detection of fasciolosis in animals and humans (O'Neill et al., 1998; Strauss et al., 1999; Cornelissen et al., 2001; Salimi-Bejestani et al., 2005; Gonzales Santana et al., 2013; Sugiyama et al., 2021).

In this study, we evaluated the performance of the most highly expressed members of the *F. hepatica* cysteine peptidases, namely FhCL1, FhCL2, FhCL3 and FhCB3, to detect liver fluke infection in experimentally- and naturally-infected lambs. The aim of the study was to assess whether these assays were applicable for evaluating lambs put on pasture that act as sentinels for fluke prevalence, that could play a valuable role in the 'test and treat' approach to liver fluke control and management. Antibody levels following drug treatment with the flukicide, triclabendazole (TCBZ) were analysed to trace the effectiveness of drug treatment and the effects on anti-*Fasciola* responses.

2. Materials and methods

2.1. Ethical statement

Experimental procedures at Moredun Scientific, UK were carried out under license from the UK Home Office by the Animal (Scientific Procedures) Act 1986 (License No. PPL/60/4426) after ethical review by the Moredun Scientific Animal Ethics Committee. Euthanasia of animals at Moredun Scientific was carried out by lethal injection. Experimental procedures at Agri-Food and Biosciences Institute (AFBI; UK) were carried out under license from the Department of Health, Social Services and Public by the Animal (Scientific Procedures) Act 1986 (License No. PPL 2771; PPL 2801), after ethical review by the AFBI Animal Ethics Committee. Experimental procedures at Teagasc Athenry (Ireland) were carried out under license from Health Products Regulatory Authority (HPRA) by the EU Directive 2010/63/EU (License No. AE19132/P097), after ethical review by the Teagasc Animal Ethics Committee. Euthanasia of animals at AFBI and Teagasc sites was carried out by captive bolt.

2.2. Experimental and field infections of sheep

Trial 1 (Moredun 1): Fourteen eight-month-old Texel cross sheep (UK; male n = 2; female n = 12) were orally infected with 150 *F. hepatica* metacercariae (South Gloucester isolate: Ridgeway Research Ltd). Blood samples were collected by jugular venepuncture prior to infection, at three weeks post infection (wpi) and on the day of necropsy, at 16 wpi. At necropsy, total enumeration of fluke burden was carried out on parasites recovered from the liver parenchyma and bile ducts (as per protocols used by Hodgkinson et al., 2018).

Trial 2 (AFBI 1): Eighteen six-month-old male Dorset cross sheep (UK) were orally infected with 150 *F. hepatica* metacercariae (Italian isolate: Ridgeway Research Ltd). Blood samples were collected by jugular venepuncture on the day of infection and the day of necropsy, which occurred on 2 days post infection (dpi; n = 6), 9 dpi (n = 6) and 18 dpi (n = 6). At necropsy on 18 dpi, the visceral and diaphragmatic aspects of the liver were photographed for gross evaluation to evaluate infection.

Trial 3 (AFBI 2): Sixteen six-month-old Dorset cross sheep (UK; male n = 8; female n = 8) were orally infected with 150 *F. hepatica* metacercariae (South Gloucester isolate: Ridgeway Research Ltd). Blood samples were collected by jugular venepuncture on the day of infection, at three wpi and at necropsy, at 16 wpi. At necropsy, total enumeration of fluke burden carried was out on parasites recovered from the liver parenchyma and bile ducts.

Trial 4 (AFBI 3): Sixteen six-month-old male Dorset cross sheep (UK) were orally infected with 120 *F. hepatica* metacercariae (Italian isolate: Ridgeway Research Ltd). Blood samples were collected by jugular venepuncture on the day of infection and at 3, 7, 11, 15, 20, 21, 23, 24 and 25 wpi. At 17 wpi, six animals were treated with one dose of TCBZ, following the dosage rates recommended by the manufacturer (Fasinex 5%; 1 mL per 5 kg; Triclabendazole 5.00 % w/v). At necropsy (25 wpi), total enumeration of fluke burden was carried out on parasites recovered from the liver parenchyma and bile ducts.

Trial 5 (Moredun 2): Thirteen eight-month-old male Texel cross sheep (UK) were orally infected with 120 *F. hepatica* metacercariae (Italian isolate: Ridgeway Research Ltd). Blood samples were collected by jugular venepuncture on the day of infection, at three wpi and at necropsy, 16 wpi. At necropsy, total enumeration of fluke burden was carried out on parasites recovered from the liver parenchyma and bile ducts.

Trial 6 (Teagasc): Twenty six-month-old sheep of three breeds (Texel, Suffolk and Belclare; Ireland; male n = 9, female n = 11) were put onto *F. hepatica*-contaminated pasture for eight weeks, after which the animals were housed for a further seven weeks prior to necropsy. Blood samples were collected by jugular venepuncture at the start of the study and at 4, 9 and 15 weeks post-liver fluke exposure (wpe). Rectal faecal samples were collected for analysis by faecal egg count (FEC) and coproantigen-ELISA at 9, 11, 13 and 15 wpe. At necropsy (15 wpe), total enumeration of fluke burden was carried out on parasites recovered from the liver parenchyma and bile ducts.

2.3. Recombinant protein production

F. hepatica cathepsin L (FhCL1, FhCL2, FhCL3) and cathepsin B (FhCB3) cysteine peptidases were expressed as functionally active recombinant forms in the methylotrophic yeast *Pichia pastoris* as

previously described by Collins et al. (2004). Soluble recombinant proteins secreted into the culture supernatant were purified and buffer exchanged into phosphate buffered saline (PBS, pH 7.4) using Nickle-chelate affinity chromatography. Protein concentration and purity were verified by Bradford Protein Assay (Bio-Rad) and by 4–20 % SDS-PAGE gels (Bio-Rad) stained with Biosafe Coomassie (Bio-Rad), respectively. The gels were visualised using a G:BOX Chemi XRQ imager (Syngene).

2.4. Analysis of anti-F. hepatica cathepsin-specific antibodies in sheep sera samples by ELISA

Flat-bottom 96 well microtitre plates (Nunc™ Maxisorp ™, ThermoFisher Scientific) were coated in triplicate with 100 μL of 1 μg/mL of the recombinant antigens (rFhCL1, rFhCL2, rFhCL3 or rFhCB3) 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, with shaking at 160 rpm. After washing three times with PBST, 100 μL of serum 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 1 h at 37 °C with shaking at 160 rpm. 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, with shaking at 160 rpm. 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 (OD_{450}) in a PolarStar Omega spectrophotometer (BMG LabTech). For the analysis of the three week time-point from trials 1, 3, 4 and 5, a cut-off point was calculated based on the mean optical density plus 3.09 standard deviations (SD, as per Valero et al., 2012; Devanarayan et al., 2017) of the negative animals.

2.5. Coproantigen-ELISA

F. hepatica-specific antigen in the faecal samples from sheep in Trial 6 were detected using the commercially available *F. hepatica* coproantigen-ELISA kit BIO K 201/2 (Bio-X Diagnostics; Martínez-Sernández et al., 2016), according to the manufacturer's instructions for sheep samples. All faecal samples were weighed out on the day of collection (0.5 g per animal) and stored overnight at 4 °C prior to processing. Positive samples were determined by expressing their net optical density as a percentage and comparing it with the provided cut-off as indicated in the kit instructions.

2.6. Faecal egg count (FEC)

FEC were performed on 5 g of faeces per animal within two weeks of collection. Faecal samples were homogenised in water prior to sieving through 710, 150 and 38 μm stainless steel Endecott sieves (200 Mm diameter) in sequence. The sediment retained on the 38 μm sieve were back-washed into a glass beaker and diluted to a volume of 500 mL with water. The suspension was left for 4 min to allow sedimentation of the *F. hepatica* eggs, after which the supernatant was decanted and the sediment re-suspended in 500 mL of water. This process was repeated until the supernatant was clear. The final sediment was re-suspended in 75 mL of water to which 1 % (w/v) methylene blue was added before it was transferred to a petri dish for microscopy at 40x magnification using a Kyowa Optical stereo microscope (SDZ-PL).

2.7. Statistical analyses

Statistical analyses were carried out using GraphPad Prism version 5. Two-way ANOVA including Bonferroni post-tests were performed to determine the significance of the differences between levels of total IgG in serum samples detected using every antigen (values were significant if $P < 0.05$). Correlation analyses were carried out following a nonparametric correlation approach to calculate the Spearman r.

3. Results

3.1. Comparison of recombinant cathepsin antigens for use in serological test for fasciolosis

Recombinant expression of three *F. hepatica* cathepsin L peptidases, FhCL1, FhCL2, and FhCL3, and a cathepsin B peptidase (FhCB3) was carried out using the *P. pastoris* yeast expression system. Proteins were purified from the yeast culture supernatant by one-step Nickle-chelate affinity chromatography which resulted in high yields ranging from four to ten mg protein per litre of culture medium. All four recombinant proteins were analysed by SDS-PAGE and were shown to migrate as single bands at the expected molecular weight (~37 kDa) and be of high purity (Fig. 1). Furthermore, all four proteins were demonstrated to be functionally active using fluorogenic peptide substrates (as per De Marco Verissimo et al., 2020) and, hence, structurally folded in native form.

Using an ELISA-based assay, the efficacy of the four recombinant cathepsin peptidases for serodiagnosis of fasciolosis was evaluated. The primary and secondary antibodies were titrated before defining optimal dilutions of 1:100 for the primary antibody and 1:50,000 for the secondary HRP-conjugated donkey anti-sheep IgG. Using these conditions, antibodies present in serum collected at three and 16 wpi from seven animals experimentally infected with *F. hepatica* (Trial 1) were compared to serum samples collected pre-infection (Fig. 2). For all the cathepsin L and B peptidases tested, antibody-specific responses could be detected at three wpi. The highest responses were observed for rFhCL1 and rFhCL3, which were not statistically different from each other but were greater compared to values obtained for rFhCB3 and rFhCL2 ($P < 0.001$). OD values obtained at 16 wpi were higher than at three wpi for all three cathepsin L proteases (Fig. 2). By contrast, no change was observed for the level of anti-rFhCB3 antibodies at 16 wpi compared to three wpi, and these were statistically lower than those observed for the three anti-cathepsin L antibodies ($P < 0.001$).

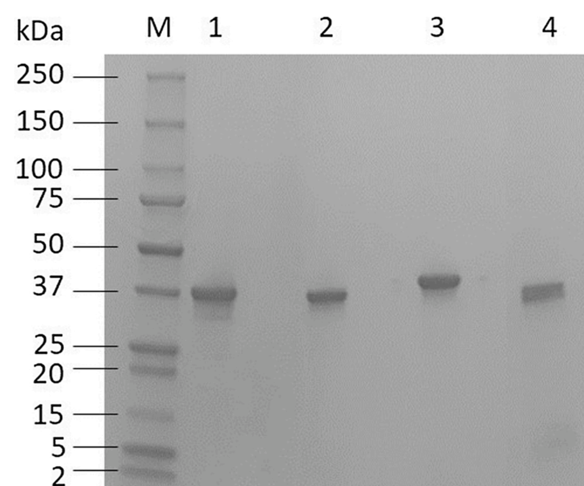

Fig. 1. Purification of recombinant cathepsin peptidases. SDS-PAGE analysis of the yeast-expressed recombinant *Fasciola hepatica* cathepsin L1 (rFhCL1; lane 1), cathepsin L2 (rFhCL2; lane 2), cathepsin L3 (rFhCL3; lane 3) and cathepsin B3 (rFhCB3; lane 4). M, molecular size markers.

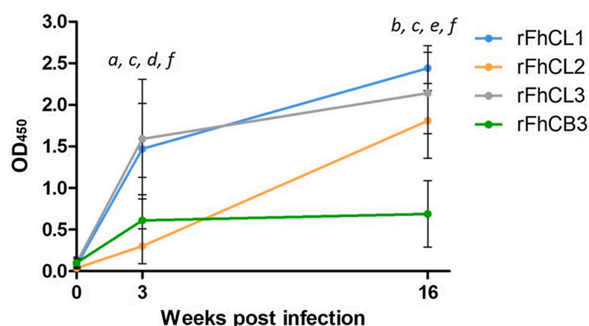

Fig. 2. Antibody responses to the *Fasciola hepatica* recombinant cathepsin peptidases. Mean optical density values for total IgG antibodies to the four recombinant cathepsin peptidases (rFhCL1, rFhCL2, rFhCL3 and rFhCB3) during an experimental infection trial of 16 weeks (n = 7; mean ± standard deviation is represented). Statistical differences highlighted as follows: *a.* FhCL1 & FhCL2, P < 0.001; *b.* FhCL1 & FhCL2, P < 0.01; *c.* FhCL1 & FhCB3, P < 0.001; *d.* FhCL2 & FhCL3, P < 0.001; *e.* FhCL2 & FhCB3, P < 0.001; *f.* FhCL3 & FhCB3, P < 0.001.

3.2. Early detection of immune responses to F. hepatica using recombinant cathepsin cysteine peptidases

Using sera obtained from sheep infected for 2, 9 and 18 days (Trial 2) each protease was assessed for their ability to detect liver fluke infection prior to three-weeks post infection (Fig. 3). For all the four recombinant antigens, antibody responses above the baseline level pre-infection could only be detected at 18 dpi. Although the highest antibody response detected at 18 dpi appeared to be against rFhCL3, this was not statistically significant compared with the other recombinant antigens because of the high variability between the six animals used per time point (P = 0.6581).

Therefore, to further probe the utility of the various cathepsin cysteine peptidases to detect *F. hepatica* infection in sheep at three wpi, and to examine inter-individual variability of the cathepsin based ELISA, serum samples collected at this time point from 59 sheep (these animals were part of four separate experimental trials; Trials 1, 3, 4 and 5) were analysed (Fig. 4A). Elevated antibody responses were observed using rFhCL1 and rFhCL3 when compared to serum collected pre-infection (P < 0.001). rFhCL3 was the best antigen recognising IgG in serum samples, with a mean OD_{450} of 1.91, followed by rFhCL1 as the second best antigen with an OD_{450} of 1.46. Antibody responses at three wpi to the recombinant antigens rFhCL2 and rFhCB3 were also elevated but not to the same extent as the responses observed against rFhCL1 and rFhCL3 that were significantly higher in comparison (P < 0.001). Ultimately, we found that the detection rate for the 59 animals evaluated at three wpi was 91 % (rFhCL1), 40 % (rFhCL2), 95 % (rFhCL3) and 41 % (rFhCB3).

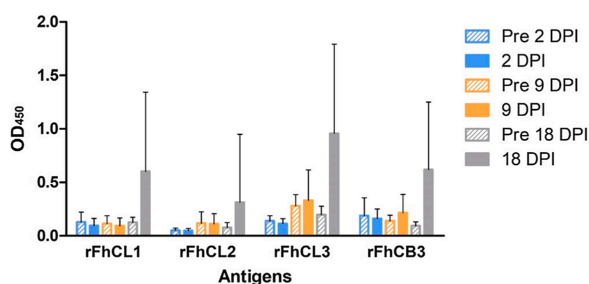

Fig. 3. Early detection of anti-cathepsin antibodies. Optical density values for total IgG antibodies to the four recombinant cathepsin peptidases (rFhCL1, rFhCL2, rFhCL3 and rFhCB3) were compared across three groups of six animals infected for 2, 9 and 18 days. Serological analysis was carried out before (represented by the Pre 2, Pre 9 and Pre 18 dpi samples) and after infection (represented by 2, 9 and 18 dpi samples). Error bars represent ± standard deviation.

The animals used in these experimental infections were male and female of two different breeds (Dorset cross and Texel cross). Furthermore, two different *F. hepatica* isolates (South Gloucester and Italian isolates) were used for infections depending on the trial. However, we did not observe any correlations between these variables and antibody levels. A wide range of adult flukes (12–142) were recovered at necropsy (16 wpi), although the animals received an experimental dose of 120 or 150 metacercariae depending on the trial. Analysis of the antibody responses at three wpi to all three cathepsin L recombinant peptidases showed a low but significant positive correlation with the number of flukes observed at necropsy (rFhCL1: r = 0.4220, P = 0.002; rFhCL2: r = 0.3473, P = 0.011; rFhCL3: r = 0.3749, P = 0.006) (Fig. 4B). By contrast, no correlation was found with rFhCB3 (r = 0.2134, P = 0.125) (data not shown).

3.3. Assessment of anti-cathepsin peptidase antibody responses in naturally-infected lambs

The efficacy and sensitivity of the recombinant cathepsin protease-based ELISA were evaluated using samples from 20 lambs naturally exposed to *F. hepatica*-contaminated pastures for eight weeks and then brought in-doors for a further seven weeks (Trial 6). *F. hepatica* infection in these animals was verified by FEC, coproantigen ELISA and by enumerating total adult flukes in livers at necropsy, which identified a total of eight infected animals (Table 1). Only seven sheep were positive for *F. hepatica* infection on the basis of the detection of parasite eggs within the faecal samples and/or within the gall bladder at necropsy. Adult liver flukes were recovered from three of these egg positive animals but the parasite burden was extremely low (one to three adult parasites). In addition, adult flukes (n = 2) were also recovered from an animal where no eggs were detected in the faeces. Of the eight infected animals, six were positive based on the coproantigen ELISA at 13 wpe.

Antibody responses to the four cathepsin cysteine peptidases determined by ELISA in the serum of all 20 animals at 0, 4, 9 and 15 wpe are shown in Fig. 5. A cut-off point was determined as the mean optical density plus 3.09 standard deviations (SD) of the negative animals at 4 wpe based on the parasitological data (dotted line in Fig. 5). This time point, rather than time 0, was chosen for the cut-off calculation to take into account the likelihood of animals being exposed to infections other than liver fluke (e.g. nematode parasites) which could affect the baseline antibody concentration in serum.

Of the four recombinant cathepsin cysteine peptidases employed, the rFhCL1-ELISA could reliably distinguish between non-infected and infected sheep at both 9 and 15 wpe based on the cut-off value calculated above. This ELISA corroborated our parasitological data by identifying all eight infected animals amongst the 20 sheep (Fig. 5A). Moreover, the rFhCL1-ELISA identified one further infected animal that had no parasite eggs nor adult flukes at time of necropsy and, therefore, we speculate that it represents a recent infection. One of the confirmed infected sheep was detected as early as 4 wpe (Fig. 5A; Table 1). The rFhCL2 and rFhCL3-ELISA identified eight (100 %) and seven (88 %) of the infected animals, respectively, predominately at 15 wpe but the discrimination between infected and non-infected animals was less clear with these assays (Fig. 5B and C). The rFhCB3 was the least effective at determining antibodies in the serum of naturally-infected sheep and did not reliably distinguish infected from non-infected lambs (Fig. 5D).

The results of the rFhCL1-ELISA were compared with the coproantigen ELISA and FEC (Table 1). Unlike the ELISA assay, neither of these faecal assays detected any infected animals at 9 wpe; however, the coproantigen ELISA and FEC method identified infected animals in our cohort beginning from 11 wpe and 13 wpe, respectively.

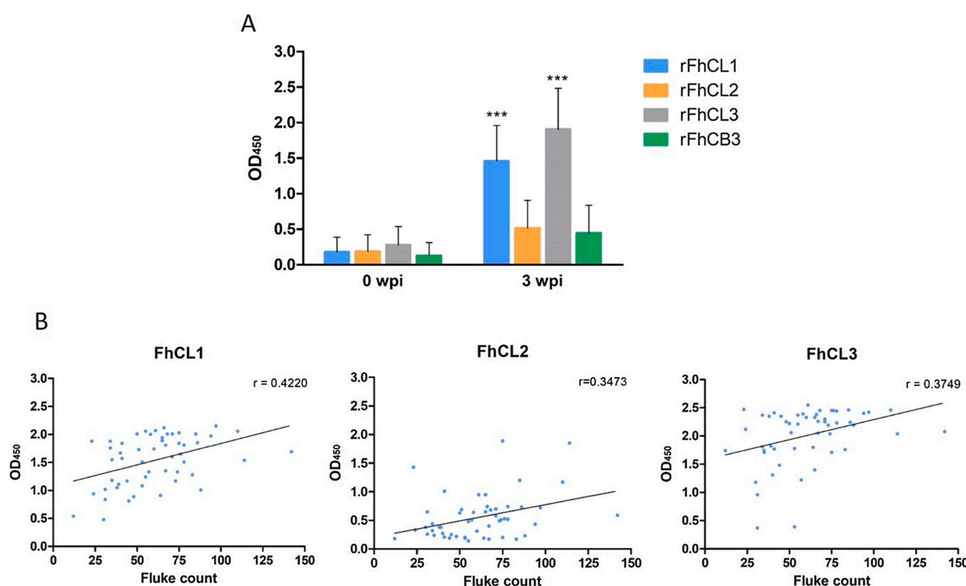

Fig. 4. Analysis of antibody responses to cathepsin peptidases at 3 weeks post-infection. (A) Comparison of the optical density values for total IgG antibodies to the four recombinant cathepsin peptidases (rFhCL1, rFhCL2, rFhCL3 and rFhCB3) prior to infection (0 wpi) and 3 weeks later (3 wpi). Antibody responses statistically different at 3 wpi compared to the start of the experiment are shown (*** P < 0.001). Error bars represent ± standard deviation. (B) Scatter plot and Spearman correlation coefficient of total adult fluke count at necropsy and the optical density values for total IgG anti-rFhCL1, anti-rFhCL2 and anti-rFhCL3 antibodies.

Table 1
Comparative analyses of the three diagnostic methods for fasciolosis at three time points following exposure to naturally infected pastures (weeks post exposure, wpe); serological rFhCL1-ELISA (ELISA), faecal egg count (FEC) and copro-antigen ELISA (Copro). Positive result shown by shaded X or by the number of eggs/gram.

Animal	Number of adult flukes at necropsy	4 wpe			9 wpe			15 wpe		
		ELISA	FEC	Copro	ELISA	FEC	Copro	ELISA	FEC	Copro
1	2				X			X	12.6	X
2	2				X					
3	3				X			X	3.4	X
4	1	X			X			X	1.2	
5	0							X		
6	0				X			X	2.8	X
7	0				X			X	0.8	X
8	0				X			X	2.6	X

3.4. Analysis of the effect of anthelmintic treatment on serological responses to the recombinant cathepsin cysteine peptidases

Seropositivity of experimentally infected animals pre- and post-treatment with the flukicide, TCBZ, was assessed based on eleven animals, six of which were administered one dose of TCBZ at 17 wpi. Successful drug treatment of these sheep was verified by faecal egg counts 10 days after treatment (Trial 4). Pre-TCBZ treatment the FEC was 58 ± 18.9 eggs/gram, which post-TCBZ treatment was reduced to 1.67 ± 2.88 eggs/gram. By 20 wpi, no eggs were recovered from the TCBZ-treated group.

Antibody responses were monitored throughout infection and after drug treatment. All sheep displayed increasing antibodies responses to rFhCL1, rFhCL2 and rFhCL3 during the course of infection until 15 wpi (Fig. 6; Supplementary Fig. 1). As previously shown, sheep exhibited a much lower response to rFhCB3, and this peaked between seven and 11 wpi before steadily declining over the following 14 weeks (Fig. 6D). Following administration of TCBZ all six animals in the treatment group exhibited a sharp decline in anti-rFhCL1, anti-rFhCL2 and anti-rFhCL3 antibodies over the subsequent eight weeks and reached levels comparable to those observed between three and seven wpi (Fig. 6). Statistical differences between the anti-rFhCL2 responses of the TCBZ-treated and non-treated animals were observed from 21 wpi (Fig. 6B; P < 0.05 - P < 0.001), whereas the decline in the anti-rFhCL1 and anti-rFhCL3 responses was more gradual, with significant differences only observed from 23 wpi (rFhCL3; P < 0.01 - P < 0.001; Fig. 6C) and 24 wpi (rFhCL1; P < 0.01 - P < 0.001; Fig. 6A). Analysis of the percentage reduction in the ELISA OD values pre- and post-drug treatment revealed that independent of specific cathepsin peptidase used for the ELISA, by 25 wpi all the OD values were decreased following TCBZ treatment, with a mean reduction between 44.9–65.5 % (Supplementary Table 1). By contrast, the control untreated group displayed stable antibody levels in all three recombinant cathepsin L ELISA, and these did not vary from 15 wpi to 25 wpi (Fig. 6).

4. Discussion

During the migration of *F. hepatica* through the organs of its mammalian host, the parasite secretes copious amounts of hydrolytic enzymes, most notably the cysteine peptidases cathepsin B and cathepsin L, which facilitates the destruction of tissue and formation of a migratory path. The expression and secretion of these peptidases are strictly regulated during development such that the newly excysted juvenile (NEJ) that penetrates the intestine primarily produces the

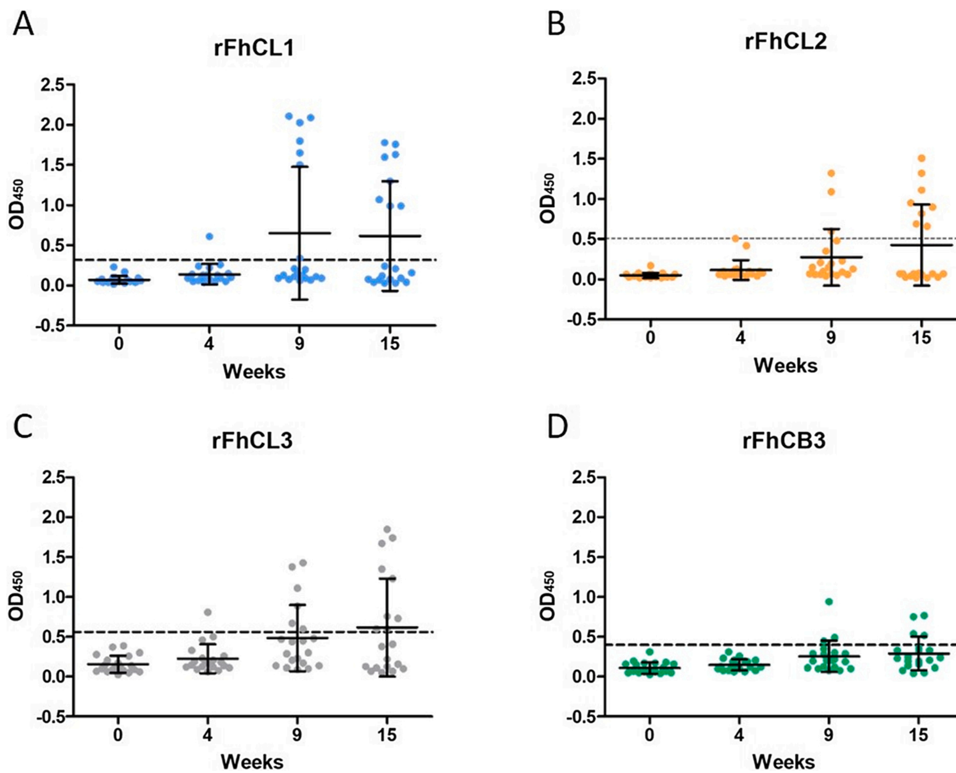

Fig. 5. Detection of total IgG in serum samples of naturally infected sheep after first exposure to *Fasciola hepatica*. Using four recombinant antigens (rFhCL1, rFhCl2, rFhCL3 and rFhCB3), total IgG (quantified as optical density) was detected by serum ELISA in samples taken before and at three time points after first exposure to *F. hepatica*. Data represented as mean ± standard deviation. The cut-off point, based on the mean optical density plus 3.09 times the standard deviation (SD) of the negative animals at 4 wpe, is shown by the dotted line.

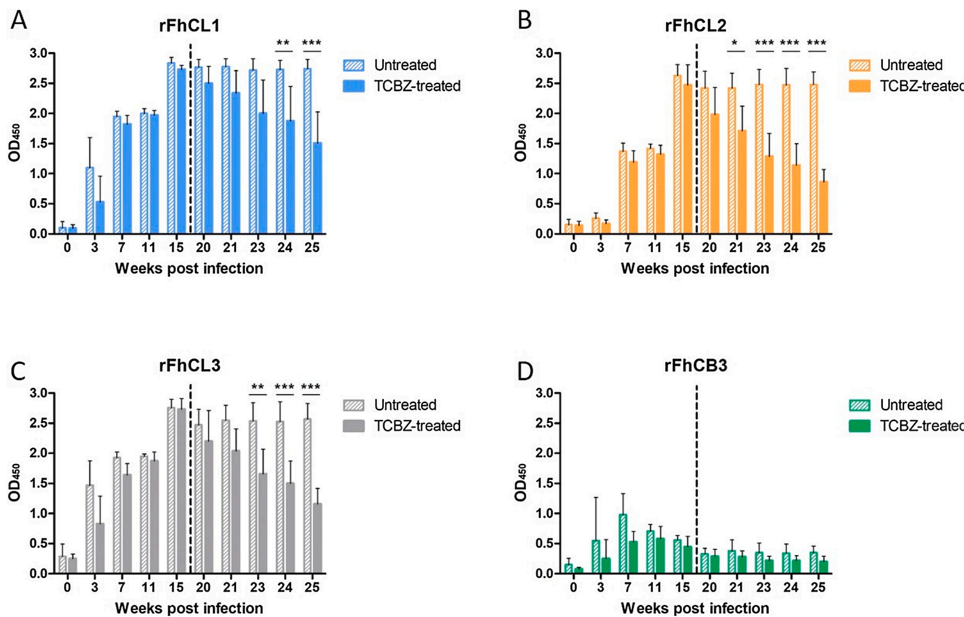

Fig. 6. Analysis of seropositivity pre- and post- triclabendazole treatment. Mean optical density values for total IgG antibodies to four recombinant cathepsin peptidases, (A) rFhCL1, (B) rFhCL2, (C) rFhCL3 and (D) rFhCB3 during an experimental infection trial of 25 weeks. Eleven animals were separated into two groups, (1) Untreated, control *Fasciola hepatica* infected group (n = 5) and (2) Triclabendazole (TCBZ) treated group (n = 6). The dotted line represents 17 wpi when TCBZ treatment was given. Data represented as mean ± standard deviation. Statistical differences observed between the untreated and treated groups are shown (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

isoforms cathepsin B1, B2 and B3 and cathepsin L3 and L4 (Cwiklinski et al., 2018). However, as the parasite migrates through the liver parenchyma, the secretion of these peptidases decline while the synthesis and secretion of isoforms cathepsin L1 and L2 increase (Cwiklinski et al., 2021). Moreover, within the bile ducts the mature parasites predominantly secretes cathepsin L1, while the expression of cathepsin L2 and L5 are less dominant (Collins et al., 2004; Robinson et al., 2008; Cwiklinski et al., 2019). The cathepsins B and cathepsins L peptidase families share only ~27 % identity and no cross reactivity was observed between them. However, members within each family exhibit high structural similarity (ranging between 72–80% identity) and therefore they exhibit immunological cross-reactivity (Law et al., 2003; Garza-Cuartero et al., 2018; Cwiklinski et al., 2019). This, and the timely and large expression of the various cathepsin B and cathepsin L peptidases, imply that they represent good candidates for developing immunodiagnostics assays which depend on the detection of parasite-specific antibody responses in the serum of infected animals.

Given the differences between the cathepsin cysteine peptidases and their differential expression during the parasites migratory life cycle, an objective of this study was to compare the performance of four different enzymes, which we have shown by proteomic studies to be predominantly excreted/secreted by NEJ (FhCL3 and FhCB3) and immature/

adult parasites (FhCL1, FhCL2), in a diagnostic ELISA for fasciolosis. The four parasite enzymes were produced as recombinant proteins in the methylotrophic yeast *P. pastoris* using protocols that we have previously described for the production of recombinant *F. hepatica* cathepsin L1 (Collins et al., 2004). In this system the recombinant proteins are secreted into the yeast culture medium and each can be readily isolated in the same manner by Nickle-chelate affinity chromatography. This provided ample quantities of high quality, properly folded (as judged by functional enzymatic activity) products of ~37 kDa that represent the stable zymogenic form of the enzymes. ELISA assays with low background (OD < 0.1) were developed for each antigen using 100 ng protein per well that was used to screen serum from sheep (at 1/100 dilution).

It was immediately clear from our studies that all cathepsin L peptidases, FhCL1, FhCL2 and FhCL3 performed better in the ELISA when compared to the cathepsin B3. This is perhaps not surprising given that the cathepsin B enzymes are produced by the NEJ stages, mainly during their passage through the intestine wall, and their expression is gradually switched off over the first three to four weeks of parasite development (Cwiklinski et al., 2018, 2021). The immune responses in experimentally-infected sheep correlated with this molecular off-switch since we observed that antibodies to cathepsin B3 gradually increased following infection to approximately seven weeks post-infection, after which they steadily declined over the course of the infection (see Fig. 6). By contrast, the antibody responses to the cathepsin L peptidases increased rapidly in early infection, surpassing those of cathepsin B within the first four weeks, and continued to escalate until week 15 when they remained high during the course of the 25-week infection. The continued increase of antibodies was helped, no doubt, by the re-stimulation and expansion of B-cells responsive to epitopes that are shared by the enzymes as their predominance in the secretome switches from FhCL3 to FhCL2 to FhCL1 as the parasite develops.

Amongst the cathepsin L peptidases, FhCL3 and FhCL1 consistently performed better than FhCL2 at the early stages of experimental infection although no significant difference could be observed between the three enzymes at the latter stages of infection. While FhCL3 elicited the strongest antibody response in the first three weeks of infection, this was not significantly different from the antibody response recognising FhCL1 due to the high variability of responses. As infection develops, expanding B-cell responses would tend to be directed against shared cathepsin L epitopes rather than unique regions and thus the type of cathepsin L used in the ELISA became less discriminating. On the other hand, reflective of the relatively low antibody responses elicited to FhCB3, this antigen showed a sensitivity of approximately 70 % between seven and 11 weeks after infection but was not reliable after 20 weeks post-infection when titres had declined to background levels.

Because *F. hepatica* causes extensive tissue pathology and haemorrhaging during the migration of the immature flukes through the liver parenchyma, with the consequential animal production losses, it is important that diagnostic tests pick up infections as early as possible so that control interventions can be made quickly. We found that the earliest time point at which antibodies could be detected in experimentally-infected sheep using our ELISA was at 18 days post infection when either rFhCL3 or rFhCL1 were employed as the detecting antigen, albeit at this time point, diagnosis was not consistent due to the very high variability of antibody responses between animals. However, the detection was more robust at three weeks post-infection (21–22 dpi) with 91 % of the 59 infected animals tested at this time point deemed positive by the rFhCL1-ELISA and 95 % by the rFhCL3-ELISA. Interestingly, a low but significant relationship was observed between the antibody readings obtained at the three-week time point and the number of flukes recovered at necropsy 13 weeks later (16 weeks post-infection) which suggested, unsurprisingly perhaps, that there was a correlation between greater fluke burden and higher titres early IgG antibodies soon after infection. From seven weeks post-infection, all three cathepsins L assays detected fasciolosis in sheep with 100 % sensitivity. The time of seroconversion and the extent of antibody induced did not appear to be influenced by the sex or breed of the sheep, nor by the strain of parasite used for infection, albeit this is something that needs to be investigated with even greater animal numbers.

To evaluate if our ELISA could detect fasciolosis in naturally-infected animals, 20 lambs were exposed to pastures contaminated with liver fluke metacercariae for eight weeks before housing for a further seven weeks. ELISA were performed on serum samples taken at 0, 4, 9 and 15 wpe. Overall, we found that rFhCL1 and rFhCL3 were again the best performing antigens in our ELISA, and exhibited a similar pattern of antibody responses to what was observed in experimental infection. However, in the case of natural infection, the IgG levels were lower (overall highest average OD_{450} of 0.65 for rFhCL1 at nine wpe, compared with an overall maximum OD > 2.0 for the same antigen in animals experimentally infected in Trial 1). This was not unexpected given that only eight of the field-exposed animals became infected, and these harboured very low sub-clinical parasite burdens; at necropsy no more than three adult flukes were recovered from any of the infected animals. Encouragingly, all animals that were infected, regardless of the low parasite burden, were detected by the rFhCL1-ELISA by nine weeks after exposure and one animal was deemed positive after four weeks of field exposure.

Our data is consistent with that of Martínez-Sernández et al. (2018) who found that FhCL1, FhCL2 and FhCL5 exhibited 100 % sensitivity in assessing chronic infection in naturally infected sheep and cattle, although they did observe that FhCL2 exhibited a greater sensitivity in detecting fasciolosis in cattle. The recombinant proteins employed by Martínez-Sernández et al. (2018) were produced as insoluble proteins in the prokaryotic system, *Escherichia coli*, before being subjected to a re-folding process that resulted in antigens that performed similarly to the peptidases produced by us. In addition, the studies by Kuerpick et al. (2013) and Walsh et al. (2021) show that recombinant FhCL1 expressed in yeast (using similar methods as described here) effectively detected *F. hepatica* experimental infections in sheep and cattle as early as four weeks after infection. However, Walsh et al. (2021) suggested that reactivity to recombinant FhCL1 in naturally-infected animals was weaker and more variable than the one observed in experimentally infected animals.

The rFhCL1-ELISA was compared to two standard methods of diagnosis, FEC and coproantigen-ELISA. FEC has been used for many years for fasciolosis detection although it is time consuming, labour intensive and dependent on the consistency of egg shedding in faeces. The original version of the coproantigen-ELISA kit, based on the work of Mezo et al. (2004), had some described problems regarding sensitivity due to fluctuation in coproantigen levels (Valero et al., 2009; Novobilský et al., 2012), but this issue was overcome by introducing a combination of biotinylated secondary antibody and a peroxidase-labelled streptavidin for detection (Martínez-Sernández et al., 2016). Despite these advances, the method is still limited in detecting disease in animals before flukes have matured in the bile ducts and when animals are infected with low fluke burdens (George et al., 2017, 2019). While seven of the eight field-exposed sheep that picked up infection in our study were detected by the rFhCL1-ELISA at nine wpe, neither FEC nor the coproantigen ELISA test deemed these as positive at this time point. However, at 15 wpe all three tests were comparable, with the exception of one animal (no. 4, Table 1) that was not positive by coproantigen ELISA, and another animal (no. 5, Table 1), which was positive by rFhCL1-ELISA at 15 wpe but not positive by FEC or coproantigen ELISA and was, therefore, likely infected with immature flukes. The corollary of these findings are that the rFhCL-ELISA can detect subclinical infections of as low as one to three flukes in sheep within the first nine weeks of infection and as early as three weeks, for sheep with higher fluke burdens.

Finally, the antibody responses of experimentally-infected sheep throughout the acute and chronic stages of the disease and following TCBZ treatment at 17 weeks after infection were examined. In non-treated animals the antibody responses to rFhCL1, rFhCL2 and rFhCL3

increased during the acute phase of infection and after peaking at about 15 weeks post-infection, remained constant for the subsequent 10 weeks. By contrast, antibody responses to all three peptidases declined rapidly in the animals administered TCBZ treatment, consistent with that observed by Munita et al. (2019a) in cattle using four commercially available ELISA kits. The data demonstrates that adult parasites residing in the bile ducts must continually stimulate the host adaptive immune systems, likely by way of their blood-feeding activity through the punctured bile duct and their regurgitation of secreted proteinases, and other antigens. Presumably, the delivery of an array of immunomodulatory molecules present in their digestive secretions (Ryan et al., 2020) into the host bloodstream maintains an immuno-suppressive or regulatory control that provides a benefit to the parasite, despite its location in what is perceived to be an immunologically-protected site (Farina et al., 2009). Of greater importance to the health of the infected animal would be the possibility that continued suppression of immune responses would make them more susceptible to co-infections with microbes (e.g. tuberculosis) or other parasites (e.g. rumen fluke) (Cwiklinski et al., 2016; Howell et al., 2019; Munita et al., 2019b). While drug efficacy is best monitored using FEC reduction tests, which can provide definitive assessments within two weeks of treatment, the observed correlation between clearance of the parasites and decline of antibodies suggest the possibility of using the rFhCL-ELISA as an additional assay to monitor the success of drug treatments by comparing antibody responses before and after treatment.

5. Conclusion

Since our first characterisation of the cathepsin L family, many laboratories have exploited these enzymes in the development and commercialisation of diagnostic tests for detecting fasciolosis in sheep, cattle (Carnevale et al., 2001; Selemetas et al., 2014; Bloemhoff et al., 2015; Munita et al., 2016; Mokhtarian et al., 2018) and humans (O'Neill et al., 1998; Gonzales Santana et al., 2013; Gottstein et al., 2014). These antigens are amongst the most immunogenic molecules secreted by the parasite and different isoforms are produced from the moment parasites excyst in the duodenum to their long-term residence in the bile ducts. Although, all tested isoforms of the cathepsins L perform with 100 % sensitivity for the detection of sheep fasciolosis after seven weeks of infection, rFhCL1 and rFhCL3 showed a slightly better performance than rFhCL2, particularly in the acute stages of disease. The reason for this difference is unclear but could be related to the relative abundance at which each enzyme is secreted by the parasite, the amount of cross-reactive epitopes, and the affinity of specific epitopes for specific B-cell receptors. Antibodies measured in the rFhCL-ELISA were shown to remain high throughout a chronic infection but decline after drug treatment with the flukicide, TCBZ, implying that the test could be adapted to trace the effectiveness of drug treatment. Cathepsin B3 may not be as useful for the serological diagnosis of fasciolosis because of the low levels of antibodies elicited and their decline from seven weeks post-infection, although it could prove to be a useful marker of acute infection.

A number of methods to diagnose fasciolosis in livestock have been described (reviewed by Alvarez Rojas et al., 2014), for example FEC (Graham-Brown et al., 2019; Kamaludeen et al., 2019), coproantigen-ELISA (Martínez-Sernández et al., 2016), serum-ELISA (Cornelissen et al., 1999, 2001; Salimi-Bejestani et al., 2005; Mezo et al., 2007; Sugiyama et al., 2021; Walsh et al., 2021), PCR (Robles-Pérez et al., 2013; Calvani et al., 2018; Shi et al., 2020) or LAMP techniques (loop-mediated isothermal amplification; Martínez-Valladares and Rojo-Vázquez, 2016) but only serum-ELISA, PCR and LAMP have demonstrated an ability to diagnose early acute stages of infection (Martínez-Pérez et al., 2012; Mazeri et al., 2016). The rFhCL-ELISA developed here were very effective assays in detecting subclinical liver fluke in lambs exposed to natural infection on pastures. Testing of lambs each year as sentinel animals would be a rapid and straightforward way to assess the extent of infection on sheep farms during various seasons and annually, which is also important for understanding the prevalence of *F. hepatica* in cattle in areas where sheep and cattle co-graze. By taking a 'test and treat' approach to disease management farmers would save time and money which would lessen the prospect of drug resistant parasites emerging and reduce the environmental contamination of pastures with chemical treatments.

Author statement

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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.

Acknowledgments

This work was supported by a European Research Council Advanced Grant (HELIVAC, 322725) and Science Foundation Ireland (SFI) Professorship grant (17/RP/5368) awarded to J.P. Dalton.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2021.109517.

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