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Fasciola hepatica infection in sheep: Current and novel diagnostic tests

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Declaration

The work presented in this thesis is my own work, unless otherwise stated, and has not been submitted for any other degree or professional qualification.

The studies within this thesis have been published in part, and the publications have been reproduced, with permission from the publishers and co-authors, in Appendix 6.

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Abstract

Fasciola hepatica infections cause morbidity and mortality in sheep and have a significant economic impact on farmers. The commonly used diagnostic tests; faecal egg count (FEC), anti-*Fasciola* antibody ELISA (AbELISA) and the biochemical assays (measuring GLDH and GGT) all have limitations, particularly in detection of pre-patent infections in sheep. A coproantigen ELISA (cELISA) is reported to detect low burdens of infection from 4 weeks post-challenge (wpc) and to only detect current infection. A faecal PCR has been used for early detection of infection, but is limited by inhibitory factors in faecal samples. Loop-mediated isothermal amplification (LAMP) is more resistant to inhibitory factors and has the potential to be a pen-side assay. Triclabendazole (TCBZ) is the drug of choice to treat immature *F. hepatica* but there have been increasing reports of TCBZ treatment failure in the UK. Treatment outcome is determined using a FEC reduction test (FECRT). A cELISA reduction test (CRT) has recently been proposed.

Within this thesis the cELISA, along with FEC, and where feasible the AbELISA and the use of GLDH and GGT concentrations, are evaluated in (1) an experimental challenge model in sheep, (2) individual naturally exposed sheep, in early infection, pre- and post-treatment situations, (3) groups of naturally exposed sheep, including composite samples, in pre- and post-treatment situations and evaluating the FECRT and CRT, lastly a LAMP assay is developed for the detection of *F. hepatica*, and evaluated against cELISA, FEC and PCR based detection.

Two groups of 6 sheep were challenged with *F. hepatica* metacercarial cysts. In both studies, AbELISA was first to detect infection (3-4 weeks post-challenge (wpc)), followed by cELISA (3-10 wpc) and then FEC (9-10 wpc). Minor fluctuations were seen in both FEC and cELISA levels over both studies and a transient increase in cELISA levels was seen in the first study at 3-8 wpc. All animals were dosed with TCBZ 2 weeks prior to slaughter. The highest FECR was 37% and all sheep had live fluke present in their livers *post-mortem*.

27 lambs were sampled monthly between June and November with AbELISA, GLDH, GGT, FEC and cELISA tests performed. GLDH and GGT concentrations were above reference ranges from June. AbELISA detected infection in most animals by September and in all but one animal by November. FEC and cELISA both detected some very early positive results, most likely false-positive results, but the majority of animals became positive in November. Twelve lambs were followed to slaughter and all had low burdens of fluke (≤10).

A cross-sectional study was conducted including 36 British farms, comprising 812 and 528 sheep pre- and post-treatment, respectively. Low FEC and cELISA results were seen, with better agreement between the two tests pre- than post-treatment. Disagreements between the two tests were more frequently seen where the FEC detected infection but the cELISA did not. This was true both before and after treatment.

80 animals from 2 Scottish farms were confirmed to be infected with liver fluke and given either a TCBZ or closantel treatment and followed for 56 days. A closantel treatment was given to animals that were still infected at 21 days post-treatment (dpt). The highest FECR and CR of the TCBZ-treated groups was 60.3% and 56.4%, respectively, and the lowest FECR and CR of the closantel-treated groups was 83.7% and 94.9%, respectively. A small proportion of closantel-treated animals maintained a low FEC following treatment. Both the FECRT and CRT indicated treatment outcome from 7 dpt.

In a postal survey, 41 sample packs were sent to British farmers, of which 25 farmers participated. Samples from 44 and 36 groups were submitted pre- and post-treatment, respectively. Individual and composite faecal samples from each group were tested by FEC and cELISA. Group mean FECs were low and prevalence of infection on farms did not follow a normal distribution. The composite cELISA was more sensitive than the average cELISA, whilst the opposite was true for FEC. The composite cELISA was less sensitive than the composite FEC in low burden situations. A modified version of the composite CRT showed good agreement with

the composite FECRT and appears promising in situations where burden was sufficiently high.

A faecal LAMP assay, specific to *F. hepatica,* was developed and evaluated using samples from one of the groups of 6 experimentally challenged animals described above. FEC, cELISA and PCR testing were also performed and compared to the LAMP results. LAMP first detected infection at 3 wpc, followed by cELISA (7 wpc), FEC (10 wpc) and PCR (13 and 14 wpc).

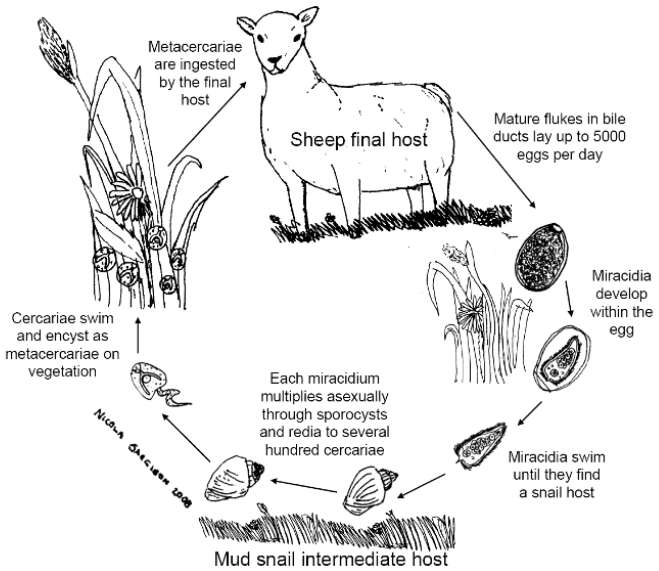
The studies within this thesis (1) confirm that cELISA can detect experimental infection of sheep with *F. hepatica* later than AbELISA but earlier than FEC, and confirm the TCBZ resistant status of a British isolate (Moredun isolate), (2) demonstrate that in animals naturally exposed to *F. hepatica*, the cELISA does not have an advantage of earlier detection over FEC and is not as sensitive as FEC in established infections (3) show that the modified CRT and composite CRT appear to give a good indication of treatment outcome from 7 dpt, but is of limited use in flocks with a low burden of infection, and (4) demonstrate that a faecal LAMP can detect *F. hepatica* infection at 3 wpc.

Chapter 1: Introduction

1.1 Liver fluke biology

The liver fluke, *F. hepatica*, has a complex life-cycle including free-living stages and an intermediate snail host (Figure 1.1). The adult parasites reside in the large bile ducts of the host, shedding eggs which are released into the gall bladder and travel along the intestines to be excreted in the host's faeces. In the environment, once the eggs have been freed from faecal matter, a free-swimming miracidium hatches. The miracidia locate and penetrate a suitable snail intermediate host (*Galba trunculata* in the UK) and migrate to the digestive gland, where a transformation to the sporocyst stage occurs. Each sporocyst undergoes asexual reproduction, giving rise to many rediae; each redia then undergoes asexual reproduction resulting in the cercarial stage. This process of multiple rounds of asexual reproduction results in large numbers of cercariae (estimates range from the hundreds to thousands) being produced from just one miracidium. The free-swimming cercariae are released from the snail host and migrate to vegetation where they form resistant cysts (or metacercariae). These metacercariae are ingested by a mammalian host, excyst in the duodenum and migrate through the peritoneal cavity to the liver. Here they move through the liver as immature fluke, developing into adults in the bile ducts.

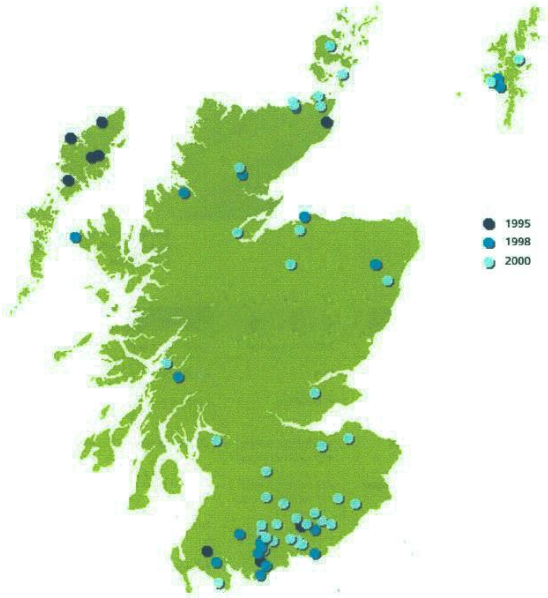
Figure 1.1 *F. hepatica* life cycle. Image used with kind permission of Nicola Sargison



1.2 Distribution

F. hepatica has a worldwide distribution, occurring on all continents except Antarctica (Mas-Coma, 2005). In the UK, *F. hepatica* was historically found in the wetter West of the country. More recently, cases of liver fluke infection have been reported in areas which were historically 'fluke-free' (Figure 1.2) (Mitchell, 2002). This change in distribution is likely due to, amongst other factors, changing climate, increasing animal movements and changes in land management practices (Kenyon et al., 2009).

Figure 1.2 Reported Scottish *F. hepatica* cases in 1995, 1998 and 2000 (Mitchell, 2002)



1.3 Clinical effects

Fasciolosis, the disease caused by liver fluke infection, can present in three different forms depending on the extent of exposure.

1.3.1 Acute and sub-acute

The immature stages of fluke, which burrow through the liver parenchyma, cause a significant amount of damage early on in the infection (Sánchez et al., 2001). If a large number of infective stages are ingested over a short period of time, the animal can suffer from acute fasciolosis, which very quickly presents with haemorrhagic anaemia, hypoalbuminaemia and sudden death. A sub-acute infection occurs when large numbers of parasites are ingested over a period of weeks and presents with symptoms such as acute haemorrhagic anaemia, hypoalbuminaemia and submandibular oedema (bottle jaw) 1-2 weeks prior to death (Mitchell, 2002). Cases of both acute and subacute fasciolosis are traditionally seen between late autumn and early winter.

1.3.2 Chronic

The chronic form of the disease is caused by extended low level exposure. The immature forms do not cause such damage in this presentation, rather the number of adults accumulating and feeding in the liver cause symptoms such as anaemia, weight loss, eosinophilia and hypoalbuminaemia (Mitchell, 2002). Submandibular oedema can also be seen in some cases. These symptoms lead to lower weight gain, lower wool yields, reduced milk production, reduced fertility and reduced twinning rates in ewes (Hope Cawdery, 1984). Chronic fasciolosis cases appear in winter and early spring.

1.4 Economic impact

The Scottish sheep industry suffers significant losses due to the effects of fasciolosis, although there are few exact figures in the literature. An individual farm was reported to lose £19,200 (£8.73 per ewe) due to a single outbreak of liver fluke, which was compounded by treatment failure (Sargison and Scott, 2010). These losses

occur not only from the death of animals, reduced productivity and treatment costs, but also from a loss of income at slaughter where livers are condemned due to fluke infection or damage. It was estimated that 7% of livers are rejected (Froyd, 1975), although as the incidence of fluke has increased, it is likely that this figure has also. Indeed, anecdotal reports suggest that in bovines liver condemnation rates may be as high as 78% (Anon, 2011). Quality Meat Scotland (QMS) report that of 499,826 cattle and 1,531,945 sheep seen at abattoirs in 2009, 26.85% and 10.05%, respectively, suffered liver condemnation (Kathy Peebles, personal communication, 2011).

1.5 Diagnosis

There are a variety of tests available for the diagnosis of *F. hepatica* in animals. These require a variety of samples, some more practical to obtain than others, and test for a range of direct and indirect indicators of infection. Farmers require diagnostic tests which are convenient, rapid, cost effective and allow them to make efficient use of the treatments available. For farmers, it is a question of the existence of a current or historic fluke infection, when to treat, which drugs to use and how to determine the effectiveness of said drug.

1.5.1 Faecal egg count (FEC)

The FEC is the traditional test for liver fluke which has been adapted over time. This involves the detection of eggs in faeces via either flotation or sedimentation (Dennis et al., 1954; McCaughey and Hatch, 1964), and provides a determination of the number of eggs per gram (epg). This has remained a popular test for a variety of reasons, including the ease of sample collection, of the test itself and of egg identification.

By its very nature, this test is only able to detect a fluke infection after patency (egg production). This means an infection cannot be diagnosed until 9-15 wpi (Valero et al., 2006), which may result in false negatives in early infections. Due to the biology

of fluke infections, eggs are released from the adult parasite and pass into the bile duct, where they are sequestered and released sporadically into the intestines and passed out with the faeces (Mezo et al., 2004). This pattern of excretion means that there is not a constant release rate of eggs and they are seldom evenly distributed in faecal matter, which potentially allows for false negatives in mature infections. In addition, sequestering of eggs in the bile duct allows for eggs to be seen in faeces up to 3 weeks post successful treatment (Chowaniec and Darski, 1970), making this a poor test for determining treatment outcome, due to the potential for false positives. It has been stated that epg has a poor correlation with adult fluke burden in cattle (Abdel-Rahman et al., 1998), but that a relationship is present in sheep, with an increasing adult burden leading to a lowered epg (Happich and Boray, 1969).

1.5.2 Liver investigation

Another frequently used method of diagnosis is the investigation of livers at slaughter. This is routinely performed at abattoirs to ensure the quality of the liver, but is also carried out at *post-mortem* to determine cause of death. It involves the slicing of the liver to look for the presence of parasites or view the pathology caused by an infection, previous or current. This has the potential to be used as a surveillance tool for prevalence studies (Froyd, 1975), however, it must be noted that livers may have been condemned due to reasons other than fluke infection. Although this information can be useful to the farmer to adapt future management practices through knowledge of current or historic infection, the damage has already been done to that year's income in terms of loss of liver, low body weight, low wool yield or death of animals.

1.5.3 Serum antibody ELISA (AbELISA)

Various enzyme-linked immunosorbent assays (ELISAs) have been developed to detect anti-*Fasciola* antibodies in serum (Zimmerman et al., 1982). Whilst these can detect an infection very early on, 4-8 wpi (Valero et al., 2009; Zimmerman et al.,

1982), the antibody titres persist at high levels following successful treatment (Ibarra et al., 1998; Sánchez et al., 2001). This serves to make the test useful as an indicator of past exposure but unreliable for indicating current infection status past the first year of life. In addition, the requirement of a blood sample is invasive which makes this a less practical test for farmers, especially sheep farmers, than those which require faecal samples.

1.5.4 Serum antigen ELISA

The detection of *F, hepatica* antigens, rather than anti-*Fasciola* antibodies, in serum by ELISA was first demonstrated in mice (Langley and Hillyer, 1989), and sheep (Rodríguez-Pérez and Hillyer, 1995). This allows for a direct detection of parasites, thus only indicating current infection. The test also benefits from allowing a very early detection of infection, 1-2 wpi (Duménigo et al., 2000; Rodríguez-Pérez and Hillyer, 1995). However, as the host develops an immune response, antibodies bind to epitopes of the antigen forming an immune complex, reducing the ability of the antibody in the ELISA to bind to the antigen (Rodríguez-Pérez and Hillyer, 1995). This lowered sensitivity limits the use of this test in the field. The length of time taken for the amount of free circulating antigen to drop below detectable levels varies in the literature, whilst it has been reported to be absent from 5 wpi, other reports state that it does not decline until 10 wpi (Langley and Hillyer, 1989; Rodríguez-Pérez and Hillyer, 1995).

1.5.5 Biochemistry and haematology

Subacute and chronic liver fluke infection can be diagnosed indirectly through the elevation or suppression of various haematological and biochemical factors. Sheep infected with *F. hepatica* were shown to have raised white blood cell counts, eosinophils, segmented and band neutrophil counts, mean corpuscular volumes, gamma-glutamyl transferase (GGT), glucose concentrations and globulins (Matanović et al., 2007). In the same study, levels of red blood cell counts,

lymphocytes, haemoglobin, packed cell volumes, mean corpuscular haemoglobin concentrations, aspartate aminotransferase, blood urea nitrogen, creatinine and albumin were found to be lower in infected animals than the uninfected controls.

Most commonly, GGT and glutamate dehydrogenase (GLDH) are measured as an indication of bile duct and liver damage, respectively, with GLDH giving very early detection of infection, 2-3 wpi (Mitchell, 2002). However, as these are indirect tests, there is the potential for false positives, and thus mis-diagnosis, due to other underlying clinical conditions. In addition, the requirement for a blood sample also renders the test invasive.

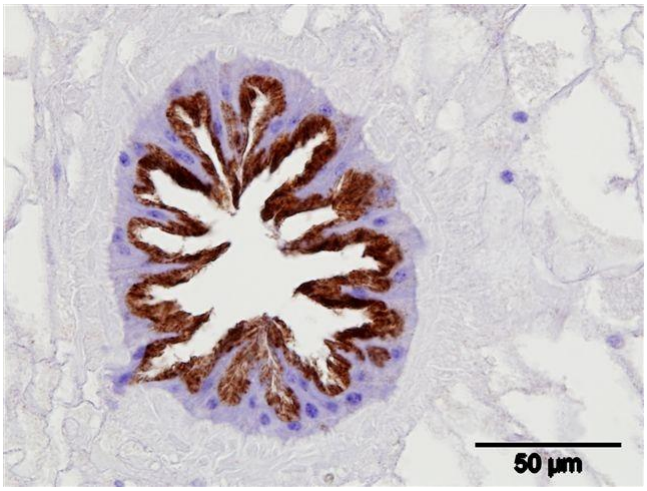
1.5.6 Coproantigen ELISA (cELISA)

As stated previously, faecal samples are the most practical test samples in terms of ease of collection. As such, the detection of parasite antigens in host faecal matter was investigated by a number of groups using various capture antibodies. Monoclonal antibodies, ES78,were used in a sandwich ELISA which was able to detect an infection of 5 adult fluke in cattle and showed no cross-reactivity with *Dicrocoelium dendriticum* (Duménigo et al., 1996), a related trematode with mammalian hosts. Moustafa et al. (1998) used purified polyclonal antibodies raised against *Fasciola gigantica*, a trematode closely related to *F. hepatica*, to detect infection in mice, rabbits and rats and found the sensitivity of the test to be host species-specific, achieving 83.3% sensitivity in mice. In cattle, Abdel-Rahman et al. (1998) found a monoclonal antibody ELISA to be 100% sensitive and 90% specific in infections of greater than 10 fluke at *post-mortem*, detecting infection at 6 wpi and showing no cross-reaction with *Paramphistomum microbothroides*, a trematode parasite of livestock. The same study also reported a correlation between parasite burden and ELISA titres. This finding was also reported when using the monoclonal antibody coproantigen ELISA (Duménigo et al., 2000).

A commercial cELISA, (BIO K201, Bio-X Diagnostics), based upon the MM3 monoclonal ELISA (Mezo et al., 2004), was developed to detect parasite antigens in

faeces. This ELISA involves the use of a monoclonal antibody to bind gut secretions released from the parasite during normal metabolic function (Figure 1.3). The coproantigen used has been shown to be specific to the gastrodermal cells of adult and immature fluke (Flanagan et al., 2011b). The target antigens of the MM3 monoclonal antibody were shown to be several *Fasciola* cathepsins L1 and L2 as well as a Kunitz-type protein (Muiño et al., 2011) Experimental infections in sheep have indicated that the test can detect antigen from 5 wpi (Flanagan et al., 2011b), considerably earlier than FEC and can detect antigen from a single adult parasite (Mezo et al., 2004). Whilst there is indication that coproantigen concentrations correlate with adult fluke burden in cattle (Brockwell et al., 2013; Charlier et al., 2008; Mezo et al., 2004), this has not been seen in sheep (Valero et al., 2009). There have been reports of this test performing well in sheep (Flanagan et al., 2011a; 2011b; Valero et al., 2009), cows (Brockwell et al., 2013; Mezo et al., 2004) and humans (Ubeira et al., 2009), although there is no information regarding its use in natural sheep infections.

Figure 1.3 Cross-section of an adult *F. hepatica* gut branch stained with MM3 monoclonal antibody from the BIO K201 kit.



Initial investigation into the effects of sample storage on the consistency of the coproantigen ELISA has shown good agreement between freshly prepared supernatants and those that had been frozen for 12 weeks (Flanagan et al., 2011b). The same study showed that the storage of faecal samples at room temperature (20°C

and 26˚C) for 4 days increased the OD in some samples but decreased the OD in other samples when compared to samples processed on arrival, but the overall result (positive or negative) remained the same. It is worth noting that only a small number of samples were compared.

1.5.7 DNA-based methods

DNA-based detection methods are not yet used for the routine diagnosis of *F. hepatica.* These methods are still under development and, in their current form, may be cost-prohibitive to farmers. Despite this, DNA-based detection methods have the potential to directly detect infection with high sensitivity and specificity.

1.5.7.1 Polymerase chain reaction (PCR)

PCR is a commonly used method of DNA amplification. In PCR a DNA sequence is exponentially amplified using thermal cycling, opposing primer pairs, *Taq* polymerase, a proprietary buffer and dNTPs. PCR has been used to successfully amplify DNA extracted from both *F. hepatica* adult parasites and infected snails (Ai et al., 2010a; Caron et al., 2011; Cucher et al., 2006; Kozak and Wedrychowicz, 2010). DNA has also been successfully amplified using PCR when extracted from metacercarial cysts and from eggs of other helminth species (McNally et al., 2013; Sugiyama et al., 2002). It is possible to quantify the amount of DNA amplified using real-time PCR (Alasaad et al., 2011).

There are limited reports of successful amplification of DNA extracted from the faecal samples of animals infected with *F. hepatica*. Martínez-Pérez et al. (2012) used primers targeted to the cytochrome C oxidase 1 gene (cox 1) to amplify DNA extracted from faecal samples using a standard and a nested PCR. The standard PCR amplified DNA from 3 wpi, whilst the nested PCR amplified DNA from 2 wpi. Robles-Pérez et al. (2013) were able to use the same primer set to amplify DNA extracted from faecal samples from 2 wpi. As yet, this assay has not been developed for real-time PCR applications.

Despite the potential for sensitive and specific faecal-based diagnosis using PCR, the assay is limited. Faecal samples are known to contain substances which are inhibitory to *Taq* polymerase and this may affect test sensitivity (Wilson, 1997). In addition, PCR requires the use of a thermocycler and a DNA detection method such as the use of agarose gel electrophoresis. There is also the potential for contamination of samples if the user is not careful, affecting specificity. Lastly, there is the lengthy process of DNA extraction prior to PCR, which can be costly and time-consuming. These factors raise the cost of the assay and require laboratory conditions for the test to be run. This may put the test out of the price range of most farmers.

1.5.7.2 Loop Mediated Isothermal Amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is an alternative DNA amplification method. It uses 3 primer sets, a master mix containing a DNA polymerase, which has strand displacement activity, and operates under isothermal conditions to amplify extracted DNA (Notomi et al., 2000). The process of amplification is more complex than that used in PCR and is illustrated in Figure 1.4. The F3/B3 primer set gives specificity to the assay and forms a structure recognised by the FIP/BIP primer set (Figure 1.4 (a)). This primer set binds to the structure formed by F3/B3 primer set and allows for amplification (Figure 1.4 (b)). The FLP/BLP primer set then binds to the structures formed by the FIP/BIP primer set and allows for further amplification (Figure 1.4 (c)).

Unlike PCR, LAMP is more resistant to the inhibitory substances found in faecal samples. It is also faster than PCR, with amplification possible in 45 minutes or less, only requires a single temperature for the reaction, and the results can be visualised by use of a fluorescent dye, a colour changing dye or by eye, so there is no requirement for sophisticated equipment (Notomi et al., 2000). These factors make LAMP attractive as a potential diagnostic test.

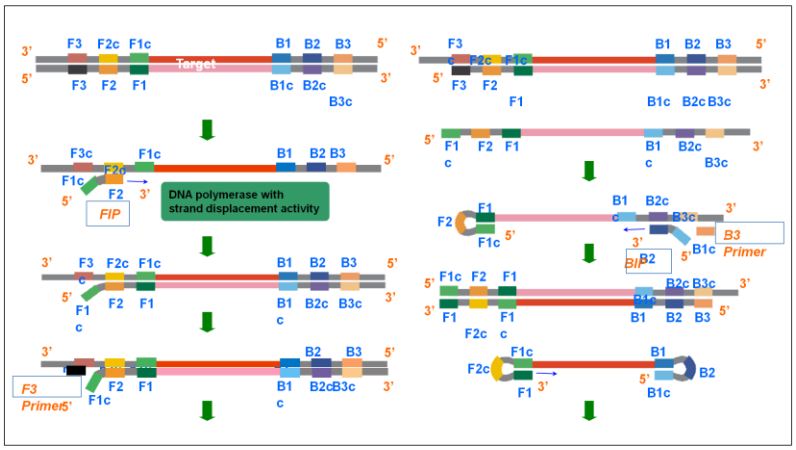
LAMP has been used to successfully amplify DNA from *F. hepatica* adults and was shown to be more sensitive than PCR (Ai et al., 2010b). In this assay, amplification

was achieved in 55 minutes, using DNA extracted using sodium dodecyl sulphate/proteinase K treatment. It has also been reported that LAMP can be used to amplify DNA extracted from snails infected with other trematode species and eggs of nematode species (Chen et al., 2013; Hamburger et al., 2013; Melville et al., 2014). The method has yet to be used on metacercarial cyst or trematode egg samples. Successful amplification of DNA extracted from faecal samples has been shown in *Clonorchis* (trematode), *Opisthorchis* (trematode) and *Strongyloides* (gastrointestinal nematode) infections (Arimatsu et al., 2012; Cai et al., 2012; Watts et al., 2014). Recent developments in LAMP chemistry have allowed real-time LAMP applications on multiwell microtitre plates e.g. for DNA extracted from the eggs of the gastrointestinal nematode *Haemonchus contortus* (Melville et al., 2014).

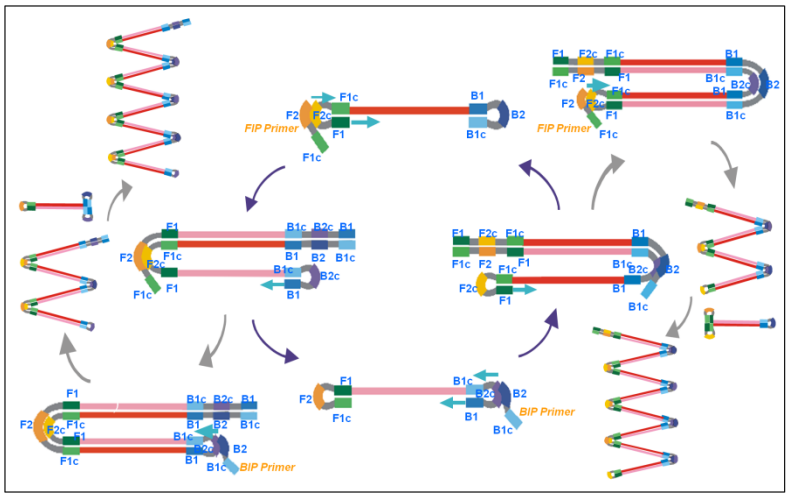
In its current form, LAMP is unlikely to become a routinely used diagnostic test. The reagents are expensive, not thermostable at ambient temperatures and the lengthy DNA extraction requiring laboratory conditions remains. DNA extraction may be possible using a freeze/thaw method similar to that used by Mukhopadhyay et al. (2012) for faecal samples infected with parvovirus. The company which supplies the LAMP reagents is currently developing a lyophilised version of the reagents, which would be more thermostable and has been shown to give results in 10 minutes when used in a real-time LAMP assay (I. McElarney, personal communication to P. Skuce, 2014). Visualisation of results using a lateral flow device has been shown for parasite, bacterial and viral DNA samples (Kiatpathomchai et al., 2008; Njiru, 2011; Rigano et al., 2010). With these developments LAMP could become a sensitive and specific pen-side faecal based test.

Figure 1.4 Process of DNA amplification using LAMP using (a) F3/B3, (b) FIP/BIP and (c) FLP/BLP primer sets. Images courtesy of Eiken Chemical Company Ltd reproduced by kind permission of Iain McElarney, MAST technologies

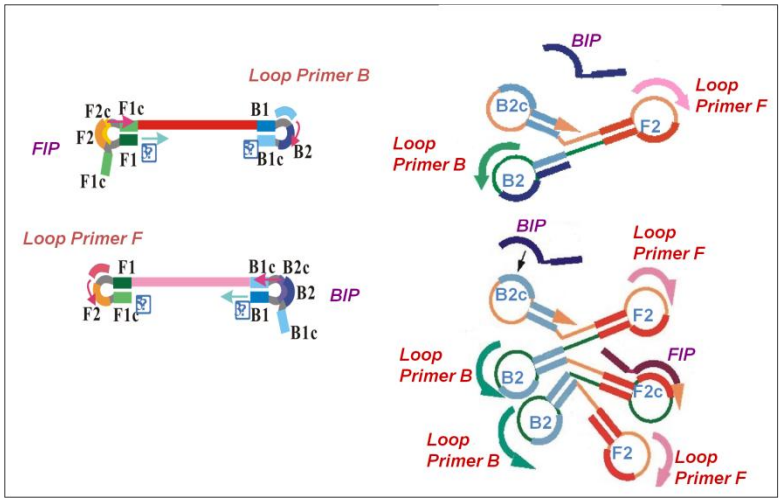
(a)



(b)



(c)



1.6 Treatment

1.6.1 Chemotherapy

Flukicidal anthelmintics are licensed for use in the UK but vary in the life-cycle stages they affect. Farmers require drugs which have a short meat or milk withdrawal period, affect the widest range of life stages possible (to limit the effects of immature fluke) and have high efficacy values. The ability to target immature fluke is of particular importance to sheep farmers due to the devastating effects of acute and sub-acute fasciolosis in sheep. In order to reduce the number of times animals need to be treated, combinations of anthelmintics have been released onto the market. These can be both combinations of different flukicides and combinations of flukicides and anthelmintics against nematode worms. Concern has been raised regarding the use of combinations targeting both nematodes and fluke as the ideal timings of treatments against these helminths would typically be different (Fairweather and Boray, 1999). Although there are 5 chemical families from which all flukicides are derived; halogenated phenols, salicylanilides, benzimidazoles, sulphonamides and phenoxyalkanes, only the two most commonly used families are described below.

1.6.1.1 Benzimidazoles

The benzimidazoles include triclabendazole (TCBZ), albendazole and netobimin. Of these, TCBZ affects the widest range of life stages; targeting very young immature fluke just 1 wpi in sheep and >2 weeks in cattle (Boray et al., 1983), whilst albendazole and netobimin are only effective against adult parasites (Bishop, 2005).

1.6.1.2 Salicylanilides

Closantel, nitroxynil and oxyclozanide are the available salicylanilides. Both closantel and nitroxynil are able to target young fluke (Sargison and Scott, 2010), effective in sheep at 6-8 wpi and 8 wpi, respectively (Fairweather and Boray, 1999).

Closantel acts later in cattle infections, >12 wpi, making it unable to target pre-patent fluke (Fairweather and Boray, 1999).

1.6.2 Treatment outcome

1.6.2.1 Controlled efficacy test (CET)

Due to the lack of a standardised FEC reduction test (FECRT) for trematode infections, the only World Association for the Advancement of Veterinary Parasitology (WAAVP) approved way to determine treatment outcome is by use of a controlled efficacy trial (CET), also known as dose and slaughter (Coles et al., 2006). The method of CET for fluke is described by Coles et al. (2000) and Coles and Stafford (2001). Briefly, two groups of 6 to 8 animals were dosed with 200 *F. hepatica* metacercariae, a treatment group and a control group. The treatment group was given a fasciolicidal treatment at 12 wpi, whilst the control group remained untreated. All animals were killed at 2 weeks post-treatment (wpt) and livers examined for fluke. The formula below is used to calculate the efficacy of the treatment used in the treatment group, where FB_C is the arithmetic mean fluke burden of the control group and FB_T is the arithmetic mean fluke burden of the treatment group. However, performing a CET on a working farm is highly impractical.

$$\text{Efficacy (\%)} = \left(\frac{(FB_C - FB_T)}{FB_C} \right) \times 100$$

1.6.2.2 Faecal egg count reduction testing (FECRT)

A FECRT has been developed to determine treatment outcome. This does not require an absence of eggs but rather a 95% or greater reduction in the number of eggs being seen between the treatment date and 14 days post-treatment (dpt) (Coles et al., 2006). This allows for a small number of sequestered eggs to be seen without affecting the

interpretation of treatment outcome. Although this is a widely used test, it is based upon the criteria developed for nematode infections and there has been no standardisation of this test for trematode infections (Coles et al., 2006).

1.6.2.3 Coproantigen reduction testing (CRT)

A coproantigen reduction test (CRT) has been proposed (Flanagan et al., 2011a) as an alternative to the current FECRT. As with the FECRT, faecal samples are taken at the point of treatment and 14 dpt but all animals must be negative at 14 dpt for the treatment to be deemed successful. This has been reported in one study to date in which sheep were experimentally infected with either TCBZ resistant (TCBZ-R) or TCBZ susceptible strains of fluke and the animals in each group were either treated with TCBZ or left untreated as a control. Fluke were cleared according to the coproantigen ELISA by 14 dpt in all groups bar one of the TCBZ-R strains treated with TCBZ (Flanagan et al., 2011a). However, it is possible that the time taken for antigen from live parasites to be cleared varies between populations of sheep and fluke and, as such, further studies are required to validate this CRT and the time points used.

1.6.3 Drug resistance

Due to the range of fluke stages treated by TCBZ, it has been a popular drug for many years, generating a significant selection pressure on the parasite population. As such, there have been a number of reports of treatment failure and suspected or confirmed TCBZ resistance in sheep and cattle. These reports have been worldwide, including Australia (Brockwell et al., 2014; Overend and Bowen, 1995), Scotland (Mitchell et al., 1998; SAC, 1998), Ireland (Lane, 1998; Mooney et al., 2009), Wales (Thomas, 2000), The Netherlands (Gaasenbeek et al., 2001; Moll et al., 2000), Spain (Álvarez-Sánchez et al., 2006), South America (Olaechea et al., 2011) and Peru (Ortiz et al., 2013).

The currently used indicator of drug resistance is a FECRT. However, a <95% reduction only indicates a reduction of eggs in the faeces viewed post-treatment, compared to the number of eggs in the faeces viewed pre-treatment. This may be due to the release of eggs which had been sequestered in the gall bladder, or due to live parasites remaining and continuing to shed eggs. At best the FECRT can be used to indicate if a treatment has failed or succeeded at killing adult parasites. A distinction must be made between failure of a drug and resistance of a parasite population to a drug. Treatment failure can occur for a variety of reasons including under-dosing, which may be due to faulty dosing equipment, underestimation of animal weight or reduced bioavailability of the drug (potentially due to reduced metabolism caused by parasite damage in the liver). Other explanations for treatment failure include inadequate diagnostics or poor drug formulation (Fairweather, 2011a). Most commonly, if TCBZ treatment appears to fail, animals will be treated with an alternative flukicide such as closantel and, if that treatment is successful, TCBZ 'resistance' (TCBZ-R) is declared. There is currently debate in the veterinary research community as to the usefulness of these declarations (Fairweather, 2011b; Sargison and Scott, 2011). Whilst it is important to be aware of the existence of TCBZ resistant fluke and alternative treatments, more harm can be caused by incorrect reporting and the subsequent needless abandonment of a very effective flukicide.

Due to the difficulties surrounding the determination of true drug resistance, it is difficult to determine the true extent of TCBZ-R. However, strains of fluke believed to be resistant to TCBZ have been isolated in Australia, Ireland, The Netherlands and Spain, although the resistant status of the Spanish isolate has been called into question when used in experimental infections (Álvarez-Sánchez et al., 2006; Coles et al., 2000; Flanagan et al., 2011a; Moll et al., 2000; Walker et al., 2004). The Irish isolate (Sligo) has been shown to be resistant both *in vivo* and *in vitro.* The mechanism of resistance is not fully understood and was previously believed to be associated with β-tubulin (Brennan et al., 2007), although further investigation found no difference in the and α- and β-tubulin isotypes between TCBZ-susceptible and TCBZ-R isolates (Ryan et al., 2008). Other studies, reviewed by Fairweather (2011c)

indicate that there may be a number of different mechanisms contributing to the development of TCBZ-R. These include an increase in the metabolism of TCBZ to TCBZ.SO and TCBZ.SO_2 as well as a reduced uptake of TCBZ and TCBZ.SO, this may indicate the involvement of P-glycoprotein-linked drug efflux pumps (Alvarez et al., 2005; Mottier et al., 2006; Robinson et al., 2004). Due to the observation that the Australian isolate (Oberon) does not appear to suffer any fitness costs, it is unlikely that a reversion to susceptibility will be seen and indeed this has been illustrated in The Netherlands (Borgsteede et al., 2005; Brennan et al., 2007). Of particular concern is the reduced efficacy (69.3%) of closantel seen in the Oberon strain (Fairweather, 2011a).

Closantel, whilst being a suitable alternative to TCBZ when used correctly (Coles et al., 2000), has also had resistance reported in Australia (Boray et al., 1990), indicating that mismanagement of this drug, or over-use in the treatment of *H. contortus* could lead to closantel-resistant strains of parasite developing as has been seen for TCBZ-R parasites.

1.7 Control

Traditionally, the management of fluke included activities such as regular treatment of stock with flukicides in the autumn and at lambing, drainage of boggy pasture and use of molluscicides (Hope Cawdery, 1984). With growing concern regarding damage to the environment and knock-on effects on ecosystems, the use of drainage and chemical molluscicides is no longer an option. Coupled with increasing treatment failure, farmers have had to adapt their management strategies.

Fluke management now includes avoidance rather than elimination of snails, the invertebrate host (Sargison and Scott, 2010). It is recommended that boggy areas, which may contain the snail, and thus infective metacercariae, should be fenced off to avoid animal grazing. However, this is not always a practical solution as on many farms a large proportion of the pasture would need to be fenced off in order to achieve this.

Reliance on anthelmintics is still a crucial aspect of successful control programmes (Sargison and Scott, 2010). As such, it is also recommended that farmers monitor the efficacy of their treatments by use of a FECRT to ensure that parasites have been cleared from their animals. Regarding the treatment of sheep, there has been a drive to encourage farmers to treat according to individual weight or to the heaviest animal in the flock rather than the average weight or an estimation of individual weight (Fairweather and Boray, 1999). Indeed this is one of the take home messages of the Sustainable Control of Parasites of Sheep (SCOPS) group's best practice advice to farmers and practitioners. This is to reduce the potential for under-dosing, a recognised major risk factor in the development of anthelmintic resistance.

If farmers are to follow these guidelines, they require diagnostic tests that are practical and rapidly produce reliable results that can indicate treatment outcome. If there was a greater understanding of infected snail distribution on pasture, and how animals interact with these areas, it may enable farmers to manage their pastures more effectively, which would greatly benefit those farms with particularly boggy, low-lying fields.

1.8 Intermediate snail host

Galba trunculata, the intermediate host of *F. hepatica* in the UK, is a mud snail of the family *Lymnaeidae* and the only known snail host of fluke in Scotland. *G. trunculata* can inhabit small pools of slow moving water with slightly acidic soils, often co-habited by plants of the *Juncus* genus commonly known as rushes (Mitchell, 2002; Rondelaud et al., 2011).

As these snails are hermaphroditic, the introduction of a few infected snails can quickly create new foci of infection. In addition, it is has been shown that only a small percentage of snails, 4-11%, need be infected in order to create stable fluke transmission (Abdel-Rahman et al., 1999; Caron et al., 2011; Mage et al., 2002; Manga-Gonzalez et al., 1991; Mekroud et al., 2004; Schweizer et al., 2007). This has an impact on control strategies that would target snails. It is not sufficient to

remove a large number of snails as only a small number of snails are required for the infection to become re-established.

Whilst it is possible to detect infected snails on pasture, this is no longer widely performed in the UK. It does, however, still play a role in control programmes in The Netherlands. There are a variety of tools to detect infected snails including dissection, polymerase chain reaction (PCR), real time PCR (RT-PCR) and multiplex PCR (Caron et al., 2011; Kozak and Wedrychowicz, 2010; Schweizer et al., 2007). These have the potential to be used to gain a better understanding of the distribution of infected snails on pasture and potentially indicate pasture burden to farmers, allowing them to better manage their pastures and treatment plans.

1.9 Other trematodes in Scottish Sheep

1.9.1 Rumen fluke

There are many different species of rumen fluke, 3 of which are of relevance in the UK; *Paramphistomum cervi*, *P. leydeni* and *Calicophoron daubneyi*. Historically, it was believed that *P. cervi* was the sole rumen fluke of livestock in the UK (Willmott, 1950). At the time, identification was based on morphology and the actual identification was of *P. scotiae* and *P. hiberniae*, both of which have since been recognised as *P. cervi* (Kamburov 1976, cited in Eduardo (1982)). Rumen fluke have undergone several reclassifications over the years, typically based on morphology (Eduardo, 1982). Recent molecular genetic studies, using sequencing of the ITS-2 region of ribosomal DNA, have identified only *C. daubneyi* to be present in UK livestock, with *P. leydeni* being found in Irish deer (Gordon et al., 2013; O'Toole et al., 2014)(unpublished data). All three rumen fluke species use a snail as the intermediate host. The species of snail varies between parasite species and location, with *C. daubneyi* being found to utilise *G. truncatula* as an intermediate host in France (Abrous et al., 1999).

The pathogenicity of rumen fluke is a controversial topic (Sanabria and Romero, 2008). This is despite the mechanism of pathogenicity of large infections of

immature fluke being demonstrated by Horak (1971). It was found that immature rumen fluke in the small intestine cause severe enteritis, resulting in malnutrition, dehydration and death (Horak, 1971). Horak (1971) also summarises several cases where rumen fluke were thought to be the cause of clinical signs in animals. More recently, several possible and confirmed clinical cases of paramphistomosis were reported in sheep and cattle in the UK (Foster et al., 2008; Mason et al., 2012; Millar, 2012). The rarity of clinical cases of paramphistomosis may be due to the large number of immature fluke required (Horak, 1971). It is also possible that many cases of enteritis go undiagnosed or are mis-diagnosed due to veterinarians not considering rumen fluke as a cause. The number of reported cases may increase with greater awareness and better diagnosis (Foster et al., 2008). Paramphistomosis is treated with oxyclozanide, which is licensed in the UK for the treatment of *Fasciola spp*. in sheep and cattle, but not for the treatment of rumen fluke.

The presence of rumen fluke in the UK has implications for the diagnosis of *F. hepatica*. Co-infections of both fluke species have been seen and, as the eggs are morphologically similar, this could lead to incorrect egg counts being recorded. A rumen fluke infection may be wrongly diagnosed as a *F. hepatica* infection, or a mixed infection could be reported as having a higher FEC if rumen fluke eggs are not identified. As most treatments for *F. hepatica* do not target rumen fluke, it is likely that, in a mixed infection liver fluke may be successfully treated but incorrectly identified rumen fluke eggs could give the impression that treatment was not effective.

1.9.2 Lancet fluke

The lancet fluke, *Dicrocoelium dendriticum,* is another liver fluke which is rarely seen in the UK. This liver fluke has a complicated life-cycle involving two intermediate hosts (an ant and a snail). It has only been reported in a few locations in the UK and is endemic on the Island of Coll in the Outer Hebrides (Anon, 2012; Cranwell et al., 2010; Sargison et al., 2012; Tarry, 1969). Large infections of these small liver fluke can cause disease, with clinical signs including photosensitivity, as

well as leading to the condemnation of livers (Sargison et al., 2012). *D. dendriticum* infections are treated with albendazole, although this is not wholly effective (Sargison et al., 2012).

D. dendriticum infections do not have as many implications for liver fluke diagnosis as rumen fluke infections do. This is due to the rarity and small geographic area over which infections are seen in, the fact the eggs are morphologically distinct and that there is no cross-reaction with the cELISA (Gordon et al., 2013). There is, however, a potential for cross-reaction in DNA-based diagnosis, due to genetic similarities between these trematode parasites.

1.10 Thesis aims

This thesis aims to evaluate currently available and novel diagnostic tests for *F. hepatica*. To achieve this the cELISA, along with FEC, and where feasible the AbELISA and the use of GLDH and GGT concentrations, are evaluated in (1) an experimental challenge model (Chapter 3), (2) individual naturally exposed sheep, in early infection, pre- and post-treatment situations (Chapter 4), (3) groups of naturally exposed sheep, including composite samples, in pre- and post-treatment situations and evaluating the FECRT and CRT (Chapter 5), and lastly a LAMP assay is developed for the detection of *F. hepatica*, and evaluated against cELISA, FEC and PCR based detection (Chapter 6).

Chapter 2: Materials and Methods

2.1 Sample collection

2.1.1 Faeces

Faecal samples from live animals were either collected from paddock/pen floors or per rectum. When possible, faecal samples were collected from the field in screw top pots. If samples were received in bags or gloves, they were transferred to screw top pots for storage. For rectal faecal sampling, animals were restrained and faeces removed from the rectum using gloved fingers, index finger ± middle finger. Fresh gloves were used for each animal. In the case of deceased animals, the rectum was cut away from the carcass and squeezed to recover faeces. On arrival at the laboratory faeces were stored at 4˚C. Samples were prepared for coproantigen ELISA (cELISA) testing within 7 days and faecal egg counts (FEC) were performed within 23 days.

2.1.2 Blood

Blood sampling was carried out either by a qualified veterinarian or a personal license holder registered for the procedure in question. 20G x 1" PrecisionGlide™ needles (BD Vacutainer®, UK) and plastic serum tubes with Red BD Hemogard™ closure (BD Vacutainer®, UK) were used. Samples were either stored at room temperature overnight to allow the clotting of the blood or centrifuged at 1,275 g for 10 minutes to obtain the serum. If storage was required for archive purposes, serum samples were frozen in 1.5 ml microcentrifuge tubes at -20˚C. Frozen samples were not needed for any studies within this thesis.

2.1.3 Livers

Livers were collected at slaughter or *post-mortem*, each liver was double-bagged in polythene bags to ensure that no fluke were lost. The animal identifier was written on both bags and, where possible, the ear tag was included in the bag with the liver. On arrival at the laboratory, livers were stored at 4˚C if they were to be processed on the

same day (<24 hours). If processing was to be delayed, livers were frozen at -20˚C for no more than 7 months.

2.2 Faecal testing

2.2.1 Faecal egg counting (FEC)

2.2.1.1 SAC method

FEC was performed by Scottish Agricultural College Consulting: Veterinary Services (SAC C VS) Dumfries, using a sedimentation method. Briefly, 3 g of faecal samples were weighed and washed through 710 µm, 150 µm and 38 µm sieves in sequence, with the faecal material collecting on the 38 µm sieve. The contents of the 38 µm sieve were washed into a 250 ml beaker which was then filled to the 200 ml mark with water. The contents of the beaker were allowed to sediment in water for 4 minutes. The supernatant was then poured off and the sediment re-suspended in water to the 200 ml mark and allowed to sediment again. This was repeated as necessary until the supernatant was clear. The final sediment was transferred to a petri dish, stained with 1 or 2 drops of 0.1 - 0.5% (w/v) methylene blue, viewed under a light microscope at x8 magnification and any fluke eggs observed counted.

2.2.1.2 Moredun method

The Moredun FEC method consisted of a modified version of the method outlined by McCaughey and Hatch (1964). Briefly, 3 g of each sample was homogenised in 42 ml of cold tap water and poured through a strainer into a 250 ml beaker. The filtrate was then poured through a 150 µm sieve into a 250 ml conical measure. The beaker was half-filled with water and the contents poured through the sieve again to wash any remaining eggs through to the conical measure. The conical measure was allowed to sit at a slight angle, ~20˚, for precisely 3 minutes at which point the supernatant was siphoned off using a vacuum line. One drop of 1% (w/v) methylene blue was added to the sediment, which was examined on a marked petri dish (7 cm diameter) at x16 magnification using a light microscope. Each sample dish was counted twice and an average taken, this was divided by 3 to calculate the number of

eggs per gram (epg). An animal was considered positive when at least 1 egg was detected. When less than 3 g of faeces were available the final result was divided by the amount of faeces tested to calculate the epg. Samples with less than 1 g available were considered to be insufficient for an accurate FEC to be performed and were thus excluded. FECs were performed by Danielle Gordon-Gibbs, Margaret Oliver, Lynsey Melville and Gillian Mitchell.

2.2.2 Faecal egg count reduction (FECR)

Faecal egg count reduction testing (FECRT) was used to determine treatment outcome. The reduction in the FEC, from 0 days post-treatment (dpt) to a subsequent post-treatment sampling point, was calculated using the formula below (Coles et al., 1992). This was originally developed for nematodes, where FEC_0 is the group arithmetic mean FEC at 0 dpt and FEC_t is the group arithmetic mean FEC at the post-treatment sampling. In this thesis, group sizes of a minimum of 9 animals for naturally exposed animals, and 6 animals for experimentally challenged animals, were used to calculate FECR.

$$FECR = \left(\frac{(FEC_0 - FEC_t)}{FEC_0} \right) \times 100$$

2.2.3 BIO K201 *Fasciola* Coproantigen ELISA (cELISA)

The BIO K201 *Fasciola* Coproantigen ELISA (cELISA) (Bio-X Diagnostics, Belgium) was performed according to manufacturer's guidelines, with the following modifications. All samples were homogenised prior to weighing out the 0.5 g (±0.03 g) required for the cELISA and all subsamples were suspended in buffer and vortexed for 10 seconds prior to centrifugation. A full protocol with modifications is listed in Appendix 1. Samples were prepared to supernatant stage and supernatants were stored in 1.5 ml microcentrifuge tubes at -20°C until testing. Optical densities

were read at 450 nm on an ELx808 IU Absorbance Microplate Reader (BioTek® Instruments Inc, USA).

For each plate run, the positive control had to be equal to or greater than a minimum optical density (OD), which was batch specific and ranged from 0.805 to 1.236, in order for the plate to be considered valid. Initially, the manufacturers stated that a test sample with an OD of ≥0.15 was to be considered positive by the BIO K201 kit criteria. Subsequently, the kit criteria outlined that a result should be interpreted as a percentage of the OD of the kit's positive control. A sample was considered positive if this percentage was greater than a set value which was batch specific (ranging from 6.07% to 7.49%). The formulae used to calculate the OD and PD are shown below.

$$OD = \text{well 1 value} - \text{well 2 value}$$

$$PD = \left(\frac{\text{sample OD}}{\text{Positive control OD}} \right) \times 100$$

The composition of the kit was also altered twice during this study. In the first instance, a concentrated chromogen tetramethylbenzidine solution (TMB) was provided with the kit which had to be diluted with a provided substrate solution. When the results interpretation changed, the TMB was provided in a ready to use form. The third change in kit was the addition of a yellow colouring to the dilution buffer. The cELISA was performed by Danielle Gordon-Gibbs, Margaret Oliver, Lynsey Melville, Lisa Imrie and Gillian Mitchell.

2.2.4 Coproantigen reduction (CR)

A novel coproantigen reduction test (CRT) to determine treatment outcome was used in studies within this thesis, which differed to that proposed by Flanagan et al. (2011b). Instead the FECRT formula described in Section 2.2.2 was applied to pre- and post-treatment cELISA OD/PD values and a CR calculated. A CR of ≥95% was

deemed to indicate successful treatment. An additional criterion was added in Chapter 5, which automatically deemed treatment successful if the post-treatment cELISA PD indicated that no fluke infection was present. In this thesis, group sizes of a minimum of 9 animals for naturally exposed animals, and 6 animals for experimentally challenged animals, were used to calculate cELISA reduction (CR).

2.3 Serum testing

2.3.1 Biochemistry

Serum testing for the determination of glutamate dehydrogenase (GLDH) and gamma-glutamyl transferase (GGT) concentrations was conducted by SAC C VS (Dumfries). GLDH was measured using a commercial biochemical assay kit (Randox Ltd) which measures the decrease in absorbance at 340 nm in the following reaction which is catalysed by GLDH: a-oxoglutarate + NADH + NH4+ → glutamate + NAD+ + H_2O. GGT was measured using another commercial biochemical assay (Instrumentation Laboratory UK Ltd) which measures the rate of increase in absorbance at 405 nm in the following reaction which is catalysed by GGT: L-γ-glutamyl-3-carboxy-4-nitroanilide + glycylglycine ↔ L-γ-glutamylglycylglycine + 5-amino-2-nitrobenzoate. Both assays were viewed on an IL600 random access analyser using standard methods.SAC C VS reference ranges for normal liver and bile duct function used in this thesis were 2-10 IU/L and 27-31 IU/L, respectively.

2.3.2 Pourquier fasciolosis antibody ELISA (AbELISA)

The Pourquier bovine fasciolosis serum and milk verification ELISA, P05120-5 (IDEXX Laboratories, USA) was performed by SAC C VS (Dumfries) or Biobest Laboratories Ltd (UK) for samples in this thesis. The AbELISA was performed and interpreted according to the manufacturer's guidelines, which state that a sample is positive if the S/P % of the sample is >30. The SP % is calculated by dividing the OD of the test sample by the OD of the positive control and multiplying this number by 100. Biobest Laboratories Ltd gave further interpretation of the results stating that

an S/P % of 31-79 indicated a light infestation, 80-149 indicated a moderate infestation and ≥150 indicated a heavy infestation.

2.4 Dose and slaughter trial

2.4.1 Metacercarial cyst maintenance

Metacerarial cysts were received from Ridgeway Research, Gloucestershire (UK) on plastic sheeting. These sheets were transferred to 500 ml capacity plastic tubs with screw top lids, which were filled with ~400 ml distilled water. Two or three sheets were stored in each tub. The tubs were stored at 4ºC. The water was changed weekly; this was done by pouring out the existing water into a jug and replacing with fresh distilled water. The tubs were gently swirled to ensure that the sheets were not stuck to each other and the fresh water was able to circulate around each sheet. The waste water was disposed of following heat inactivation of any metacercarial cysts that may be present (heated to 70˚C for several hours and then allowed to cool prior to disposal). Cyst maintenance was performed by Danielle Gordon-Gibbs and Gillian Mitchell.

2.4.2 Fluke challenge preparation

Infectious doses of metacercarial cysts were prepared for a dose and slaughter trial and experimental infections. The cysts were examined under a light microscope to check for viability (Figure 2.1). Using a metal spatula, cysts were scraped into a small volume of 0.5% agar, which was stirred with a metal spatula to obtain an even distribution. 100 µl aliquots of this stock solution were then taken and again examined under a light microscope to determine the concentration of viable cysts. Additional 0.5% agar could then be added to the stock solution to obtain the desired concentration of viable cysts. Doses were taken up into 20 ml syringes, capped and stored at room temperature for oral administration the same day.

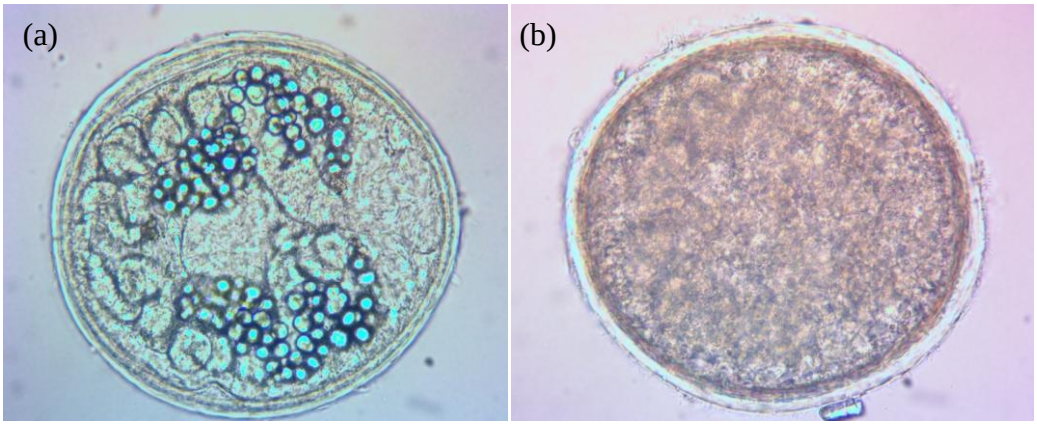


Figure 2.1 Viable (a) and non-viable (b) *F. hepatica* metacercarial cysts.

2.4.3 Liver processing

2.4.3.1 SAC method

The gall bladder and main bile ducts of livers obtained from slaughter were opened with scissors and any fluke present removed. Livers were then cut into ~1 inch slices and pressure applied to remove fluke. Liver slices were soaked in water overnight and pressure applied to each slice the next day. Water from the overnight soaking was passed through a 355 µm sieve and the debris examined under a dissecting microscope for the presence of any whole or partial fluke.

2.4.3.2 Moredun method

Fluke were extracted from livers obtained at slaughter or at *post-mortem* following the method outlined by Clery et al., (1996), with an additional maceration of liver slices. The full protocol used is presented in Appendix 1. Livers were processed by Danielle Gordon-Gibbs, Margaret Oliver, Lynsey Melville, Lisa Imrie and Gillian Mitchell. In some cases, for reasons of practicality, livers were frozen at -20˚C on receipt for investigation at a later date, as examination was not always possible on the day of collection. Retrieved fluke were temporarily placed in water and examined under a light microscope to determine fluke age. Fluke length and width of shoulders were noted and also used to determine fluke age. For partial flukes recovered, the

numbers of anterior and posterior ends were counted and the larger figure added to the number of whole fluke seen in order to determine liver burden. After examination fluke were stored in 75-100% ethanol.

2.4.4 Egg recovery

In some instances, sequestered eggs from the gall bladder were required, either for molecular work or to propagate the isolate. In these cases, the gall bladder was clipped off and contents collected on the surface of a sieve. Contents were washed with distilled water, any adult fluke removed and eggs allowed to sediment in a universal tube. Eggs were then re-suspended in either distilled H_2O and stored at 4˚C or ethanol and stored at room temperature depending on their intended use, hatching or DNA extraction, respectively. Eggs that were to be hatched were kept at 4˚C overnight and posted to the relevant organisation/research teams the next morning. The quantity of eggs could be estimated by re-suspending the solution and counting the eggs present in a 100 µl aliquot. This was performed by Danielle Gordon-Gibbs, Lynsey Melville and Gillian Mitchell.

2.5 DNA Extraction

DNA extractions were performed by Danielle Gordon-Gibbs and Gillian Mitchell.

2.5.1 DNeasy® Blood and Tissue Kit

The DNeasy® Blood and Tissue Kit (Qiagen) was used to extract genomic DNA from trematode tissue, trematode eggs and ovine faeces (0.2 g ±0.03 g). The kit was used as per manufacturer's instructions for animal tissues (spin-column protocol); with the exception that DNA was eluted into 100 µl of Buffer AE rather than 200 µl. The DNA extraction was performed in a UV sterilisation cabinet (Bigneat Ltd, UK). A full description of the protocol used is listed in Appendix 1.

2.5.2 QIAmp® DNA Stool Mini Kit

The QIAmp® DNA Stool Mini Kit (Qiagen) was used at 95°C to extract DNA from 0.2 g (±0.03 g) ovine faeces as described by the manufacturers for stool pathogen detection, with the exception that samples were vortexed with the InhibitEX Tablet for 2 minutes rather than 1 minute and DNA eluted into 100 µl Buffer AE rather than 200 µl. The DNA extraction was performed in a UV sterilisation cabinet (Bigneat Ltd, UK). A full description of the protocol used is listed in Appendix 1.

2.5.3 QIAmp® Fast DNA Stool Mini Kit

The QIAmp® Fast DNA Stool Mini Kit (Qiagen) was used at 95°C to extract DNA from 0.2 g (±0.03 g) ovine faeces as described by the manufacturers for stool pathogen detection, with the exception that DNA eluted into 100 µl Buffer ATE rather than 200 µl. The DNA extraction was performed in a UV sterilisation cabinet (Bigneat Ltd, UK). A full description of the protocol used is listed in Appendix 1.

2.5.4 Freeze/thaw DNA extraction

A freeze/thaw DNA extraction method based on that outlined by Mukhopadhyay et al. (2012) was used to extract DNA from ovine faeces. Briefly, 0.25 g (±0.03 g) of homogenised faeces were weighed out into a sterile 1.5 ml microcentrifuge tube and frozen at -20°C. Samples used for assay development were stored at -20˚C for no longer than 1 month prior to DNA extraction, whilst samples used for the evaluation of PCR and LAMP assays were stored for ~1 year prior to DNA extraction. Subsequently, these samples were diluted in 500 µl of nuclease-free (NF) H_2O (Sigma) and vortexed until mixed. Samples were then kept at 96°C for 10 minutes before immediately being placed on ice for 5 minutes. Lastly, samples were centrifuged at 12,000 g for 10 minutes and the supernatant recovered. 50 µl of this supernatant was then diluted in 450 µl of NF H_2O (Sigma). This diluted DNA could then be used in amplification techniques.

2.6 DNA amplification

2.6.1 PCR and gel imaging

DNA amplification was carried out using polymerase chain reaction (PCR) on either an Applied Biosystems® GeneAmp® PCR System 2700 (Thermo Fisher Scientific, USA) or an Applied Biosystems® 2720 Thermal Cycler (Thermo Fisher Scientific, USA). In each instance, a mastermix for 25 µl reactions was created in a DNA-free UV sterilisation cabinet (Bigneat Ltd, UK), using the Platinum® *Taq* DNA polymerase kit (Invitrogen, USA) and dNTPs (Invitrogen, USA), NF H_2O (Sigma). All primers were ordered from Eurofins MWG Synthesis GmbH (Germany), made to a 100 pmol/µl concentration using NF H_2O (Sigma) as described on the oligonucleotide synthesis report prior to being stored at -20ºC.

Briefly, 1.2% agarose gels were prepared by adding ~400 ml of distilled water and 8 ml of 50x TAE (Tris-acetate-EDTA) to 4.8 g of molecular grade agarose (Bioline, UK) and heating in a 600W (category E) microwave until the mixture bubbled. It was then allowed to cool before 40 µl of GelRed™ Nucleic Acid Gel Stain (Biotium) was added. The mixture was then swirled and poured into a gel plate with an appropriate comb. Finally, the gel was allowed to cool until it had set, at which point the comb was removed and samples could be added.

For each PCR product, 2 µl of PCR product was added to 5 µl Blue Juice™ gel loading buffer (Invitrogen, USA). These samples were run on a 1.2% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium) at 100 volts. Gels were viewed on an AlphaImager™ 2200 (Alpha Innotech) gel documentation system. PCRs and gel imaging were performed by Danielle Gordon-Gibbs and Gillian Mitchell.

2.6.2 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) was carried out using the MAST Isoplex® DNA Amp kit (MAST Group Ltd., UK). Reaction mixes and primer mixes

were made in 1.5ml microcentrifuge tubes in a UV sterilisation cabinet (Bigneat Ltd, UK). All tubes and reagents were kept on IsoFreeze® MCT racks. Fresh NF H_2O (Sigma) was used for each mastermix. The Loopamp® fluorescent detection reagent (Eiken Chemical Co. Ltd, Japan) was added to the reaction mix to visualise amplification. DNA samples were added in a separate area, again on a cool plate. A kit positive, parasite positive and at least one negative control, NF H_2O (Sigma) was included in each run. HPLC purified primers were ordered from Eurofins MWG Synthesis GmbH (Germany) and brought to 100 pmol/µl, as described in the oligonucleotide synthesis report, using NF H_2O (Sigma). Amplification was carried out on either an Applied Biosystems® GeneAmp® PCR System 2700 (Thermo Fisher Scientific, USA) or an Applied Biosystems® 2720 Thermal Cycler (Thermo Fisher Scientific, USA). Amplification was viewed directly within the reaction tubes on an AlphaImager™ 2200 (Alpha Inotech) gel documentation system. Reaction tubes were disposed of by incineration without tube lids being opened. LAMP assays were carried out by Danielle Gordon-Gibbs.

2.7 Statistical methods

Statistical analysis was performed in RStudio (R Core Team, 2014) unless otherwise specified.

The "lm()" function, from the "stats" package, was used to fit a linear model to two sets of data in order to compare tests (R Core Team, 2014). The model form was x ~ y.

The "cor()" function, from the "stats" package was used to calculate the correlation between two data sets (R Core Team, 2014). As the data were not of a normal distribution, the "spearman" method was specified in order to calculate the Spearman's rank correlation coefficient (rho).

The "confusionMatrix()" function, from the "caret" package, was used to create cross tabulations of the results of two tests, one of which was deemed to be the 'gold

standard' or reference test (Kuhn, 2014). The format of the table produced by "confusionMatrix()" is shown in Table 2.1.

Table 2.1 Format of "confusionMatrix()" function table

		Reference	
		Event	No event
Predicted	Event	A	B
	No event	C	D

From this table, the sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) of the non-reference test were calculated using the "confusionMatrix()". The formulae used to calculate sensitivity, specificity, PPV and NPV and prevalence are listed below (Kuhn, 2014).

$$\text{Sensitivity} = \frac{A}{(A + C)}$$

$$\text{Specificity} = \frac{D}{(B + D)}$$

$$\text{Prevalence} = \frac{(A + C)}{(A + B + C + D)}$$

$$\text{PPV} = \frac{A}{(A + B)}$$

$$\text{NPV} = \frac{D}{(C + D)}$$

In addition, the Cohen's unweighted Kappa for the two tests was also calculated using the "confusionMatrix()" function. Kappa values were categorised according to the degree of agreement beyond chance, ranging from none to almost perfect as described by McGinn et al. (2004) and summarised in Table 2.2.

The "wilcox.exact()" function, from the "exactRankTests" package, was used to perform a modified Wilcoxon rank sum test, which is an unpaired non-parametric t-test, that allows for ties (repeat values) in the data (Hothorn and Hornik, 2013).

Table 2.2 Kappa values and corresponding category of degree of agreement beyond chance as described by McGinn et al. (2004)

Kappa value	Degree of agreement beyond chance
0	None
0-0.2	Slight
0.2-0.4	Fair
0.4-0.6	Moderate
0.6-0.8	Substantial
0.8-1.0	Almost perfect

The FECR was performed in using the "FECRT()" function from the "bayescount" package (Denwood, 2009). The WAAVP method for the calculation of FECR was used (Coles et al., 1992), as opposed to the Bayesian MCMC or bootstrap methods also offered by the "FECRT()" function. As this function requires integers, all FECs were rounded to the nearest integer. All FECs which were <0.5 epg but >0 epg were rounded to 1.0 epg.

The CR was performed in Microsoft Excel, using the formula specified in Section 2.2.2, due to the impracticalities of rounding ODs and PDs to the nearest integer.

Chapter 3: Evaluation of current diagnostic tests following experimental challenge

3.1 Abstract

Although the faecal egg count (FEC) is commonly used to diagnose *Fasciola hepatica* infections, it cannot detect the pre-patent stages of liver fluke which cause acute and sub-acute fasciolosis. Whilst the anti-*Fasciola* antibody ELISA (AbELISA) can detect pre-patent fluke infection, it is not specific for current infection. A new test, the coproantigen ELISA (cELISA), has been reported to detect early infection, only detect current infection, and be able to detect the presence of even low numbers of fluke. Triclabendazole (TCBZ) is the drug of choice to treat *F. hepatica,* especially in sheep, as it is the only drug available which can target all stages of the parasite within the definitive host. There have been increasing reports of TCBZ treatment failure in the UK, although resistance has yet to be confirmed. The present study aimed to develop a challenge model in order to (1) evaluate the performance of AbELISA, FEC and cELISA in sheep which had been experimentally challenged with *F. hepatica,* (2) determine the TCBZ resistant (TCBZ-R) status of the *F. hepatica* isolate used and (3) profile the dynamics of this isolate using blood- and faecal-based diagnostics. Over the course of 2 years, 2 groups of 6 sheep were challenged with metacercarial cysts derived from a *F. hepatica* isolate obtained from naturally infected sheep with a history of TCBZ treatment failure. In both studies, AbELISA was first to detect infection (3-4 weeks post-challenge (wpc)), followed by cELISA (3-10 wpc) and then FEC (9-10 wpc). Although minor fluctuations were seen in both the FEC and cELISA levels over both studies, a transient increase in the cELISA levels were seen in the first study at 3-8 wpc. TCBZ treatments were given to all animals 2 weeks prior to slaughter. The highest FEC reduction seen was 37% and all sheep had live, visually undamaged fluke present in their livers at slaughter, confirming the presence of fluke resistant to TCBZ. Drug efficacy could not be calculated. This study confirms that cELISA can detect experimental infection of sheep with *F. hepatica* later than AbELISA but earlier than FEC. A transient increase in cELISA values during early infection was described for the first time. In addition, TCBZ-R was confirmed for the first time for a British isolate (Moredun isolate) by means of dose and slaughter trials.

3.2 Introduction

Fasciolosis in the UK is caused by the liver fluke, *Fasciola hepatica*, and is responsible for significant economic losses to the UK sheep and cattle farming industry. Although faecal egg counting (FEC) is a useful diagnostic test for chronic fasciolosis, it is unable to detect the pre-patent acute and sub-acute forms of the disease (Valero et al., 2006). Detection of anti-*Fasciola* antibodies by means of the antibody ELISA (AbELISA) gives very early indication of infection, from 4 weeks post-infection (wpi), but it cannot differentiate between past and current infection (Sánchez et al., 2001; Zimmerman et al., 1982). Due to the severity of acute and sub-acute fasciolosis, a specific diagnostic test for this condition is urgently needed.

The BIO K201 cELISA is a relatively new diagnostic test, developed by Mezo et al. (2004) and commercialised by Bio-X Diagnostics (Belgium). The authors that developed this test reported that it could detect infection from 5 wpi, that it was specific for current liver fluke infection and that it could detect infection by a single fluke in lambs (Bio-X, 2010; Mezo et al., 2004). However, there have been few studies investigating the commercial test in sheep (Flanagan et al., 2011a; 2011b).

Triclabendazole (TCBZ) is the fasciolicidal drug of choice for the treatment of *F. hepatica*, especially in sheep, due to its unique ability to kill all stages of liver fluke within the definitive host (Boray et al., 1983). Whilst other treatments are available, they have little or no efficacy against immature fluke, younger than 6 weeks old, which can cause acute and sub-acute fasciolosis (Fairweather and Boray, 1999). As acute and sub-acute fasciolosis are caused by pre-patent fluke and often result in sudden death, they can be difficult to diagnose and, as such, TCBZ is often given prophylactically i.e. without a diagnosis (Boray, 1985). This reliance on a single drug, for so many years, has placed a strong selection pressure for TCBZ resistant (TCBZ-R) fluke.

The potential for TCBZ-R was first demonstrated in Australia by Overend and Bowen (1995). Since then, TCBZ-R isolates have been identified in Australia,

Ireland, The Netherlands and Spain (Álvarez-Sánchez et al., 2006; Coles et al., 2000; Moll et al., 2000; Walker et al., 2004). There have also been numerous reports of TCBZ treatment failure in the UK (Anon, 1998; Lane, 1998; Mitchell et al., 1998; Thomas, 2000). It is important to note that cases of treatment failure do not automatically indicate resistance. Treatment failure can occur for a number of reasons, including poorly stored medications and under-dosing. Under-dosing can be caused by underestimating animal weights, poorly calibrated dosing equipment or the dose not being completely swallowed or absorbed.

To confirm that an isolate of *F. hepatica* is truly resistant to a drug, a dose and slaughter trial is required (Coles and Stafford, 2001). Whilst this may appear unnecessarily extreme, it has been shown that *F. hepatica* isolates, believed to be resistant based on FECRT results, have proven to be susceptible when evaluated in dose and slaughter trials (Fairweather, 2011a). This mis-classification of isolates gives a false impression of the extent and spread of resistance. It could also have implications for future research into the detection and management of resistant fluke populations. For resistance to be better understood at a genetic level, isolates of known resistance status are essential.

This chapter aims to establish a *F. hepatica* challenge model in sheep in order to (1) evaluate the performance of AbELISA, FEC and cELISA, (2) to determine the TCBZ resistant status of a British *F. hepatica* isolate (Moredun isolate) and (3) to profile the dynamics of said isolate using blood- and faecal-based diagnostic tests.

3.3 Materials and methods

3.3.1 Isolation of Moredun isolate

Two in-lamb Blackface x Blue Leicester ewes, henceforth referred to as donor ewes, were selected from a Scottish farm, which had a history of repeated TCBZ treatment failure. These donor ewes were identified as being infected with liver fluke according to FEC and cELISA testing. The donor ewes were weighed and faecal sampled prior to treatment with TCBZ, according to the manufacturer's recommended dose rate, 1

ml per 5 kg bodyweight (Fasinex®, Novartis Animal Health). Sampling was repeated at 0, 1, 2, and 3 weeks post-treatment (wpt). Both ewes were FEC and cELISA positive at each sampling, with limited reduction in FEC from pre-treatment levels (no greater than 69% FECR). At 3 wpt, they were again weighed and dosed with TCBZ (Fasinex®, Novartis Animal Health), according to the manufacturer's recommendations. The animals were sampled again 35 days later, at which point they were still FEC and cELISA positive. The ewes were considered to be sufficiently healthy, allowed to lamb and a third TCBZ (Fasinex®, Novartis Animal Health) treatment given once the lambs had been weaned. FEC and cELISA results from each sampling point can be seen in Figure 3.1. Treatment was often followed by a reduction in FEC but not cELISA results (Figure 3.1) and at no point did the FEC reduction (FECR) exceed 66% for either animal. Full details of the FECR can be seen in Appendix 2.

The donor ewes were killed 7 days following the third treatment. At this point, 46 adult fluke were recovered from ewe A1055 and 75 from ewe A1087, as described in Section 2.4.3.2. In addition, fluke eggs were recovered from the ewes' gall bladders, as described in Section 2.4.4. These eggs were combined into one sample and posted to Ridgeway Research (Gloucestershire, UK) where they were hatched into miracidia and used to infect *Galba truncatula* snails. The metacercarial cysts that were shed by the infected snails were collected on cellophane sheets and sent back to Moredun Research Institute, UK (MRI). The metacercarial cysts were maintained at 4˚C in ultra pure water, with weekly water changes for 5 months in the case of challenge experiment 1, and 2 months in the case of challenge experiment 2, until the point of challenge, as described in Section 2.4.1.

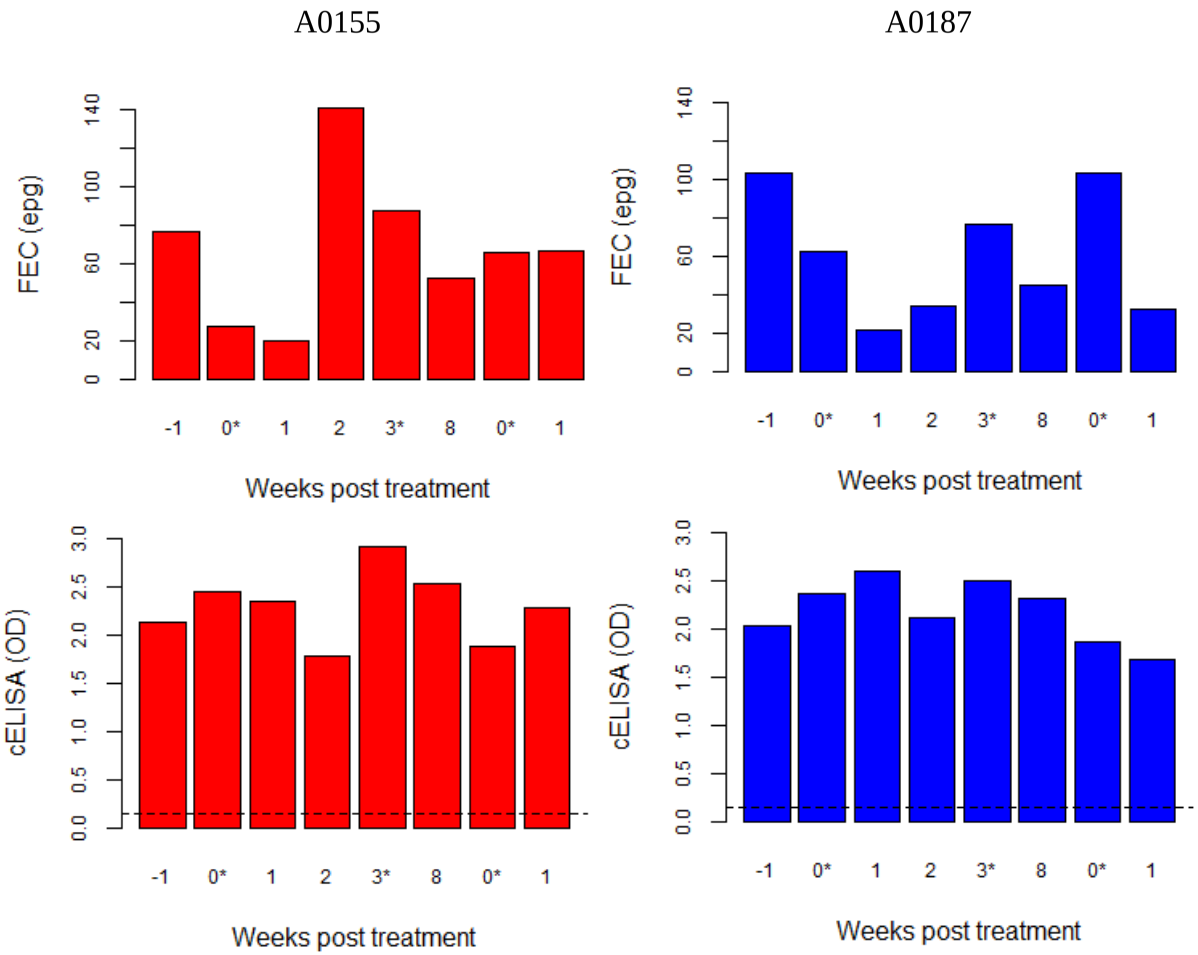


Figure 3.1 Results of FEC (epg) and cELISA (OD) performed on donor ewe A1055 (red) and donor ewe A0187 (blue) at each sampling point *indicates that a TCBZ treatment was given. ---- indicates the cELISA positive cut off (0.15).

3.3.2 Challenge animals

In both challenge experiments, six 1 year-old mule wethers (cross-breed castrated male sheep) were used. These were raised worm-free and had not been grazed on pasture or knowingly exposed to gastro-intestinal parasites or liver fluke. The liver fluke-free (and unexposed) status of the animals was confirmed by FEC, cELISA and AbELISA testing prior to starting the study. In the second challenge experiment, the animals were the control group for a vaccine trial (unpublished). As such, they were injected with QuilA adjuvant at -6, -3, 0 and 3 wpc (weeks post challenge). For both challenge experiments, sheep were housed and fed on hay and concentrates with water available *ad lib* for the 18 or 20 week duration of the studies.

3.3.3 *F. hepatica* challenge

All experimental procedures were carried out at MRI (UK) by either personal license holders or veterinarians. Ethical approval was obtained from the Institute's Experiments and Ethical Review Committee, in accordance with the Animals (Scientific Procedures) Act 1986. Animals were health checked daily for the duration of the studies.

The Moredun isolate was used for both challenge experiments. In the first challenge experiment, each animal was given an oral dose of approximately 160 viable *F. hepatica* metacercarial cysts suspended in 18 ml 0.5% agar, on the first sampling date. In the second challenge experiment, each animal was given an oral dose of approximately 90 viable *F. hepatica* metacercarial cysts suspended in 9 ml 0.5% agar, 6 weeks into the vaccine trial. In both cases, doses were administered via 20 ml syringe. Full details of dose preparation can be seen in Section 2.4.2.

3.3.4 Sampling and testing

Blood and faecal samples were collected weekly for the duration of the studies as described in Section 2.1 (Figure 3.2). Serum samples were sent to Biobest Laboratories Ltd. (UK) for AbELISA testing, as outlined in Section 2.3.2. The FEC and cELISA were performed at MRI (UK), as described in Sections 2.2.1.2 and 2.2.3. Samples were considered cELISA positive if the percentage difference (PD) was ≥6.07 or ≥7.49 for the first and second challenges, respectively.

Figure 3.2 Set up of weekly sample collection from challenged animals

The reduction in FEC (FECR) was calculated as described in Section 2.2.2, using a reduction of ≥95% as the criterion for successful treatment. A treatment was considered successful according to the cELISA if the cELISA result was negative post-treatment, as proposed by Flanagan et al. (2011b).

3.3.5 Post-mortem

In both challenge studies, animals were killed by captive bolt gun stunning followed by exsanguination. Livers were removed and the gall bladder clipped off prior to investigation for fluke and collection of fluke eggs from the gall bladder, as described in Sections 2.4.3.2 and 2.4.4 (Figure 3.3). As for the initial isolation, the eggs from all 6 animals were combined into one sample and posted to Ridgeway Research (Gloucestershire, UK) to maintain the isolate.

Figure 3.3 Sample collection set-up for challenge animals' *post-mortem*

3.3.6 Statistical analysis

All statistical analyses were performed in RStudio (R Core Team, 2014). The "lm()" function was used to fit a linear model to compare faecal- and serum-based tests to liver burdens at slaughter (R Core Team, 2014), whilst the "cor(x,y,method="spearman")" function was used to calculate the Spearman's rank correlation coefficient (rho) (R Core Team, 2014). Because of the low sample size in each experiment, statistical significance was set at $P = 0.10$. FECRs were calculated as described in Section 2.2.2.

3.4 Results

3.4.1 Challenge experiment 1

Individual AbELISA, FEC, cELISA, and fluke burden results can be seen in Appendix 2.

3.4.1.1 Challenge experiment 1: AbELISA

Antibody titres remained negative at both the group and individual level until 3 wpc, at which point 4 of the 6 animals were antibody positive. By 4 wpc, all animals were

antibody positive and remained so until the end of the study. A summary of the individual antibody titres throughout the study can be seen in Figure 3.6. At both 5 and 6 wpc, two animals had antibody titres which were distinctly separate from the rest of the group's. In both cases, it was A1079 which had a lower antibody titre and A1076 which had a higher antibody titre than the rest of the group. An increase in antibody titre was seen at 9 wpc, followed by a decrease at 13 wpc and another increase at 14 wpc.

3.4.1.2 Challenge experiment 1: FEC

A positive FEC was first seen at 9 wpc, with 3 animals having a positive egg count and a group mean FEC of 0.5 epg. At 10 wpc, all but one animal had a positive FEC with a group mean of 4.1 epg. By 11 wpc all animals had a positive FEC, with a mean of 17.3 epg, and remained positive until the end of the study. A summary of the FEC results can be seen in Figure 3.4. From 9 wpc, A1074 consistently had the highest FEC, being distinctly separate from the rest of the group. Within each individual animal, fluctuations in FEC were observed (Figure 3.6). FECRs at 2 wpt ranged from -430 to 30%. The lowest FECR (-430%) came from animal A1076, which had a FEC of 5 epg pre-treatment but a FEC of 26.5 epg post-treatment, indicating a rise of 430% in FEC epg rather than a reduction.

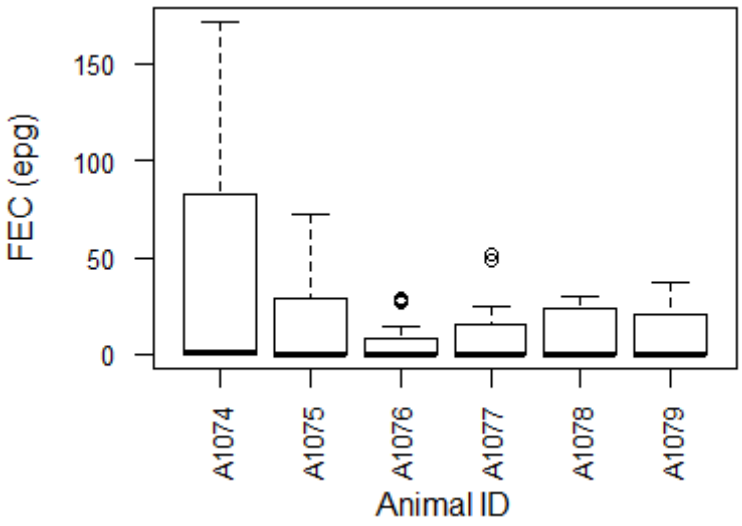


Figure 3.4 Individual FEC (epg) ranges of challenge experiment 1 wethers

3.4.1.3 Challenge experiment 1: cELISA

At 3 wpc, all animals except A1076 were positive for liver fluke infection according to cELISA, with the mean group PD being 9.82%. There was a transient increase in cELISA level between 3 and 5 wpc where animals tested positive for infection. This was followed by a transient decrease between 4 and 7 wpc where animals tested negative for infection. At 8 wpc all animals were cELISA positive with a mean PD of 36.68% (Figure 3.6). All animals remained positive until the end of the study. A summary of cELISA results between 3 and 8 wpc is shown in Table 3.1. No animal maintained a positive cELISA result through 3 to 8 wpc. After becoming positive at 3 or 4 wpc, each animal was negative at least once, usually at 6 wpc, before becoming positive again by 8 wpc. The peak value of the early rise in PD was 17.48%, whilst the peak value of the second rise in PD was 117.62%. There was some natural fluctuation in cELISA readout at an individual animal level once the PD has begun to plateau.

Table 3.1 cELISA results (PD) for challenge experiment 1 between 3 and 8 wpc. Positive results are shown in bold. The positive cut-off was ≥6.07%

	Animal					
	A1074	A1075	A1076	A1077	A1078	A1079
3 wpc	**15.09**	**9.5**	-5.89	**18.59**	**15.44**	**6.18**
4 wpc	**17.48**	**10.14**	**10.55**	**13.23**	1.46	**12.47**
5 wpc	**8.57**	**7.4**	**8.92**	**17.13**	-6.06	5.36
6 wpc	2.1	3.09	5.59	1.68	1.95	**6.42**
7 wpc	**16.86**	**7.77**	5.74	**25.75**	**7.62**	**19.6**
8 wpc	**47.08**	**26.92**	**20.11**	**23.62**	**42.15**	**60.18**

3.4.1.4 Challenge experiment 1: fluke burden in the liver

Fluke burdens of challenge experiment 1 animals ranged from 22 to 80 adult fluke, with a median of 42. The recovery rate for the fluke ranged from 14 to 50% (mean = 28%). A strong correlation was seen between fluke burden at *post-mortem* and both FEC (rho = 0.77, P = 0.072) and antibody titre (rho = -0.58, P = 0.228). There was

little correlation between fluke burden and cELISA PD at *post-mortem* (rho = 0.09, P = 0.872). The AbELISA, FEC, cELISA and liver burden results can be seen in Figure 3.7. All livers had some damage visible externally, with migratory tracts and lesions evident (Figure 3.5). Liver damage appeared to be mostly confined to the left lobe.

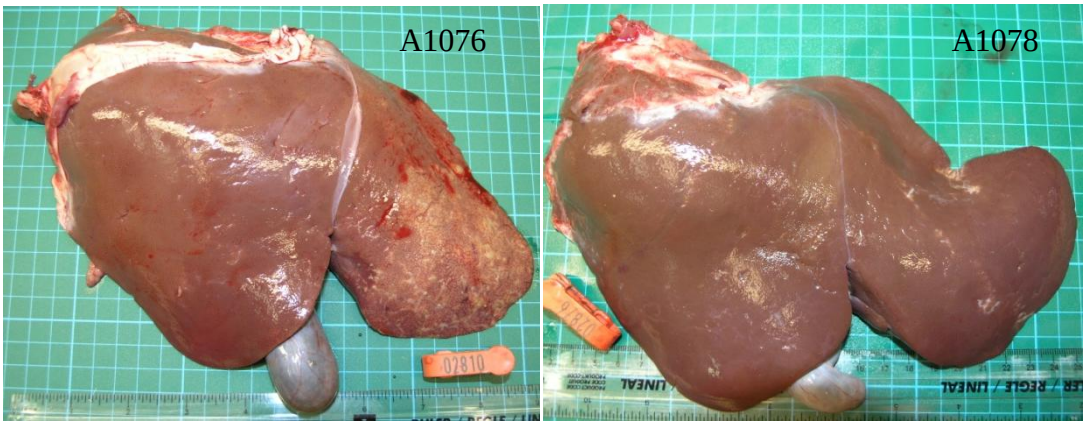


Figure 3.5 Appearance of livers from animals A1076 (22 fluke recovered) and A1078 (28 fluke recovered) at *post-mortem*

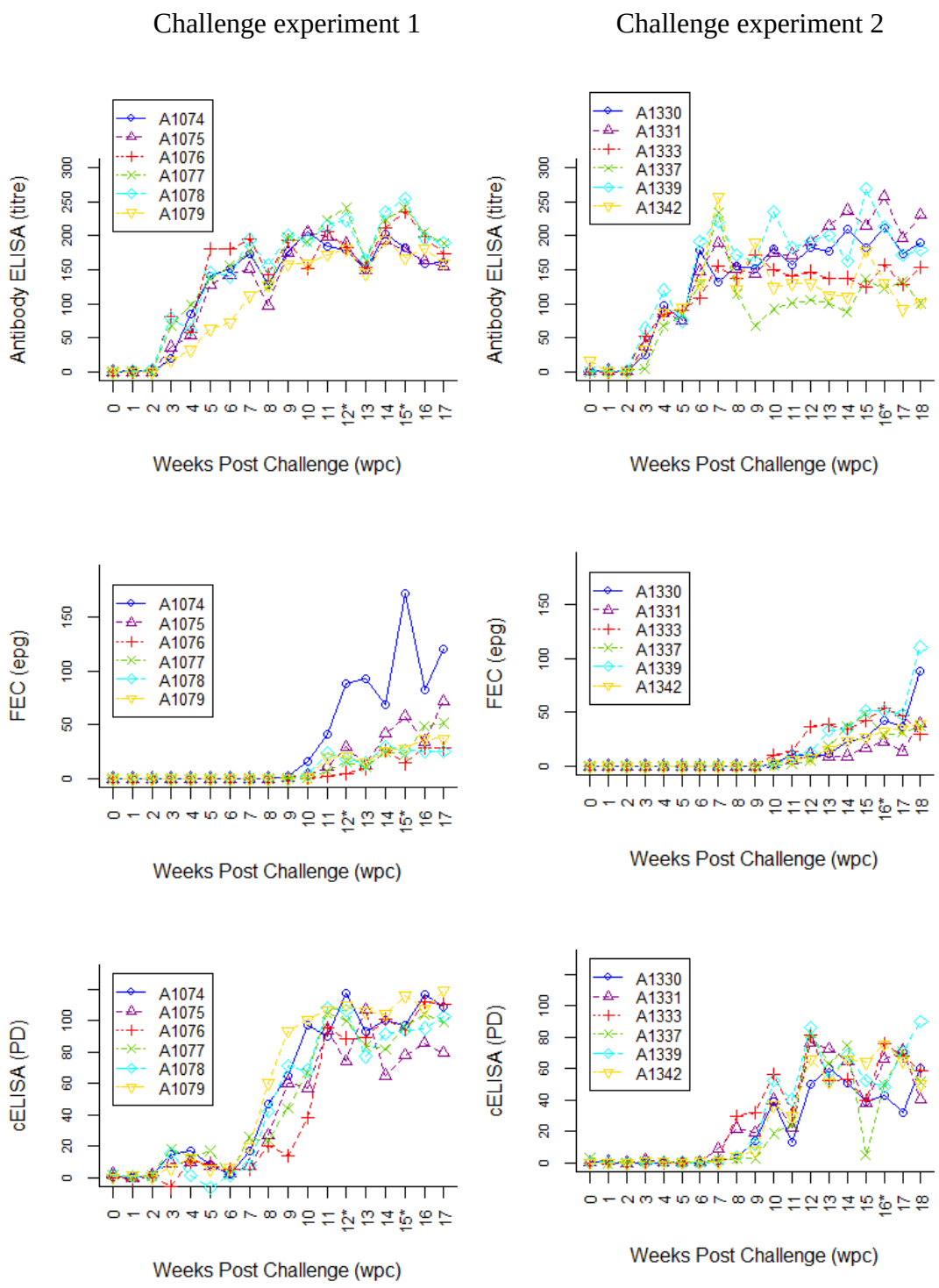


Figure 3.6 AbELISA (titre), FEC (epg), and cELISA (PD) results of challenge experiment 1 and 2 at each sampling point. * indicates a TCBZ treatment was given.

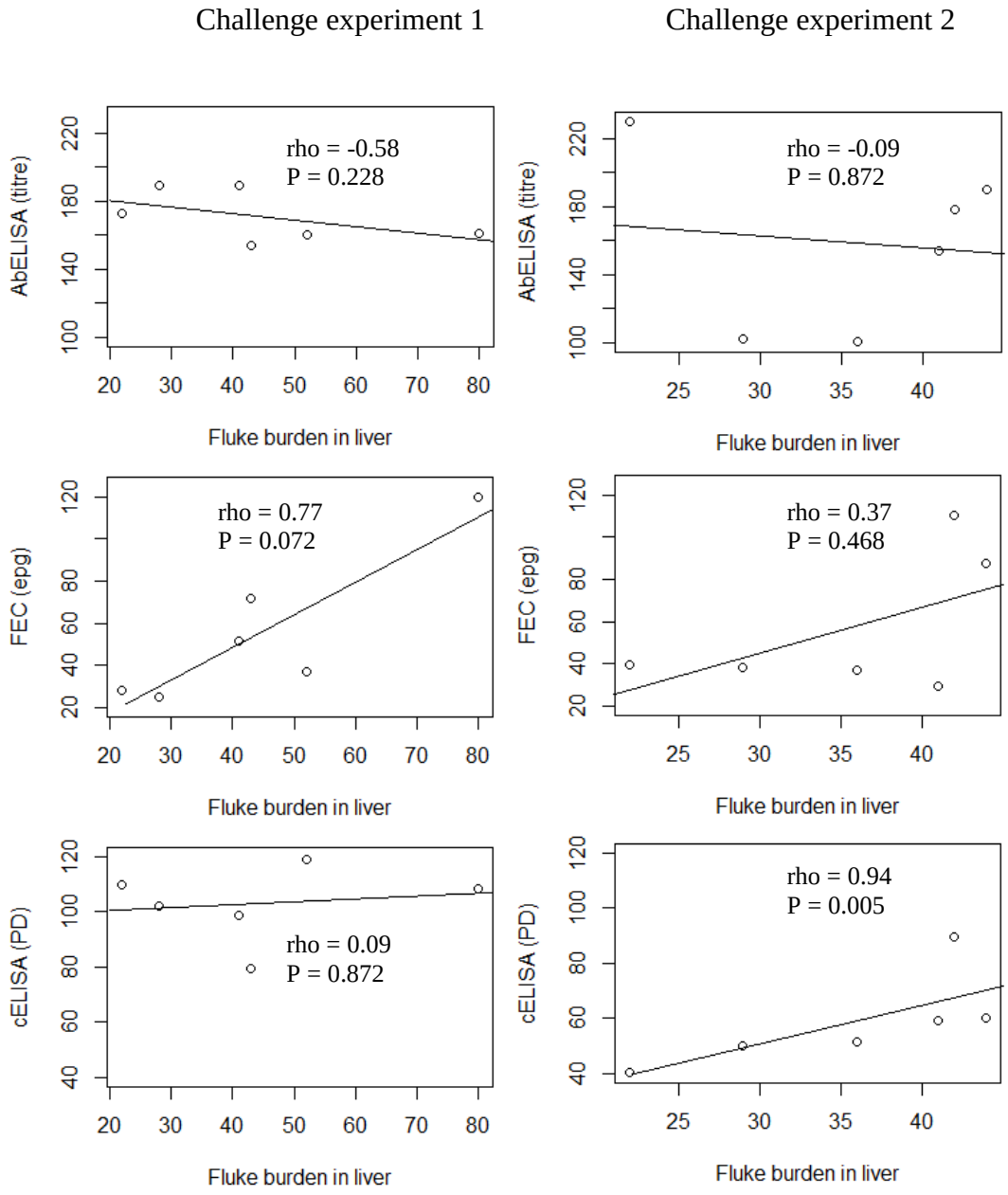


Figure 3.7 AbELISA (titre), FEC (epg), cELISA (PD) and fluke burden results at the point of slaughter for challenge experiment 1 and 2.

3.4.2 Challenge experiment 2

Individual AbELISA, FEC, cELISA, and fluke burden results can be seen in Appendix 2.

3.4.2.1 Challenge experiment 2: AbELISA

As in challenge experiment 1, antibody titres remained negative at both the group and individual level until 3 wpc, at which point 4 of the 6 animals were antibody positive (mean = 36.5, median = 35.5), and by 4 wpc, all animals were antibody positive and remained so until the end of the study, as can be seen in Figure 3.6. At 9 wpc, A1337 had an antibody titre which was distinctly lower than the rest of the group.

3.4.2.2 Challenge experiment 2: FEC

A positive FEC was first seen at 10 wpc, with all 6 animals having a positive egg count and a group mean FEC of 2.8 epg. All animals remained FEC positive until the end of the study. A summary of the FEC results can be seen in Figure 3.6. FECRs at 2 wpt ranged from -186 to 37%.

3.4.2.3 Challenge experiment 2: cELISA

At the individual level, the first positive cELISA result was seen at 7 wpc, i.e. around the same time as the persistent increase in cELISA in experiment 1, with 1 animal testing positive. The number of animals positive by cELISA increased until 10 wpc, at which point all animals were cELISA positive, with a mean PD of 40.6%. The group mean cELISA result was first positive at 8 wpc (mean PD = 10.9%, median PD = 3.85%). Once cELISA positive, all animals except A1337 remained positive until the end of the study. A1337 tested positive for liver fluke by cELISA at 14 and 16 wpc, but negative at 15 wpc. The 15 wpc sample was re-tested and remained

negative. At 17 wpc, A1330 had a cELISA PD which was distinctly lower than the rest of the group, whilst A1339 had a cELISA PD which was distinctly higher than the rest of the group at 18 wpc. The cELISA PD of all animals at all sampling points can be seen in Figure 3.6.

3.4.2.4 Challenge experiment 2: fluke burden in the liver

Fluke burdens ranged from 22 to 44 adult fluke, with a mean of 35.7 and a median of 38.5. The recovery rate for the fluke ranged from 24 to 49% (mean = 40%). A strong positive correlation was seen between cELISA PD at *post-mortem* and liver burden (rho = 0.94, P = 0.005). There was little to no correlation between fluke burden and either FEC or antibody titre (rho = 0.37, P = 0.468 and rho = -0.09, P = 0.872, respectively). The AbELISA, FEC, cELISA and liver burden results can be seen in Figure 3.7. In contrast to challenge experiment 1 damage was not evident on the surface of every liver. Where liver damage was seen it was confined to the left lobe.

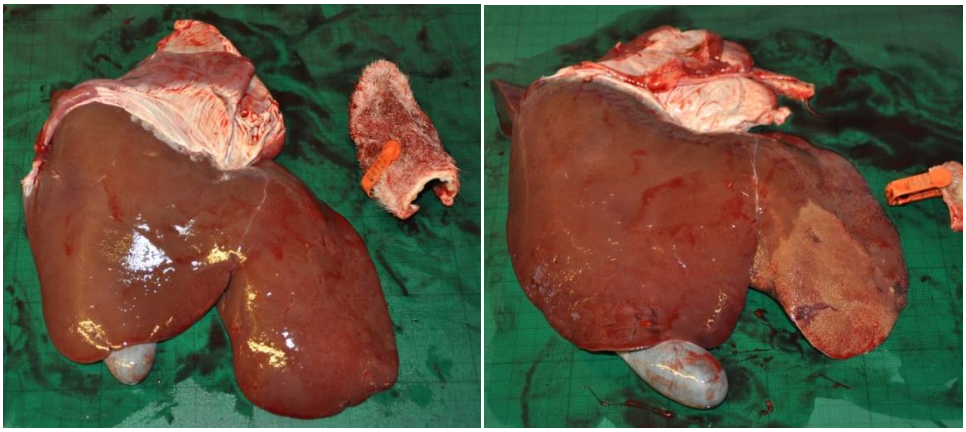


Figure 3.8 Appearance of livers from animals A1333 (41 fluke recovered) and A1342 (29 fluke recovered) at *post-mortem*

3.5 Discussion

This chapter aimed to establish a *F. hepatica* challenge model in sheep to (1) evaluate the performance of AbELISA, FEC and cELISA, (2) to determine the

TCBZ resistant status of a British *F. hepatica* isolate and (3) to profile the dynamics of this isolate using blood- and faecal-based diagnostic tests.

3.5.1 Test performance

In terms of the kinetics of the infection, as described by AbELISA and FEC, the tests performed as expected with antibodies being first detected between 3 and 4 wpc, and eggs being first detected between 9 and 10 wpc (Valero et al., 2006; Zimmerman et al., 1982). However, the cELISA performed differently, with early detection at 3 wpc in challenge experiment 1 and late detection at 7 wpc in challenge experiment 2 (Flanagan et al., 2011a; 2011b; Mezo et al., 2004). The lower metacercarial challenge (n = 90 as opposed to n = 160) may be responsible for the later detection in challenge experiment 2 compared to challenge experiment 1 although the average number of adult fluke recovered from animals was remarkably similar across experiments (38.5 and 42 for experiment 2 and 1, respectively).

In the initial isolation and both challenge experiments, there were minor fluctuations in FEC and cELISA OD/PD. This has been reported in the literature in regards to FEC (Valero et al., 2011) and cELISA (Brockwell et al., 2013). At no point in challenge experiment 1 did the FEC stabilise or plateau, although the AbELISA and cELISA stabilised at ~7 and 10 wpc, respectively. In challenge experiment 2 AbELISA, FEC and cELISA stabilised at ~6, 12 and 12 weeks, respectively.

A transient increase was seen in the 3 to 8 wpc cELISA results of challenge experiment 1 animals. The earliest reported detection of liver fluke infection by cELISA was at 5 wpc (Flanagan et al., 2011b). There were no reports of a transient increase in cELISA results. All animals in our challenge experiment were affected and animals maintained an early positive cELISA for 1 to 3 weeks, reducing the likelihood that these fluctuations are due to false positive results. Animals also maintained the second negative cELISA period for 1 to 3 weeks, again reducing the likelihood that these are false negative results. The cELISA samples for 3 to 8 wpc were all tested across 3 cELISA plates over 2 days.

It is possible that this early coproantigen signal originates from antigens released from the parasites prior to entry of the liver, as metacercariae or excysted fluke which failed to migrate to the peritoneal cavity. The second negative cELISA period would then indicate the time at which the parasites are within the liver and antigens are channelled through the biliary system, stored in the gall bladder and then released. This subsequent release of coproantigens would then explain the start of the second period of positive cELISAs, which continued until the end of the study.

This transient increase in cELISA values did not occur in either challenge experiment 2 animals, dosed with 90 *F. hepatica* metacercariae, or published reports, which use up to 250 metacercarial cysts (Flanagan et al., 2011a; 2011b; Mezo et al., 2004). Thus, it is unlikely that the fluctuations in infection status are related to challenge dose or early fluke burden. In challenge experiment 2, a single animal had 1 negative cELISA result at 15 wpc, which was preceded and followed by positive cELISA results. In the case of this animal and its faecal sample, it is highly likely that this is a false negative result, surrounded by true positive results. The false negative may be due to an error in the test, poor storage of the faecal sample prior to the formation of a supernatant, or user error, relating to the labelling or handling of samples.

At the end of challenge experiment 1 (17 wpc), a correlation was seen between FEC (epg) and the number of fluke recovered from the liver and both FEC (epg) and AbELISA. Only the correlation between liver burden and FEC was statistically significant. However, this correlation was not seen at the end of challenge experiment 2 (18 wpc), with a correlation being seen between cELISA (PD) and the number of fluke recovered from the liver. This correlation was also statistically significant. The lack of consistency of the FEC and fluke burden relationship between challenge experiments may be due to the fluctuations in egg shedding often seen in liver fluke infections (Valero et al., 2011). The relationship between cELISA and fluke burden would be expected to be relatively stable, as there is no evidence that coproantigens are stored in the gall bladder. Although the challenge dose was significantly higher in challenge experiment 1, the resulting fluke burdens were similar between challenge experiments, making it unlikely that burden played a role

in the changing cELISA/fluke burden relationship at *post-mortem*. Despite the burdens being similar between the two experiments, the cELISA PDs were higher in experiment 1 than in experiment 2. If the cELISA plateaus at a certain point the PD may no longer be able to correlate with liver burden. This may be what has happened in experiment 1, as no PD was lower than ~80% whereas this is the upper end of the PDs seen in experiment 2.

If the number of fluke recovered from the liver at *post-mortem* (18 wpc) and the 17 wpc AbELISA, FEC and cELISA results from challenge experiment 2 are compared, a different relationship is seen. Now, as for challenge experiment 1, there is a correlation between FEC and fluke burden (rho = 0.77, P = 0.072) whilst no correlation is seen between either AbELISA or cELISA and liver burden. This is likely due to the apparent increase in FEC at 18 wpc in the challenge experiment 2. It is highly unlikely that the fluke burden in the liver had changed between 18 and 17 wpc as there was no evidence of dead or decaying fluke in the liver on examination at slaughter. This highlights the natural fluctuations seen in FECs and the implications they may have when attempting to estimate liver burden or determine correlations.

3.5.2 TCBZ-R status of the Moredun isolate

Due to the lack of a control group, it was not possible to calculate the efficacy of TCBZ when used against the Moredun isolate. Despite this, it is clear that TCBZ is not sufficiently effective against the Moredun isolate to clear infection at 17/18 wpc. FECRs were never greater than 37% and large numbers of visually undamaged adult fluke were recovered *post-mortem* following treatment with TCBZ. Further studies, with the use of a control group and animals treated and slaughtered at different weeks post-challenge, would further characterise the dynamics of this isolate's resistance to treatment with TCBZ. Despite the poor efficacy of TCBZ against adult fluke it may still be an effective treatment against immature fluke of the Moredun isolate. This was seen in the Oberon isolate where, despite TCBZ having 0% efficacy at 2 and 12 wpc, it had a limited efficacy at 4 wpc (Walker et al., 2004).

A preliminary sequence analysis of the region encoding part of the second nucleotide binding domain of Pgp, a candidate anthelmintic resistance gene, identified an increase in allele frequency, including an S1144R SNP (Wilkinson et al., 2012). DNA was extracted from fluke recovered in challenge experiment 2, but found not to contain the SNP proposed by Wilkinson et al. (2012) to identify TCBZ-R fluke (unpublished data). This indicates that in the Moredun isolate at least, this SNP is not involved in the ability to survive treatment with TCBZ. Other isolates will need to be investigated to determine if this SNP is involved with their susceptibility to TCBZ.

3.5.3 Comparison with other experimental challenge models

A *F. hepatica* isolate is a strain of *F. hepatica* which has been collected from a natural infection and propagated in the laboratory for study. There are several *F. hepatica* isolates available for research purposes, which are summarised by Fairweather (2011a). These have been shown to have different characteristics in terms of fecundity, status of resistance to various flukicides, time to first positive FEC and cELISA and in recovery rates from the livers of animals. The results in this chapter only relate to the Moredun isolate. As no other isolates were tested under the same conditions, any differences between the Moredun isolate and those previously reported may be due to the conditions of the challenge models and the protocols used for FEC, cELISA and recovery of fluke from livers. The Oberon isolate has been confirmed to be TCBZ-R whilst the Leon, Fairhurst and Cullompton isolates are susceptible to TCBZ (Álvarez-Sánchez et al., 2006; Flanagan et al., 2011a; McCoy, 2005; Walker et al., 2004).

The animals became FEC positive between 63 and 77 days post-challenge (dpc), whilst cELISA positives first occurred between 42 and 77 dpc, using the data from all 12 challenged animals. In the case of the cELISA results from challenge experiment 1, the first positive was taken to be the earliest positive following the 3-8 wpc fluctuations. The only study to date which recorded the earliest FEC and cELISA detection of infection in known isolates was carried out by Flanagan et al. (2011a). In that study, animals were challenged with 200 metacercariae, a larger dose

than the 160 or 90 cysts given in the present study. If the results are compared using the earliest detection of eggs and coproantigens, the Moredun isolate had earlier detection by FEC than the Cullompton (77 dpc), Fairhurst (70 dpc) or Leon (75 dpc) isolates, but later detection by FEC than the Oberon isolate (59 dpc). The Moredun isolate also had earlier detection by cELISA than the Oberon (47 dpc), Cullompton (50 dpc), Fairhurst (53 dpc) and Leon (62 dpc) isolates (Flanagan et al., 2011a).

The recovery rate of fluke from animals in the challenge studies varied, with a mean of 28% and 40% in challenge experiment 1 and 2, respectively. The low recovery rate seen in challenge experiment 1 is likely to be due to the age of the metacercariae when the infectious dose was prepared. Whilst each dose contained ~160 viable cysts, these cysts had been stored for 5 months, and may have been compromised by this extended storage. On the other hand, for the second experiment, the cysts were only stored for 2 months. When compared to the findings of Flanagan et al. (2011a) in relation to untreated groups of sheep, the Moredun isolate had a higher recovery rate than the Fairhurst (6%), Oberon (11%) or Leon (22%) isolates but a lower recovery rate than the Cullompton isolate (51%).

Due to the lack of a control group, it is not possible to know whether this recovery rate is a true indication of the number of fluke which survived to adulthood, or is reduced in the case of a partially effective treatment.

3.6 Conclusion

In conclusion, (1) the cELISA appears to be a more useful tool for earlier indication of liver fluke infection than the standard FEC, although there may be fluctuations in very early infections, (2) a TCBZ-R *F. hepatica* isolate, the Moredun isolate, has been identified in Great Britain, further studies will reveal the extent of resistance in this isolate, and lastly (3) the Moredun isolate appears to become patent and produce coproantigens earlier than TCBZ susceptible isolates but in contrast eggs are detected later and coproantigens are detected earlier in the Moredun isolate than in the TCBZ-R Oberon isolate.

Chapter 4: Investigation of existing diagnostic tests in naturally exposed sheep: Studies at an individual animal level

4.1 Abstract

Pre-patent *Fasciola hepatica* infections cause acute and sub-acute fasciolosis. This disease has a significant economic impact on sheep farmers, and can result in the sudden death of sheep. The commonly used faecal egg count (FEC) is unable to detect the pre-patent form of liver fluke. The serum based anti-*Fasciola* antibody ELISA (AbELISA) and biochemical assays, which measure concentrations of GLDH and GGT, are capable of detecting pre-patent fluke but the AbELISA cannot differentiate between current and past infection and changes in GLDH and GGT healthy concentrations may not be specific to *F. hepatica* infection. A commercial coproantigen ELISA (cELISA) is reported to detect low burdens of infection, from 4 weeks post-infection (wpi) and to only detect current infection, but this has yet to be evaluated in sheep naturally exposed to liver fluke. The studies presented in this chapter aim to evaluate the currently available diagnostic tests for liver fluke in terms of ability to detect initial and established infection in sheep naturally exposed to *F. hepatica*. Firstly, in a longitudinal evaluation of test performance, 27 April-born lambs were monitored from June to November 2010, with blood and faecal samples taken monthly for AbELISA, GLDH, GGT, FEC and cELISA testing. A subset of 12 lambs was followed to slaughter and livers recovered. GLDH and GGT concentrations were found to be above reference ranges from the start of the study and unsuitable, on their own, for the diagnosis of liver fluke infection in these lambs. AbELISA detected infection in most animals by September and in all but one animal by November. FEC and cELISA both had some very early positive results which were most likely false-positive results, but the majority of animals became positive at the November sampling. The 12 lambs that were followed to slaughter all had low burdens of fluke (≤10) in their livers. Secondly, a cross-sectional study was conducted over 36 farms from Great Britain, comprising 812 sheep pre-treatment and 528 sheep post-treatment. Faecal samples were collected and FEC and cELISA testing was performed. Low FEC and cELISA results were seen, with better agreement between the two tests pre-treatment than post-treatment. Disagreements between the two tests were more frequently seen where the FEC detected infection

but the cELISA did not. This was true both before treatment, where cELISA would be expected to be more sensitive than FEC due to its ability to detect immature fluke, and after treatment, where shedding of residual eggs from the gall bladder may occasionally yield false positive FEC results. In animals naturally exposed to *F. hepatica*, the cELISA does not have an advantage of earlier detection over FEC and is not as sensitive as FEC in established infections, which is in contrast to results from experimental challenge studies and limits the value of cELISA for detection and management of natural infections.

4.2 Introduction

There are four main tests routinely used to diagnose liver fluke in live sheep; the faecal egg count (FEC), serum based anti-*Fasciola* antibody ELISA (AbELISA) and enzyme assays, which measure glutamate dehydrogenase (GLDH) and gamma-glutamyl transferase (GGT) concentrations. A coproantigen ELISA (cELISA) is commercially available but is not yet routinely used. The AbELISA and enzyme concentrations detect parasites indirectly, whereas the FEC and cELISA detect parasites directly.

FEC is the most commonly used diagnostic test, although it cannot detect pre-patent infections (<9 weeks post infection (wpi)), so cannot detect acute or sub-acute fasciolosis (Valero et al., 2006). This is a major drawback, because acute fasciolosis can cause sudden death in sheep, prior to any clinical signs of infection. Blood-based diagnostics can be used to support a clinical diagnosis of pre-patent infection, but the collection of blood samples is invasive and requires the presence of a veterinarian, which may limit uptake by farmers, especially in the case of sheep. In addition, although the AbELISA can detect liver fluke infection from 4 wpi, an animal can remain positive for up to 9 months after an infection has been successfully treated. As such, AbELISA does not differentiate between current or past infection (Sánchez et al., 2001; Zimmerman et al., 1982). Serum concentrations of GLDH may be elevated from 2 wpi, but normal ranges vary depending on breed, sex and age (Phiri et al., 2007; Sandeman and Howell, 1981). In addition, raised enzyme levels are not

specific to liver fluke infection. The cELISA is reported to combine early detection (5 wpi) with high specificity for current liver fluke infection, but to date has only been evaluated for sheep in experimentally challenged animals (Bio-X, 2010; Flanagan et al., 2011b; Mezo et al., 2004). Challenge studies conducted at the Moredun Research Institute, UK (MRI) with the Moredun isolate confirmed that cELISA was an earlier indicator of infection then FEC (Chapter 3). The cELISA showed a high sensitivity and specificity when used in cattle naturally exposed to *F. hepatica* (Charlier et al., 2008). However, given the potential differences in how infections progress in sheep and cattle, as well as the differences in faecal composition between the two species, these results cannot be directly applied to sheep. The next logical step in test evaluation is to assess its performance in naturally exposed sheep.

This chapter aims to evaluate the currently available diagnostic tests for liver fluke, in terms of ability to detect early and established infection in individual sheep naturally exposed to *F. hepatica.* The chapter addresses these aims in 3 ways, (1) longitudinal monitoring using invasive and non-invasive tests, (2) a cross-sectional analysis using non-invasive tests and (3) an investigation of the ability of FEC and cELISA to quantify liver fluke burden.

4.3 Materials and methods

4.3.1 Longitudinal monitoring using invasive and non-invasive tests

4.3.1.1 Animals

In 2010, 27 Blackface x Blue Leicester April-born lambs were selected by the farmer, based upon his interest in their infection status, from a commercial flock in Dumfries and Galloway in SW Scotland. The flock had a history of liver fluke infection and all lambs were grazed on the same field. Between the August and September samplings, one lamb died from non-fluke related causes.

4.3.1.2 Sampling and testing

Each lamb was blood- and rectally faecal sampled, for diagnostic purposes, at the end of the month, for 6 months, between June and November 2010 by a veterinarian from Scottish Agricultural College Consulting Veterinary Service (SAC C VS), Dumfries. Serum blood samples were assayed for GLDH and GGT concentrations and AbELISA titres at SAC C VS, Dumfries, whilst faecal samples were sent to SAC C VS, Edinburgh for sedimentation FEC (SAC method) and, subsequently, to MRI for cELISA. A cELISA result was determined to be positive if the net optical density (OD) titre was ≥0.15, according to the manufacturer's recommendations (Bio-X Diagnostics, Belgium). Detailed descriptions of the procedures can be found in Sections 2.2.1.1, 2.2.3, 2.3.1 and 2.3.2.

A convenience sample of 12 animals was followed to slaughter in November and January, where faecal samples and livers were obtained. These were lambs the farmer had elected to send direct to slaughter, rather than sell through markets. Once again, FECs were performed by SAC C VS, Edinburgh and cELISAs were performed at MRI. In addition, livers were examined for the presence of fluke as described in Section 2.4.3. The livers from the animals slaughtered in November were collected and examined by Heather Stevenson (SAC C VS) using the SAC method, and those in January by Danielle Gordon-Gibbs (MRI) using the Moredun method.

4.3.2 Cross-sectional monitoring using non-invasive tests

4.3.2.1 Sample collection

Samples used for the analysis of FEC and cELISA in this chapter originated from a number of studies. These include the evaluation of the coproantigen reduction test (CRT) and the evaluation of coprological tests when composite samples are used, both of which are described in detail in Chapter 5, as well as studies which are not described within this thesis.

Faecal samples from sheep were either collected on-farm by the team (Danielle Gordon-Gibbs, Heather Stevenson, Royal (Dick) School of Veterinary Studies students, Professor Ruth Zadoks, Dr Philip Skuce, Professor Neil Sargison and Margaret Oliver) or collected by farmers and posted to the laboratory as part of a small postal survey. Sample collection spanned from December 2010 until March 2014. The animals varied in age, breed and sex. The results from pre- and post-treatment samples were analysed separately due to the fact that whilst in a patent infection both FEC and cELISA are expected to detect infection pre-treatment, following successful treatment low levels of egg shedding can lead to false positive FEC results but this does not affect cELISA results.

812 samples were collected pre-treatment or as routine diagnostic samples (one sample per animal) from 36 different farms located across Great Britiain. Between 1 and 146 samples were collected from each farm (mean = 23.9, median = 20). For 10 of the samples, no details of origin were provided. Postal code location data were not available for 4 of the farms (Figure 4.1). In addition, 528 post-treatment samples, one per animal, were collected from 24 farms in the same manner, from the same animals, as described above. Between 7 and 72 samples were collected from each farm (mean = 22, median = 20). Timing of post-treatment sampling ranged from 7 to 56 days post treatment (dpt), as can be seen in Figure 4.2.

Figure 4.1 Map of UK showing origin of samples used in cross-sectional evaluation of FEC and cELISA. Locations in red indicate both pre- and post-treatment samples were received, locations in blue indicate only pre-treatment samples were received.

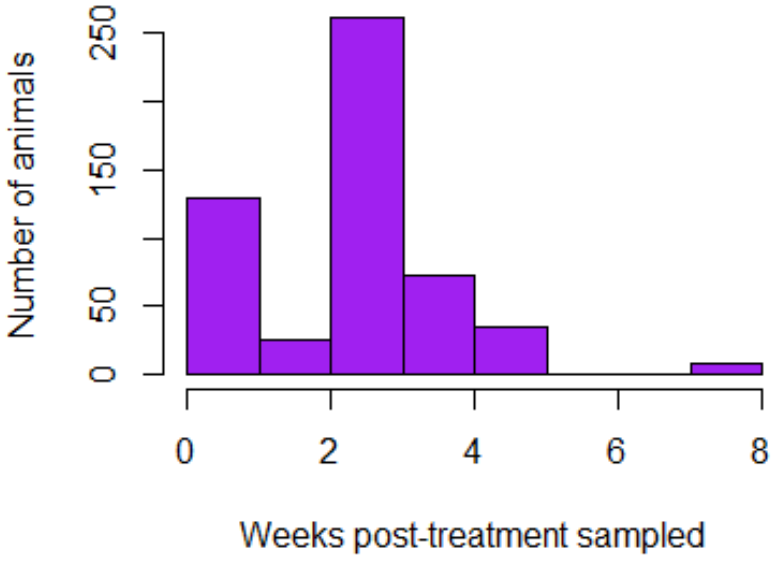


Figure 4.2 Distribution of post-treatment sample collection time points

4.3.2.2 Detection of liver fluke infection

FEC and cELISA were carried out as described in Sections 2.2.1.2 and 2.2.3 at MRI. During the period of sampling, changes were made to the cELISA kit by the manufacturers, as described in Section 2.2.3. The outcome of the cELISA for each sample was, therefore, determined according to the batch of kit used. Pre-treatment, 141 samples were analysed using the net optical density (OD), whilst post-treatment, 139 samples were analysed in this way, i.e. positive if net OD $\geq$0.15. All remaining samples were considered cELISA positive if the percentage difference (PD) between the sample's net OD and that of the positive control was higher than the cELISA kit specified. This positive cut-off was batch-specific and ranged from 6.07 to 9.32%. In a small number of instances, livers were also collected and examined as described in Section 2.4.3.2.

4.3.3 Statistical analysis

Statistical analysis was carried out in RStudio (R Core Team, 2014). The "confusionMatrix()" function of the 'caret' package was used to calculate cross-tabulations of observed and predicted results by different tests (Kuhn, 2014). From the subsequent 2x2 tables, unweighted Kappa, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and prevalence were calculated. To account for ties in the data, a modified version of the Wilcoxon rank-sum test ("wilcox.exact()" function), was used to determine if there was a statistically significant difference in FEC (epg) and cELISA PD/OD between sampling points (Hothorn and Hornik, 2013). To determine if there was a correlation between the number of fluke recovered from the liver (liver burden) and FEC (epg), cELISA PD/OD or AbELISA titre, the Spearman's rank correlation coefficient test ("cor(x,y,method="spearman")") function was used, which is denoted by rho (R Core Team, 2014). Linear models ("lm()" function) were fitted to sets of data in Section 4.4.3 and the R^2 of these models calculated ("summary(lm)"function) (R Core Team, 2014).

4.4 Results

4.4.1 Longitudinal detection of liver fluke infection

4.4.1.1 Diagnosis of infection using invasive assays

Blood samples were collected from all lambs at each sample point. GLDH concentrations are shown in Figure 4.3. Between June and November, the GLDH concentrations ranged from 1 to 365 IU/L (mean range = 18 to 109.8 IU/L, median range = 16 to 77 IU/L). The mean and median GLDH concentrations were above the SAC C VS "normal" range (2-10 IU/L) at all sampling points. At an individual level, 2 animals had GLDH concentrations within the "normal" range in June, 6 in July, 2 in August, 2 in September and 0 in October and November (Appendix 3). There was no consistency in which lamb had the highest GLDH concentration at any given sampling. The proportion of animals which had GLDH concentrations outside of the "normal" ranges is shown in Figure 4.4.

At the individual animal level, the GGT concentrations ranged from 10 to 363 IU/L (mean range = 49.33 to 103 IU/L, median range = 48 to 75 IU/L) between June and November. The mean and median GGT concentrations were above the SAC C VS "normal" range (27-31 IU/L) throughout the study (Figure 4.3). At the June and July samplings, one lamb had a GGT concentration which was distinctly higher than that of the rest of the group. No individual lamb consistently had the highest GGT concentration between August and November. The proportion of animals which had GGT concentrations outside of the "normal" ranges is shown in Figure 4.4.

Individual AbELISA values ranged from -7 to 334 between June and November (Figure 4.3). At the individual level, the number of positive animals per month was 4, 4, 3, 16, 24 and 25 for the months June to November. The infection status of three animals fluctuated between months, changing from positive to negative and back. At both the July and August samplings, one lamb had an antibody titre which was distinctly higher than the rest of the group. In November, all animals except one were positive; this animal did not have a positive test result throughout the study. The proportion of animals testing positive for liver fluke infection by AbELISA titre is

shown in Figure 4.4. The mean AbELISA values were first above the positive cut-off value at the September sampling and remained so until the end of the study (mean range = 14 to 198.7, median range = 2 to 214.5).

4.4.1.2 Faecal tests

Rectal faecal samples were collected from 27 animals in June, July and August, 26 in September, 24 in October and 23 in November, although the quantity of material available was occasionally insufficient to perform FEC and/or cELISA. The results of the FEC are shown in Figure 4.3. All animals were FEC negative in June. The first positive egg count was in July, with 3 animals having >0 epg (group mean = 0.07 epg). One animal was positive in August (group mean = 0.02 epg), 0 animals in September, 2 in October (group mean = 0.03 epg) and 15 in November (group mean = 0.61 epg).

The cELISA titres are shown in Figure 4.3. No animals were positive at the June sampling, 1 animal was positive in July, but, no animals were positive at the August and September samplings. For October and November, 3 and 15 animals were cELISA positive, respectively. The mean OD value was first above the positive threshold of 0.15 at the November sampling. The proportion of animals testing positive for liver fluke infection by cELISA is shown in Figure 4.4.

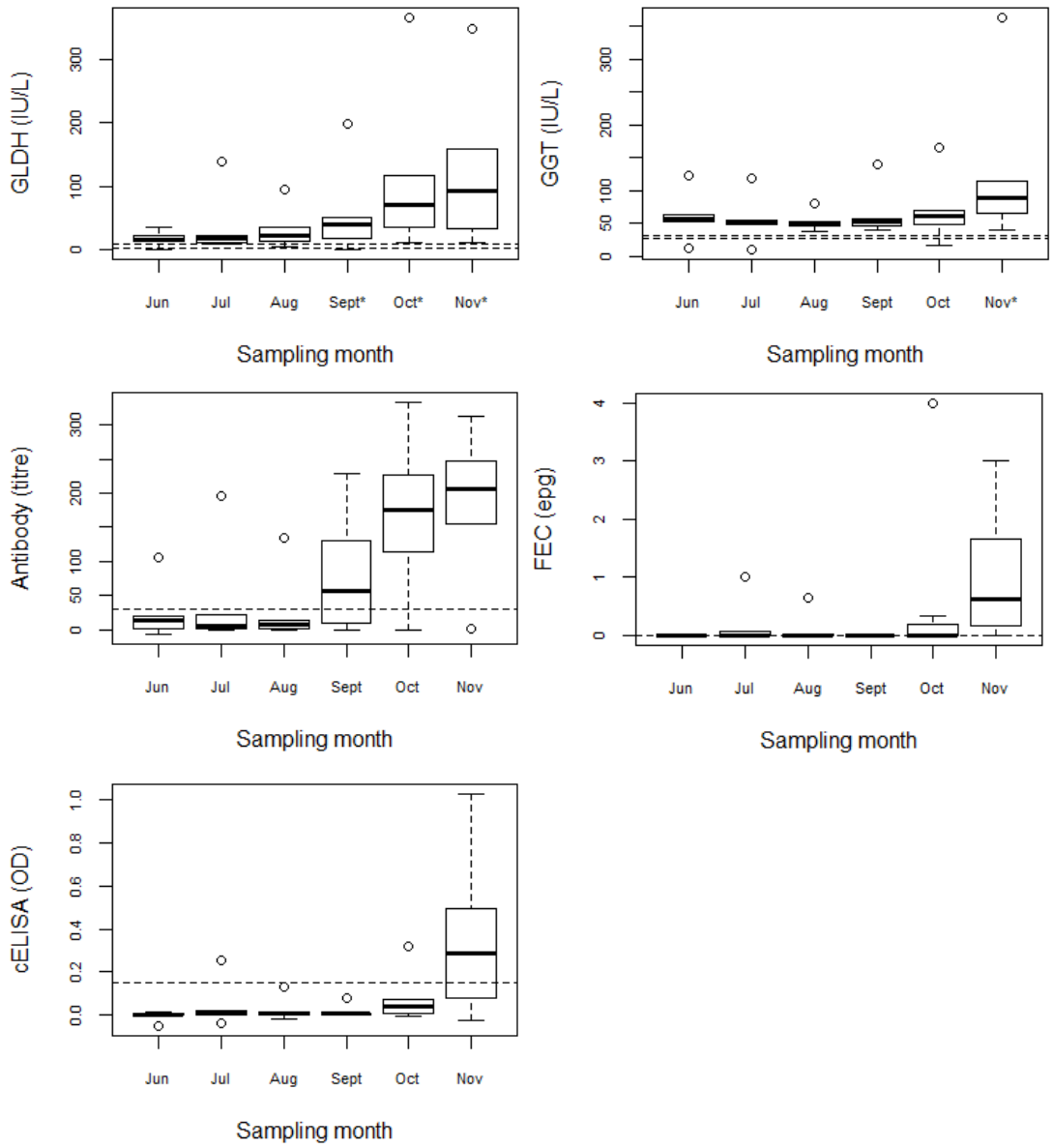


Figure 4.3 Box plots of GLDH and GGT concentrations, AbELISA titres FEC epg and cELISA OD of naturally exposed lambs at each sampling. --- indicates the "normal" ranges as defined by SAC C VS or positive cut-off value. In the GLDH and GGT plots * indicates a significant difference from the June sampling result

4.4.1.3 Determination of infection date and proportionality

The proportion of lambs testing positive by each test, at each sampling point, is shown in Figure 4.4. At the individual level, lambs would have first been infected between April and September (Figure 4.5). The first instance of a lamb testing positive by a test was taken to be a true positive; fluctuations in infection status were not taken into account. FEC gave the earliest estimate of point of infection, with positive FECs in July indicating infection in late April, 9 weeks prior to the positive FEC. The group infection status would indicate the point of infection to be between June and September, with the biochemical assays indicating early infection in June, and the cELISA indicating infection in September.

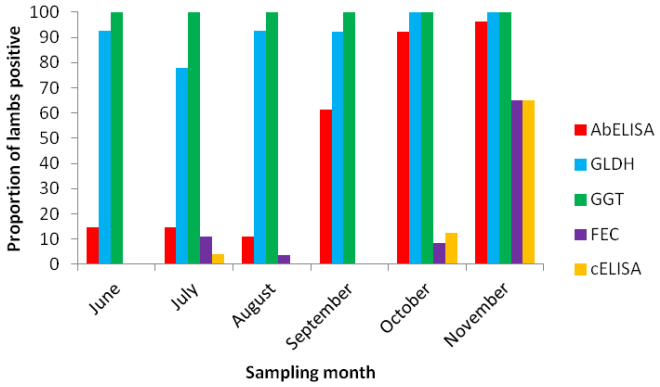


Figure 4.4 Bar chart showing the proportion of lambs positive for liver fluke infection by each diagnostic test (AbELISA, GLDH, GGT, FEC and cELISA) at each sampling month

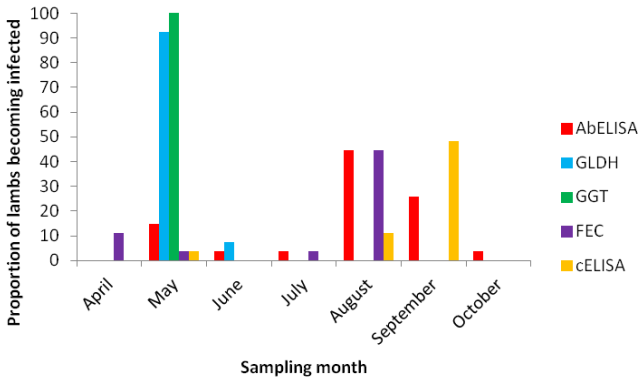


Figure 4.5 Bar chart showing the estimated point of infection of a proportion of lambs by each test, based upon the first instance of a positive result by the respective test.

4.4.2 Cross-sectional study investigating cELISA and FEC performance in sheep

4.4.2.1 Sample collection

Sample collection was carried out from December 2010 until March 2014, with the majority of samples being collected in 2011. The distribution of pre- and post-treatment sample collection can be seen in Figure 4.6.

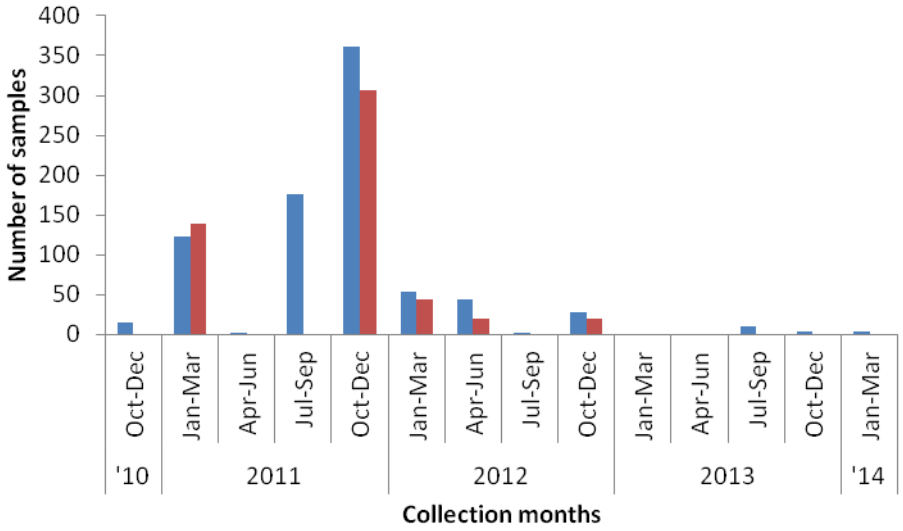


Figure 4.6 Time of collection of pre- (blue) and post-treatment (red) samples.

4.4.2.2 Determination of infection status by cELISA

Of the 280 samples tested prior to the change in cELISA kit parameters, only 3 animals would have been given a different infection status by cELISA, depending on whether the OD or PD was used. All 3 instances were with pre-treatment samples, with ODs of 0.129, 0.183 and 0.213 resulting in PDs of 6.83%, 4.95% and 5.76%, respectively. Infection status of animals used in this analysis was calculated according to the batch of test used, but plots relate to PD values.

4.4.2.3 Agreement between FEC and cELISA

4.4.2.3.1 Individual FEC vs. cELISA pre-treatment

The FEC and cELISA results from individual pre-treatment samples can be seen in Figure 4.7, with full details given in Appendix 3. FECs ranged from 0 to 408.5 epg (mean = 12.5, median = 0), whilst cELISA PDs ranged from -11.8% to 142.3% (mean = 24.7%, median = 2.8%). Substantial agreement was seen between the individual FEC and individual cELISA pre-treatment (Kappa = 0.83) (Table 4.1). Disagreement between FEC and cELISA was seen in 68 samples (8.4% of samples tested), with 56 of these being FEC positive but cELISA negative and 12 being cELISA positive but FEC negative. The median FEC of the cELISA negative but FEC positive samples was 0.83 epg, in contrast it was significantly higher for the samples which were FEC and cELISA positive, at 11.83 epg ($P < 0.0001$). If FEC is used as a gold standard, the cELISA has a sensitivity of 85.9% and a specificity of 97.1% (PPV = 96.6%, NPV = 87.8%, prevalence = 48.9%).

Table 4.1 Agreement between FEC and cELISA in individual pre-treatment samples. The Kappa value is shown.

Kappa = 0.83		FEC		
		Positive	Negative	Total
cELISA	Positive	341	12	353
	Negative	56	403	459
	Total	397	415	812

4.4.2.3.2 Individual FEC vs. cELISA post-treatment

The FEC and cELISA results from individual post-treatment samples are shown in Figure 4.7. FECs ranged from 0 to 344.8 epg (mean = 11.72, median = 0), whilst cELISA PDs ranged from -2.3% to 115.4% (mean = 13.8%, median = 0.9%). Substantial agreement was seen between individual FEC and individual cELISA post-treatment (Kappa = 0.67) (Table 4.2). Disagreement between FEC and cELISA was seen in 74 samples (14% of samples tested), with 66 of these being FEC positive

but cELISA negative and 8 being cELISA positive but FEC negative. The median FEC of the cELISA negative but FEC positive samples was 0.5 epg; in contrast it was significantly higher for the samples which were FEC and cELISA positive, at 25 epg ($P < 0.0001$). If FEC is taken as the gold standard, the cELISA had a post-treatment sensitivity of 63.9% and a specificity of 97.7% (PPV = 93.6%, NPV = 83.6%, prevalence = 34.6%).

Table 4.2 Agreement between FEC and cELISA in individual post-treatment samples. The Kappa value is shown.

Kappa = 0.67		FEC		
		Positive	Negative	Total
cELISA	Positive	117	8	125
	Negative	66	337	403
	Total	183	345	528

The majority of animals were sampled at 3 wpt (n = 262). The sensitivity of the cELISA, when using the FEC as a gold standard, ranged from 50 to 75% when animals were sampled 4 wpt or sooner. The specificity ranged from 90% to 100% when animals were sampled 4 wpt or sooner. At 5 wpt, the cELISA had a sensitivity of 0% and a specificity of 100%, when using FEC as a gold standard, as no true positives or false negatives were detected by cELISA . The sensitivity of the cELISA, using the FEC as a gold standard, could not be calculated at 8 wpt due to no animals being positive by FEC, the specificity remained 100%.

At all post-treatment sampling times, there were disagreements between the FEC and cELISA. Although disagreements were seen in both directions (cELISA positive but FEC negative and cELISA negative but FEC positive), overall the majority of disagreements were cELISA negative but FEC positive and in these instances the median FEC was low (Table 4.3). At 1, 5 and 8 wpt, there were no cELISA positive but FEC negative disagreements. The agreement between FEC and cELISA at each wpt, as well as the Kappa value and the sensitivity and specificity of the cELISA (if FEC is considered the gold standard) is shown in Table 4.4.

Table 4.3 Median FEC (epg) of cELISA negative and cELISA positive samples at each week post-treatment. * indicates a significant difference between FECs at this week (P < 0.01)

	cELISA negative	cELISA positive
1 wpt*	1.08	16.58
2 wpt*	0.42	103.17
3 wpt*	0.33	63.92
4 wpt*	0.33	5.5
5 wpt	0.75	ND
8 wpt	ND	ND

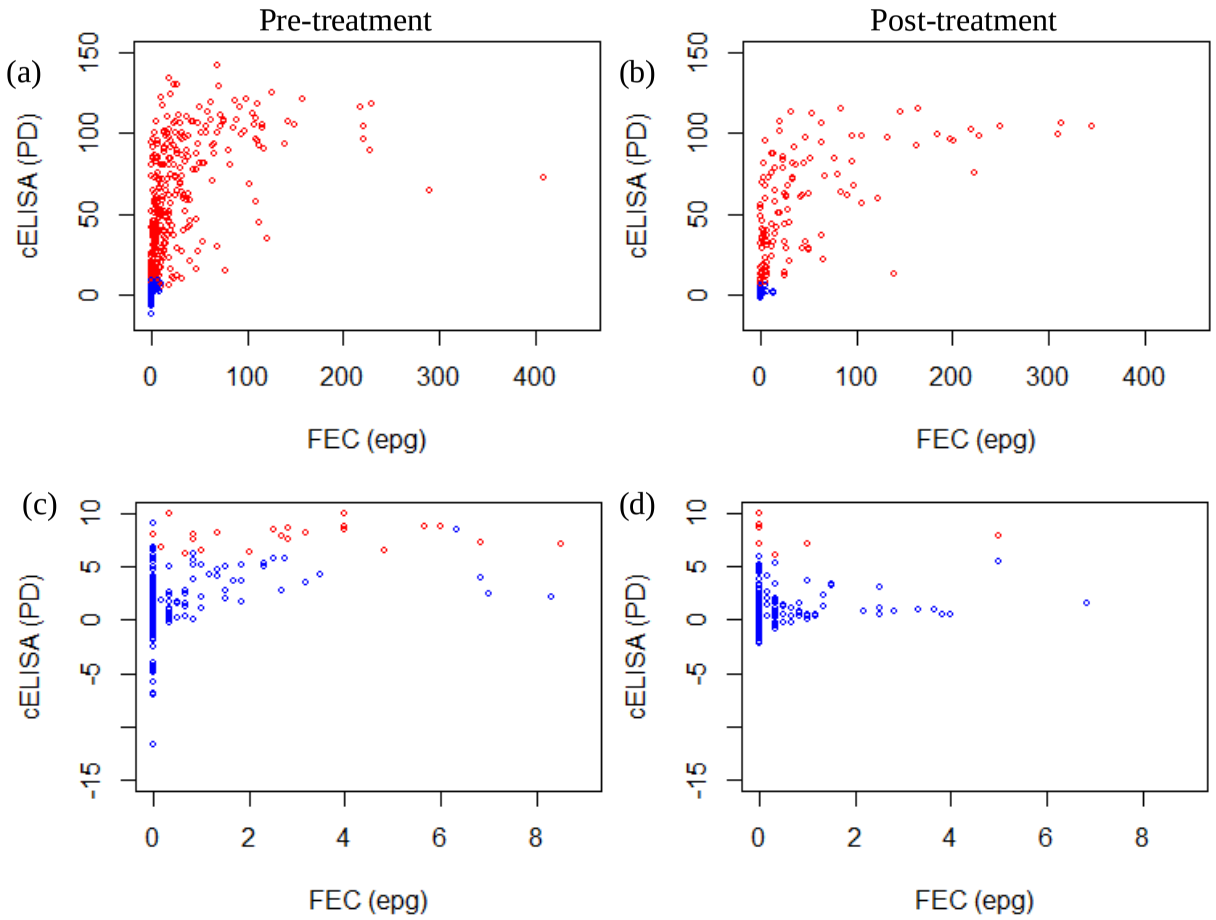


Figure 4.7 Individual FEC (epg) and cELISA (PD) results (a) pre- and (b) post-treatment with images expanded for the lower values (epg = ≤10 and PD = ≤ 10) (c) pre- and (d) post-treatment highlighted. ° = cELISA positive, ° = cELISA negative

Table 4.4 Agreement between FEC and cELISA in individual post-treatment samples. The Kappa value is shown, as well as the sensitivity (Se) and specificity (Sp) of the cELISA if FEC is used as a gold standard. ND = no data

1 wpt

Kappa = 0.66

Se = 73% Sp = 100%

		FEC		
		Positive	Negative	Total
cELISA	Positive	60	0	60
	Negative	22	46	68
	Total	82	46	128

2 wpt

Kappa = 0.44

Se = 50% Sp = 90%

		FEC		
		Positive	Negative	Total
cELISA	Positive	2	2	4
	Negative	2	19	21
	Total	4	21	25

3 wpt

Kappa = 0.58

Se = 53% Sp = 98%

		FEC		
		Positive	Negative	Total
cELISA	Positive	40	4	44
	Negative	35	183	218
	Total	75	187	262

4 wpt

Kappa = 0.75

Se = 75% Sp = 96%

		FEC		
		Positive	Negative	Total
cELISA	Positive	15	2	17
	Negative	5	50	55
	Total	20	52	72

5 wpt

Kappa = 0

Se = 0% Sp = 100%

		FEC		
		Positive	Negative	Total
cELISA	Positive	0	0	0
	Negative	2	32	34
	Total	2	32	34

8 wpt

Kappa = ND

Se = ND, Sp = 100%

		FEC		
		Positive	Negative	Total
cELISA	Positive	0	0	0
	Negative	0	7	7
	Total	0	7	7

4.4.3 Ability of FEC and cELISA to quantify liver fluke burden

4.4.3.1 Samples from the longitudinal study

Twelve livers from lambs in this study were collected at slaughter (Figure 4.8), 6 in November (examined using the SAC method), and 6 in January (examined using the Moredun method) (see Sections 2.1.3, 2.4.3.1 and 2.4.3.2). Gall bladders were not recovered for these animals. One liver was negative for liver fluke at the November sampling, this animal was positive for liver fluke by AbELISA and cELISA and GLDH and GGT concentrations indicated liver and bile duct damage. Insufficient faeces were obtained from this animal for FECs to be performed at any sampling point. For the 11 positive animals, fluke burdens ranged from 2 to 12 adult parasites (mean = 5.17, median = 4.5). No immature fluke were seen. The FEC and cELISA results for these animals, at the time of slaughter, can be seen in Table 4.5.

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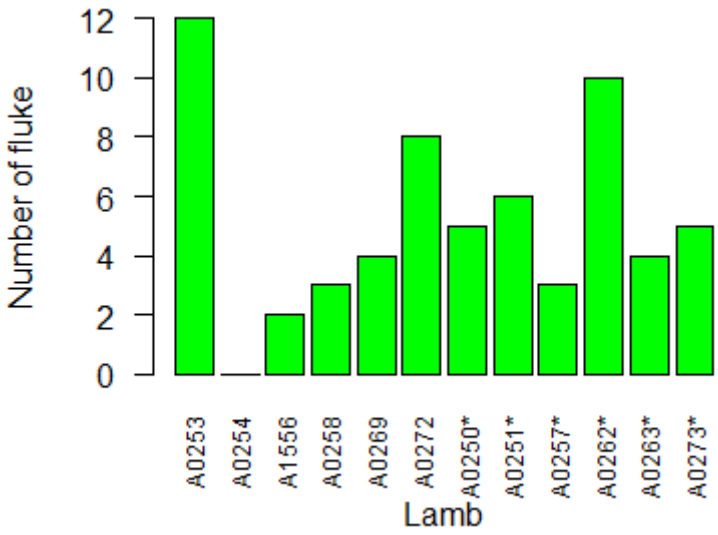

Figure 4.8 Number of fluke recovered at slaughter from the livers of naturally exposed lambs * indicates the lamb was slaughtered in January rather than November.

Table 4.5 Liver burden, FEC (epg) and cELISA (PD) results for the lambs slaughtered in November and January. * indicates livers were examined using the Moredun method. ND = no data available.

Animal	Liver burden	FEC (epg)	cELISA (OD)
A0253	12	1	8.06
A0254	0	ND	25.54
A1556	2	ND	ND
A0258	3	1	22.45
A0269	4	ND	ND
A0272	8	1.66	59.04
A0250*	5	4.16	72.53
A0251*	6	19	101.73
A0257*	3	6	28.88
A0262*	10	20.33	124.24
A0263*	4	7	12.42
A0273*	5	7.33	14.73

4.4.3.2 Samples from all studies

A total of 25 livers were collected from animals which had been grazed on pasture known to support the liver fluke lifecycle. The animals originated from 4 different Scottish farms, all of which are included in Section 4.4.2. Animals included 6 lambs, which are described in Section 4.3.1.1, and 19 sheep older than 1 year. The remaining 6 lambs from which livers were recovered from, described in Section 4.3.1.1, were not included as the method of fluke extraction differed. Gall bladders were recovered from 6 animals from 3 different farms; the details for these animals can be seen in Table 4.6. Fluke were detected in 14 of the 25 livers examined. Liver burden ranged from 0 to 75 fluke, with a mean of 7.6 and a median of 2. Full details of these 25 animals can be seen in Appendix 3.

Table 4.6 Summary of liver investigation, FEC (epg) and cELISA (PD) results for animals where gall bladders were recovered at slaughter. ND = no data

Animal	Farm	Liver burden	FEC (epg)	cELISA (PD)
A0059	10	0	0	0.758
A0060	10	2	1	5.186
A0155	22	46	66.5	132.346
A0187	22	75	32	97.619
A0498	ND	3	1.83	4.88
A0500	ND	24	40.67	-1.096

4.4.3.2.1 FEC and cELISA

The FECs ranged from 0 to 80.67 epg (mean = 12.1 epg, median = 1.7 epg), with 18 animals having a positive FEC at slaughter. The cELISA PDs ranged from -1.1% to 132.4% (mean = 30.9%, median = 5.2%), with 12 animals having a positive cELISA result at slaughter.

4.4.3.2.2 Liver burden vs. FEC

Substantial agreement was seen between liver examination and FEC in terms of determining fluke infection (Kappa = 0.84) (Table 4.7). Gall bladders were not recovered for the 4 animals which were negative for fluke in the liver but positive by FEC. Using liver examination as a gold standard, knowing that this may not be entirely accurate when gall bladders are missing, the FEC had 100% sensitivity and 63.6% specificity (PPV = 77.8%, NPV = 100%, prevalence = 56%). Gall bladders were recovered for 6 animals and, in these the FEC had 100% sensitivity and specificity. There was a positive correlation between FEC (epg) and fluke burden (rho = 0.88 $P < 0.0001$) (Figure 4.9). This positive correlation was equally as strong in animals where the gall bladder had been recovered and those where it had not been recovered (rho = 0.83, $P = 0.04$ and rho = 0.82, $P < 0.0001$, respectively).

4.4.3.2.3 Liver burden vs. cELISA

Substantial agreement was again seen between liver investigation and cELISA when determining the liver fluke infection status of an individual animal (Kappa = 0.68) (Table 4.7). One animal was negative for liver fluke infection by liver investigation but positive by cELISA. The gall bladder was not recovered for this animal and it had a positive egg count (13.3 epg). Two animals, which had a low number (2 and 3) of fluke recovered from the liver, were cELISA negative. Using liver examination as the gold standard, the cELISA had 78.6% sensitivity and 90.9% specificity. For the 6 animals which had gall bladders recovered, the cELISA had 40% sensitivity and 100% specificity. There was a positive correlation between cELISA (PD) and fluke burden (rho = 0.66, P = 0.0003) (Figure 4.9). The positive correlation was slightly weaker when looking only at animals where the gall bladder was recovered (rho = 0.54, P = 0.26), and higher where the gall bladder was not recovered (rho = 0.82, P < 0.0001).

Table 4.7 Liver examination and either (a) FEC outcome or (b) cELISA outcome in naturally exposed animals

Kappa = 0.84		Liver examination		
		Positive	Negative	Total
FEC	Positive	14	4	18
	Negative	0	7	7
	Total	14	11	25

Kappa = 0.84		Liver examination		
		Positive	Negative	Total
cELISA	Positive	11	1	12
	Negative	3	10	13
	Total	14	11	25

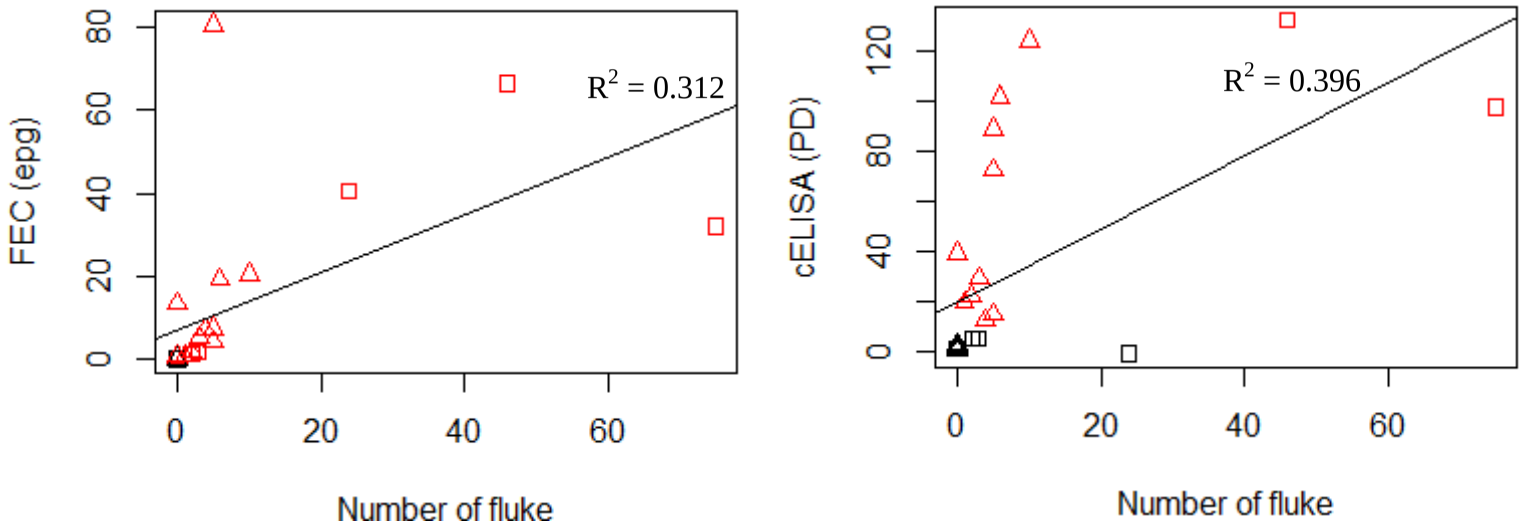

Figure 4.9 Scatterplot of number of fluke recovered from naturally exposed animals and either (a) FEC (epg) or (b) cELISA (PD). FEC or cELISA positive animals are shown in red, negative in black. △= no gall bladder recovered, ☐ = gall bladder recovered.

4.5 Discussion

This chapter aimed to evaluate the currently available diagnostic tests for liver fluke, in terms of their ability to detect both initial and established infection in individual sheep naturally exposed to *F. hepatica.* The chapter addressed these aims in 3 ways, (1) longitudinal monitoring using invasive and non-invasive tests, (2) cross-sectional analysis using non-invasive tests and (3) investigation into the ability of FEC and cELISA to quantify liver fluke burden.

4.5.1 Longitudinal monitoring

In the longitudinal monitoring of lambs, both GLDH and GGT were found to be unsuitable for the detection of *F. hepatica* infection when using the SAC C VS "normal" ranges to indicate hepatocellular damage. By SAC C VS criteria, the majority of animals were positive by both tests at the start of the study. This would indicate that animals were infected at least 6 weeks prior to the start of sampling, which would place infection in the month of May. This is very unlikely due to the animals only being one month old at this point and ingestion of infective cysts a rare occurrence. In addition, when using the SAC C VS "normal" ranges, 2 animals had elevated GGT concentrations before GLDH concentrations rose, which would suggest damage to the bile ducts prior to liver tissue damage. This would be counter-intuitive, given the known migration pathway of immature fluke. In all of the published studies consulted, a positive cut-off was not used. Instead, an animal, or a group of animals, were considered positive if the GLDH or GGT concentration was significantly higher than either that of the control group or of the animal/group in question prior to infection. Indeed, in most studies, the control or pre-infection concentration of GLDH and GGT are not within the normal (uninfected) range set by SAC C VS, with control animals/groups having GLDH and GGT concentrations of 18-20 and 70-100 IU/L, respectively (Ferre et al., 1996; Ferre et al., 1997; Raadsma et al., 2007). If this criterion of a significant increase from the control is used to determine test outcome, and the June sampling is considered to be the control, then

infection is likely to have occurred in September or October at the latest, according to GLDH and GGT concentrations, respectively. A further point to note is the fluctuating concentrations of GLDH and GGT in individual animals. This may be explained by the instability of GLDH in serum at room temperature and 4˚C (Anderson, 1977), but still suggests that GLDH and GGT are not sufficiently robust indicators of fluke infection to be used as routine diagnostic tests in the absence of other supporting evidence.

Although the AbELISA indicated early exposure, with a few animals being positive at the first sampling, the majority were not antibody positive until September, indicating an August exposure. Fluctuations between a positive and negative infection status were seen in 3 animals. This is a concerning result if the test is used at the individual animal level. *F. hepatica* antibodies are known to persist at detectable levels in an animal for several months (Ibarra et al., 1998; Sánchez et al., 2001). There is no biological reason for individual animals to have circulating anti-*Fasciola* antibodies one month, but not the following month, other than in the case of anti-*Fasciola* maternal antibodies, which wane quickly after weaning. No information could be found on anti-*Fasciola* maternal antibodies in sheep. In calves, it has been shown that the length of time anti-*Fasciola* maternal antibodies are detectable in serum varies depending on the number of cysts the mother was exposed to prior to birth. In low-dose infections, anti-*Fasciola* maternal antibodies were detectable for 7 weeks, extending to 12 weeks in high-dose infections (Mezo et al., 2010). As such, it is possible that, in June, anti-*Fasciola* maternal antibodies were at a detectable level. This would explain animal A0255, but not the fluctuations seen in A0259 and A1556. Indeed, the difference between the positive and negatives that were detected in these fluctuating animals were large and, rather than a steady increase in antibody titres over the course of the study, fluctuations are seen in nearly all animals. Two animals which maintained a positive antibody titre throughout the study may have been exposed to fluke due to grazing from an early age. This would indicate infection in May at the latest, possibly earlier. One animal, A0268, did not develop a positive antibody titre throughout the study, nor did it develop a positive FEC or cELISA result and both GLDH and GGT concentrations did not differ

significantly from the June sampling. It is highly likely that this animal remained uninfected.

Of the three animals which had early positive FECs, two maintained a positive antibody titre throughout the study. It is possible that these two animals were truly infected in the month following their birth. Unfortunately, the livers of these animals were not available for inspection at slaughter. On the other hand, it is possible that these may be false positive FECs which may arise for a variety of reasons. These include equipment contamination or a mislabelling of samples in the lab, or potentially the passage of undigested eggs (Hillyer, 1988). The fluctuations in the FEC of lambs during this study are reasonable, considering the low fluke burdens seen in the livers recovered and the nature in which eggs are stored in the gall bladder and sporadically shed (Valero et al., 2002).

Although one animal had a positive cELISA OD in July, this was not maintained in the following sampling months until November. Two other animals had cELISA ODs which changed from positive to negative between sampling, although these were October and November samples. The coproantigens detected by the cELISA are a direct indicator of infection and should be detectable until the infection is cleared (Bio-X, 2010; Mezo et al., 2004). This suggests that the positive result in July was likely a false-positive and not indicative of a true infection. In this study animals became cELISA positive the same month as they became FEC positive, contrary to the earlier detection of infection previously reported (Flanagan et al., 2011a; Valero et al., 2009).

One explanation for the negative cELISAs, fluctuations and delayed detection of infection, would be the storage of the faeces. Following collection they were taken to SAC C VS, Edinburgh, where FECs were performed, and then posted to MRI for the cELISA to be performed on the remaining sample. This delay of up to 5 days in preparation for cELISA could have resulted in degradation of the coproantigens, resulting in false negative cELISAs. Whilst rigorous studies into the stability of coproantigens at room temperature and 4˚C have yet to be conducted, an initial investigation gave conflicting results regarding storage of faeces at room

temperature, ODs falling with longer storage in 3 samples stored at 26˚C, but fluctuating in 10 samples stored at 20˚C (Flanagan et al., 2011b). In the study by Flanagan et al. (2011b) all samples maintained a positive result, but as no OD values were reported it is not possible to know if these were samples with high or low ODs. In their discussion Flanagan et al. (2011b) point out that storage conditions will have the biggest impact on samples with low ODs, potentially resulting in a positive sample testing negative after a period of storage at room temperature.

A second, equally plausible, explanation could be the low burden of fluke seen in this study, with a maximum of 10 fluke being recovered at slaughter. There are no reports in the literature of the cELISA being used in naturally infected sheep. In the majority of published studies involving experimental infections, the infective dose of metacercariae have been high, with high fluke burdens being seen at slaughter. The exception to this is the original description of the cELISA, in which lambs were infected with a small number of cysts and it was found that, in the very low infections (only 1 fluke recovered at slaughter), coproantigens were not detected until 7-8 wpi (Mezo et al., 2004). This highlights the importance of understanding the conditions under which a diagnostic test has been evaluated. It also shows that cELISA cannot be relied upon to detect infected animals with a low fluke burden.

It was not possible to ensure that gall bladders were attached to the livers which were collected from abattoirs. One animal had no fluke present in the liver tissue and bile ducts, but had positive cELISA and AbELISA results. It is highly likely that fluke were lost when the gall bladder was removed. This may have also contributed to the low burdens reported in this study, although the numbers of fluke in the gall bladder itself was likely to be low. Gall bladders should be collected wherever possible when attempting to quantify a fluke burden as fluke can be found within them *post-mortem*.

In young animals, >7 weeks old, the AbELISA appears to be the most reliable diagnostic test for liver fluke, although from a veterinary perspective it is impractical to regularly blood sample animals, especially sheep. GLDH and GGT concentrations may be valuable in older animals, which have persistent anti-*Fasciola* antibodies, but

control values are needed. The cELISA has little benefit over the FEC, in terms of earlier detection, in animals with a low burden.

4.5.2 Cross-sectional analysis

In the cross-sectional sheep study, the majority of pre- and post-treatment samples were collected during the winter months of 2011 as part of a postal survey. During the sample collection period, the Bio-X cELISA kit was altered, including the criteria for determining a positive result. The new criteria would have changed the treatment outcome of only 3 samples, however, for analysis in this chapter, the batch criteria were used to determine treatment outcome. Agreement on treatment outcome between FEC and cELISA was good, both pre- and post-treatment, with slightly better agreement pre-treatment. Biologically, in an initial infection, with a sufficient challenge, the cELISA would be expected to become positive earlier than the FEC.

Due to the animals in this study being naturally exposed to fluke, it is not possible to know when the point of infection was, nor the number of metacercariae ingested, as such it is expected that some animals would be positive by both FEC and cELISA and some positive by cELISA alone. Interestingly, the majority of disagreements pre-treatment are FEC positive but cELISA negative. Unless a flukicide treatment had been recently administered, this disagreement is not readily explainable biologically. Whilst it cannot be ruled out for some animals in this study, the majority were part of a postal survey which involved a pre- and post-treatment sampling and this greater sensitivity of the FEC was seen in pre-treatment samples. Again, fluke burden may be playing a role, all samples that were cELISA negative but FEC positive had <10 epg or <15 epg pre- and post-treatment, respectively (median = 0.83 and 0.5 epg, respectively). There was a significant difference both pre- and post-treatment between the median FEC of samples that tested positive by both FEC and cELISA and those that tested positive by FEC alone.

The reported sensitivity of the cELISA, when using FEC as a gold standard, was lower than expected, at 85.1% and 64.6% pre- and post-treatment, respectively. It is

important to note that FEC is not truly a gold standard. As discussed earlier, a negative FEC can occur in low-level infection and low positive FECs do occur following successful treatment. As all animals were naturally exposed to fluke and very few could be followed to slaughter, the true infection status of the animals remains unknown. A more recent Australian study, which had the benefit of access to a large number of samples known to be uninfected and FEC positive samples, calculated the sensitivity of the cELISA to be 88% (Palmer et al., 2014).

4.5.3 Quantifying fluke burden

Difficulties were experienced when trying to collect livers to investigate the ability of FEC and cELISA to quantify liver fluke burden in naturally exposed sheep. Miscommunication in the abattoir, in addition to an already hectic environment, meant that gall bladders were removed from the liver by the staff. Gall bladders were also not always provided when collecting opportunistic paired liver and faecal samples from local farmers and veterinarians. As such, out of the 25 livers examined, only 6 had a gall bladder attached. This had implications for using liver examination as a gold standard, as it is possible that fluke were lost either within the gall bladder or from the main bile duct once the gall bladder was removed. FEC had a sensitivity of 100% and a specificity of 63.6%, the low specificity was due to the absence of fluke in 4 livers, all of which had no gall bladders attached. Where gall bladders were recovered, the FEC was 100% sensitive and specific. The cELISA had a sensitivity of 78.6% and a specificity of 90.9%, in this case the specificity was lowered by the absence of fluke in one liver, which again did not have a gall bladder attached.

Of more interest though is the low sensitivity of the cELISA, this was due to the presence of fluke in 3 livers, but the absence of a positive cELISA result in the paired faeces. Two of these animals had low liver burdens (≤3), but did have a patent infection, albeit a low FEC (<2 epg). However, the cELISA has previously been reported to detect coproantigens from a single fluke during a patent infection in sheep (Mezo et al., 2004). The third animal in our study (A0500) had a moderate liver burden (24 fluke) and FEC (40.7 epg). It is possible that poor storage of this

sample, prior to receipt for the study, resulted in degradation of the coproantigens, although storage conditions are unlikely to have affected only one sample.

The correlation between the cELISA PD and the fluke burden was surprisingly low. It would appear that once a fluke burden crosses a certain threshold, the cELISA PD does not increase, no matter how high the burden becomes. The maximum limit for the cELISA PD would appear to be between 100 and 120%. Unfortunately, PD values of ≥100% occurred in fluke burdens as low as 5. The FEC on the other hand had a much better positive correlation with liver burden.

The low correlation seen between cELISA PD and fluke burden in sheep in this study was contrary to the good correlation seen between cELISA PD and fluke burden in cattle (Brockwell et al., 2013; Charlier et al., 2008). There are a number of potential reasons for this, including the effect of the calcification of bile ducts in cattle, the larger volume of faeces produced in cattle and the distribution of coproantigens within the faeces, the overall burden of cattle infections or the larger volume of faeces examined in the cELISA in cattle samples (2 g vs. 0.5 g). All of which may affect the relationship between cELISA PD and fluke burden.

4.5.4 Conclusion

In conclusion, (1) in young sheep, naturally exposed to *F. hepatica*, healthy GLDH and GGT concentrations may be higher than those suggested by SAC C VS, detection of infection by cELISA may be delayed in cases of low fluke burdens and care should be taken to avoid degradation of samples, (2) using FEC as a gold standard the cELISA has a sensitivity of 85.1% and 64.6% in pre- and post-treatment infections, lower than that seen in experimentally challenged animals (Chapter 3), with the majority of disagreements between FEC and cELISA being FEC positive but cELISA negative, (3) the collection of gall bladders is important when determining fluke burden, especially in low burdens, which was the case in the majority of animals seen in this study. FEC (epg) appears to correlate better with

liver burden than cELISA (PD) does, and also had a higher sensitivity (100%) and specificity (63.6%).

Chapter 5: Investigation of existing diagnostic tests in naturally exposed sheep: Studies at a group level

5.1 Abstract

The coproantigen ELISA (cELISA) is a promising diagnostic test, which has been reported to detect low burden pre-patent infections and only indicate current fluke infections; these are all potential advantages over the faecal egg count (FEC). The recently proposed cELISA reduction test (CRT) to determine treatment outcome was based on an experimental challenge infection of sheep, but has yet to be fully evaluated in animals naturally exposed to *Fasciola hepatica*. The first study presented in this chapter aimed to evaluate the CRT in natural infections on two Scottish farms with 80 animals in total included in the final analysis. Animals were followed for 56 days with either a triclabendazole (TCBZ) or closantel treatment at the start and a further closantel treatment given to any animal with a positive FEC or cELISA result at 21 days post-treatment (dpt). The second study aimed to evaluate a composite cELISA and composite CRT, with the intention of developing a simple and reliable test to determine treatment outcome which would be appealing to farmers. Both the technical performance of the test and farmer compliance with the test protocol were evaluated. Farmers were recruited through the farming press and knowledge exchange events, and participated voluntarily. In total, 41 sample packs were sent to farmers across Great Britain, of which 25 farmers subsequently participated. Farmers were asked to submit two groups of 10 faecal samples from 12 animals before treatment and at 21 dpt, based upon a published protocol. Samples from 44 pre-treatment groups were submitted whilst 36 sets of samples from post-treatment groups were submitted. Individual and composite faecal samples from each group were tested for evidence of liver fluke infection by FEC and cELISA, and mean values as obtained from individual samples (average value) were compared to values derived from composite samples (composite value). Average FECs were low but had higher sensitivity for detection of liver fluke infection in a group of sheep than composite FECs. By contrast, the composite cELISA was more sensitive than the average cELISA. The composite cELISA was less sensitive than the composite FEC in low burden situations. A modified version of the composite CRT showed good agreement with the composite FEC reduction test and appears promising in

situations where burden was sufficiently high. In conclusion, the CRT and composite CRT appear to give a good indication of treatment outcome, with the benefit of results from 7 dpt, but are of limited use in flocks with a low burden of infection.

5.2 Introduction

Sub-acute fasciolosis, caused by pre-patent *Fasciola hepatica* infection, causes significant economical losses to sheep farmers, with sudden death often being the first sign of a problem. Because this condition is caused by migrating immature fluke that do not lay eggs, it cannot be detected by faecal egg counting (FEC). In experimental challenge situations, the coproantigen ELISA (cELISA) is able to detect early stages of infection and, as such, is a promising alternative to FEC (Flanagan et al., 2011a; 2011b; Gordon et al., 2012a; Mezo et al., 2004). In individual naturally exposed animals, the cELISA was less sensitive than FEC (Chapter 4).

Although the cELISA was less sensitive than FEC when used on samples from individual animals it still needs to be evaluated at the group level. This is because most treatment decisions and evaluation of treatments are done at the flock level. In addition, FEC is prone to false positives following successful treatment due to the release of sequestered eggs from the gall bladder (Valero et al., 2002). The cELISA has been reported to only detect current infection and so may be more useful than FEC in terms of determining treatment outcome (Bio-X, 2010).

Although there is no World Association for the Advancement of Veterinary Parasitology (WAAVP) recommended test to determine fasciolicide treatment outcome in the live animal (Coles et al., 2006), the faecal egg count reduction test (FECRT), as described for nematodes, is commonly used for the evaluation of liver fluke treatments (Fairweather, 2011b; Gordon et al., 2012b). A cELISA reduction test (CRT), which was developed using samples from experimental challenge experiments, has been proposed as an alternative (Flanagan et al., 2011a; 2011b). The CRT has yet to be evaluated in naturally infected animals. In addition to being

able to give an early indication of treatment outcome in experimental challenge experiments, the cELISA has also been shown not to cross-react with rumen fluke, an emerging co-infection in UK, Irish and European livestock (Gordon et al., 2013; Kajugu et al., 2012).

This specificity for *F. hepatica* infections is of increasing importance due to the emergence of endemic rumen fluke in the UK (Foster et al., 2008; Gordon et al., 2013; Mason et al., 2012; Millar, 2012). Rumen fluke eggs are morphologically similar to those of *F. hepatica*, making diagnosis by FEC difficult if training has not been given (Rojo-Vázquez et al., 2012; Silvestre et al., 2000), even then, distinguishing the two fluke species' eggs can be relatively subjective. As rumen fluke are not affected by many fasciolicidal drugs, it can also be difficult to determine treatment outcome when an animal is infected with both types of fluke (Rolfe and Boray, 1987, 1988).

It is common practice in the UK to regularly treat sheep for liver fluke without evidence that animals are actually infected (Besier and Love, 2012). This is, in part, due to the severity of acute infections and the inability of FEC to detect this stage of infection, but it is also due to the high cost and inconvenience of diagnostic testing compared to routine treatment. Put simply, if diagnostic testing is more expensive and inconvenient than treatment, then most farmers will choose to treat without a diagnosis. Single animal testing can cost between £8 and £34 depending on the test and the company providing it (AHVLA, 2014; SRUC, 2014). The use of composite (pooled individual) samples has been proposed as a means to reduce the cost of routine diagnostic testing, thus making evidence-based decision making more appealing to farmers. A composite FEC protocol for detecting triclabendazole resistance (TCBZ-R) has been developed for use in sheep, utilising samples from 12 animals, but this has not yet been evaluated with the cELISA or CRT (Daniel et al., 2012). Initial investigation by Brockwell et al. (2013) showed that the cELISA performed well when used on composite samples from smaller groups of cattle (n = 5).

A composite CRT has the potential to reliably give an early indication of treatment outcome in sheep, even in pre-patent infections, whilst also being cheaper than individual testing.

The present study has three main aims, therefore, (1) to evaluate the CRT in sheep naturally exposed to *F. hepatica*, as part of a treatment trial; (2) to evaluate the use of a composite cELISA, in terms of (a) detecting *F. hepatica* infection status and (b) determining treatment outcome in British sheep flocks, in the form of a pilot study using group average as the gold standard; and (3) to provide a preliminary assessment of farmer compliance with the proposed composite sampling protocol for diagnosis and treatment evaluation.

5.3 Materials and Methods

5.3.1 CRT evaluation: farms and sampling

5.3.1.1 Farms and animals

Two working farms with a history of TCBZ treatment failure were selected for this study. Farm 9 was located in the south-east of Scotland, mainly kept mule (cross-breed) sheep and kept the study ewes on pasture. Farm 22 was located in Dumfries and Galloway, mainly kept Blackface x Blue Leicester sheep and housed the study ewes. Only ewes were used in this study, all of which were presumed to be in-lamb.

5.3.1.2 Sampling and treatments

A pre-screening was conducted on each farm to determine the liver fluke infection status of the flock. Following this, animals were alternately allocated to either a TCBZ (Fasinex®, Novartis Animal Health) or a closantel (Flukiver®, Janssen Animal Health) treatment group. On Farm 9, ewes were considered to weigh approximately 80 kg, as the farmer estimated that no animals exceeded this weight. Farm 22 ewes were weighed individually using a calibrated weigh crate. All animals were dosed according to dosage guidelines laid out by Novartis Animal Health and Janssen Animal Health for their respective products, with the modification that the dosage

was rounded up to the nearest ml and 1 ml added to allow for loss in the syringe during dosing. All dosing was performed orally using a 20 ml syringe to ensure accuracy. As the study took place on working farms, with in-lamb ewes, all animals in the TCBZ treatment group with evidence of liver fluke infection at 21 days post-treatment (dpt) received a closantel treatment. This ensured that the welfare of the animals was not compromised.

Rectal faecal samples were collected from ewes on both farms at each sampling point, as described in Section 2.1.1. Sample collection was carried out by Danielle Gordon-Gibbs, Professor Neil Sargison and Royal (Dick) School of Veterinary Studies students on Farm 9 and by Danielle Gordon-Gibbs, Dr Philip Skuce, Professor Ruth Zadoks, Heather Stevenson and Margaret Oliver on Farm 22, hereafter referred to as the sampling team. Samples were taken at 0, 7, 14 and 21 dpt to observe the kinetics of egg and coproantigen shedding post-treatment. Samples were also taken at 35 and 56 dpt in order to obtain 21 and 35 days post-closantel treatment samples from the TCBZ-treated group, and to determine if immature fluke survived the treatment in the closantel-treated group. The 35 dpt sampling could not be carried out on Farm 22. Samples were individually stored in 50 ml Falcon tubes at 4°C until required for testing.

5.3.2 Composite sampling evaluation: farms and sampling

5.3.2.1 Farmer recruitment

Farmers were recruited in a number of ways. The study was advertised in the farming press and at an annual Quality Meat Scotland (QMS) Press Day (2011). Interested farmers submitted their contact details and were sent a sampling pack at the start of the study. In addition, several veterinarians approached the team indicating that some clients might be interested in participating. In these instances, the veterinarian was sent 3 sampling packs at the start of the study to distribute to interested clients. Lastly, farmers who had heard about the study via 'word of mouth' approached the team requesting to participate, and were subsequently sent sampling packs.

5.3.2.2 Sampling packs and sampling

Sample packs were sent to farmers prior to treatment and prior to the 3 weeks post-treatment (wpt) sampling date. Each pack contained 2 bags of 10 sampling pots and 1 spare pot; pots were marked with a 'fill–to' line. The pack also included latex gloves, a postage paid return envelope, a cover letter and a questionnaire. In addition, the pre-treatment sample pack contained a laminated sampling instruction sheet (Figure 5.1). A simple sampling procedure was used, based on the method described by Daniel et al. (2012). Farmers were instructed to pen 12 animals and collect 10 individual faecal samples from the pen floor. These sheep were to be treated with a flukicide of the farmer's choice and marked accordingly. The same 12 sheep were to be penned 3 weeks later and a further 10 samples collected. Samples were sent by post and, upon receipt, stored at 4°C until testing. All documents included in the sampling packs may be viewed in Appendix 4.

Figure 5.1 Contents of sampling packs sent to farmers for the collection and submission of faecal samples for FEC and cELISA testing.

5.3.3 Detection of infection status

The cELISA was performed as described in Section 2.2.3. Samples were homogenised using a metal spatula and 0.5 g (±0.03 g) prepared to supernatant stage

and supernatants stored in 1.5 ml microcentrifuge tubes at -20°C until cELISA testing, which was never longer than 6 weeks following collection. The remaining faecal sample was then stored at 4°C prior to FEC.

For the samples used in the CRT evaluation, a sample was considered positive if the net optical density (OD) was ≥0.15, as outlined by the BIO K201 kit (Bio-X Diagnostics, Belgium) batch criteria used at the time.

For the samples used in the composite sampling evaluation, a sample was considered cELISA positive, if its OD was at least 6.07%, 7.46% or 9.32% of the test control positive, hereafter referred to as the percentage difference (PD). The positive cut-off used was dependent on the batch of kit used, as outlined by the BIO K201 kit batch criteria used at the time (Table 5.1). A group was considered cELISA positive if the mean PD was equal to, or greater than, the cut-off used for the individual samples in that group.

Table 5.1 Number and types of composite study samples tested at each cELISA cut-off value.

		cELISA batch cut-off value		
		6.07%	7.46%	9.32%
Sample type	Pre-treatment individual	331	78	30
	Post-treatment individual	318	0	40
	Pre-treatment composite	33	8	3
	Post-treatment composite	32	0	4

In addition, a composite sample was formed for each group in the composite sampling evaluation. Following the homogenisation of individual faecal samples for cELISA sub-sampling, 1 g of faeces was taken from each of the 10 pots in the group. The resulting 10 g was then homogenised and sub-sampled for FEC and cELISA using the same procedure as for individual samples, i.e. 0.5 g (±0.03 g) of the composite sample was used for cELISA and 3 g of the composite sample was used for FEC. Samples were tested no later than 6 weeks after collection.

In the evaluation of the CRT, the Farm 9 pre-screening samples were examined by the veterinarian (Professor Neil Sargison) for presence of liver fluke eggs but actual

counts were not performed. In all other instances, in both studies, FECs were performed as described in Section 2.2.1.2. Samples of less than 3 g were processed in the same manner, with the average number of eggs being divided by the number of grams of faeces available. Samples of <1 g were not included in either study. In the evaluation of composite sampling a group was determined to be positive for liver fluke if the mean FEC was >0 epg.

5.3.4 Evaluation of treatment outcome

The reduction in the FEC from the pre-treatment sampling to the post-treatment sampling was calculated using a standard nematode faecal egg count reduction (FECR) calculation, as described in Section 2.2.2 and proposed by (Coles et al., 1992). The FECR was also calculated using the composite FEC before and after treatment rather than the arithmetic mean. A FECR of ≥95% was considered to indicate a successful treatment.

The coproantigen reduction (CR) was calculated using the same formula used for FECR, as described in Section 2.2.4. For the CRT evaluation, the mean group OD pre- and post-treatment was used. In the case of the composite sampling evaluation, the mean group PD pre- and post-treatment was used. The CR was also calculated using the pre- and post-treatment composite PD. A CR of ≥95% was considered to indicate a successful treatment.

For the CRT evaluation, the FECR and CR were calculated at 7, 14, 21, 35 and 56 dpt, always using 0 dpt as the baseline. Post-treatment samples were requested at 21 dpt for the composite sampling study.

5.3.5 Statistical analysis

All statistical analyses were performed using RStudio (R Core Team, 2014). Because the data did not follow a normal distribution and contained ties (data entries of the same numerical value), an unpaired non-parametric t-test ("wilcox.exact()" function)

was used to compare differences between treatment groups and farms (Hothorn and Hornik, 2013). FECRs were calculated using the 'bayescount' package ("fecrt(), WAAVP method" function) on FECs rounded to the nearest integer (Denwood, 2009). FECs <0.5 but >0 were rounded up to 1.0 epg for the FECRT. CRs were calculated in Microsoft Excel, due to the impracticalities of rounding ODs and PDs to the nearest integer.

The agreement between FEC and cELISA testing, as well as between composite and group average testing, were evaluated using Cohen's unweighted Kappa test (1960) via the "confusionMatrix()" function (Kuhn, 2014). Kappa values were categorised according to the degree of agreement beyond chance, ranging from none to almost perfect (Section 2.7) (McGinn et al., 2004).

Where appropriate, the performance of a test was evaluated using an alternative test as a 'gold standard' to determine true infection status of samples/animals. The "confusionMatrix()" function was used to calculate the proportion of true positives the test could detect (sensitivity) and the proportion of true negatives the test could detect (specificity) (Kuhn, 2014). In addition, the probability that positive results were truly positive (positive predictive value) and the probability that negative results were truly negative (negative predictive value) were also calculated using the "confusionMatrix()" function.

5.4 Results

5.4.1 Evaluation of the CRT

5.4.1.1 Treatment trial

In total, 394 and 407 faecal samples were collected from 86 and 70 ewes on Farm 9 and Farm 22, respectively. Despite samples being collected by the sampling team rather than by farmers, not all animals were available to be sampled and insufficient samples were retrieved on occasions at each sampling point. This was most often seen on Farm 9, where the number of animals sampled ranged from 47 to 68, compared with Farm 22, where 67 or 68 animals were sampled (Figure 5.2). On

Farm 9, 30 samples from 19 animals had to be excluded from the study for a variety of reasons. These included insufficient faeces being obtained to calculate an epg, the animal lacking an ear tag and thus being unidentifiable between samplings, and/or the treatment given not being disclosed by the farmer. On Farm 22, only 4 samples from 4 animals had to be excluded for some of the same reasons. Of the samples collected pre-treatment, each farm had 68 samples which were suitable for analysis. Lastly, only animals which tested positive for liver fluke by both FEC and cELISA at 0 dpt, and were successfully sampled at each subsequent sampling point, were included in the analysis of samples collected post-treatment (including 0 dpt). This resulted in 174 faecal samples from 29 animals on Farm 9 and 255 faecal samples from 51 animals from Farm 22, in total. Of these animals, 14 and 25 were treated with TCBZ whilst closantel treatment was given to 15 and 26 ewes on Farm 9 and Farm 22, respectively.

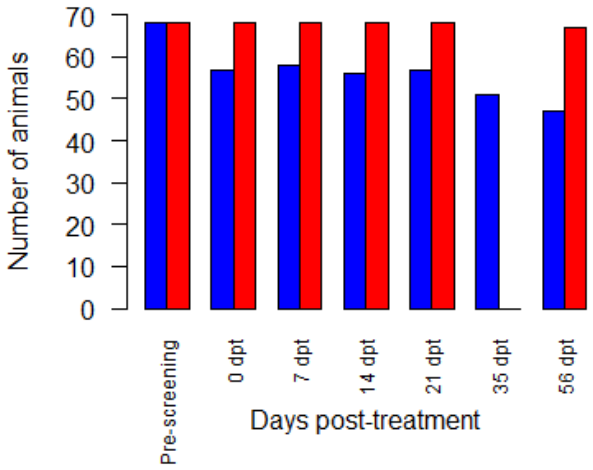

Figure 5.2 Number of ewes sampled at each sampling point on Farm 9 (blue bars) and Farm 22 (red bars) during the course of the evaluation of the CRT.

5.4.1.2 Infection levels at the pre-screening sampling

At the pre-screening sampling, 66 ewes were cELISA positive and 56 were FEC positive of the 68 sampled on Farm 9, with 56 being positive by both tests. On Farm 22, 63 and 57 of the 68 ewes tested positive for liver fluke by cELISA and FEC, respectively, with 57 being positive by both tests. FECs on Farm 22 ranged from 0 to

132.3 epg (mean = 23.2 epg, median = 9 epg) (Figure 5.3). The cELISA ODs ranged from 0.042 to 1.961 (mean = 1.106, median = 1.154) and from 0.011 to 2.090 (mean = 1.126, median = 1.210) on Farm 9 and Farm 22, respectively (Figure 5.4).

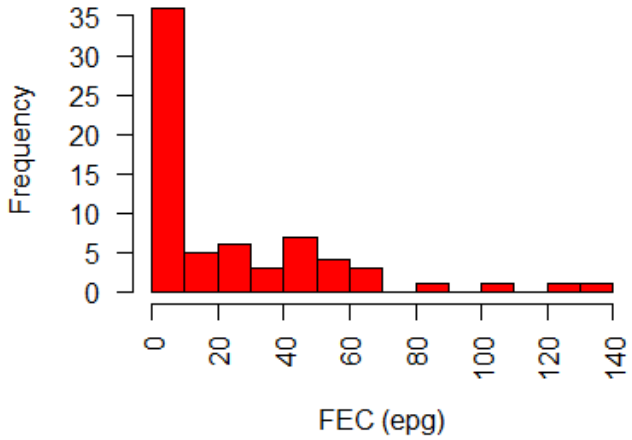

Figure 5.3 Distribution of FEC (epg) of ewes on Farm 22 at the pre-screening sampling. 11 samples had 0 epg in the first bar.

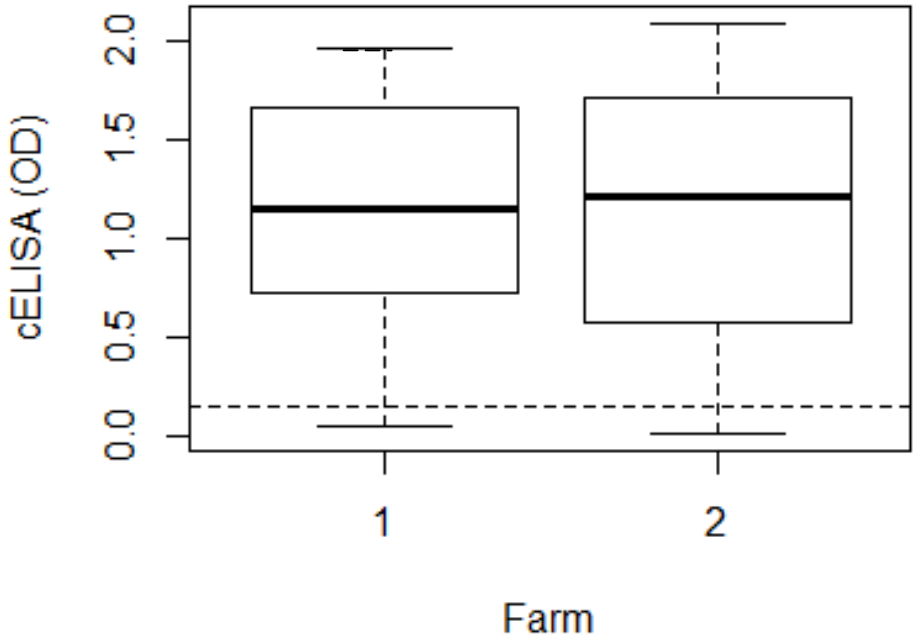

Figure 5.4 Distribution of cELISA OD for Farm 9 and 22 at the pre-screening sampling. ---- indicates the positive cut-off (0.15).

5.4.1.3 FEC and FECRT

Only animals which were both FEC and cELISA positive at the 0 dpt sampling were included in the post-treatment analysis. FECs of the 0 dpt samples ranged from 1.5 to 229.6 epg (mean = 37.7, median = 19.5) on Farm 9 and from 0.2 to 104.8 epg (mean = 18.8, median = 14) on Farm 22. Group mean pre-treatment epg did not differ significantly between the two treatment groups on either Farm 9 (P = 0.22) or Farm 22 (P = 0.28). The FEC results of all groups included in the study can be seen in Figure 5.5 and Table 5.2. All TCBZ-treated ewes retained a positive egg count, until they were treated with closantel 21 days after the initial TCBZ treatment. A proportion of closantel-treated ewes, ranging from 7 to 40%, also retained a positive, albeit low, egg count. At 7 dpt, on both farms, a number of ewes in the closantel-treated groups maintained a positive, albeit low, FEC (Farm 9, n = 6; Farm 22, n = 7). At 14 dpt, a number of closantel-treated animals retained a positive FEC, 2 and 4 ewes on Farm 9 and Farm 22, respectively. At 21 dpt, on Farm 9, only 1 closantel-treated ewe had a positive FEC, whilst 2 ewes on Farm 22 were FEC positive. Once again, at 35 dpt in Farm 9, low FECs were seen, with 3 animals in each group showing positive FEC. At the final sampling, 56 dpt, no closantel-treated ewes on Farm 9 had a positive FEC, whereas 3 TCBZ+closantel-treated ewes did. On Farm 22, 6 ewes in each treatment group had a positive FEC. The proportion of animals in each treatment group testing positive at each sampling point on each farm is shown in Table 5.2.

The results of the average FECRT for all groups on both farms at all sampling points are shown in Table 5.2. On Farm 9, a FECR of >95% was seen in the closantel-treated group at 7, 21, 35 and 56 dpt. At 14 dpt, the FECR was 83.7%, 1 ewe had a positive FEC of 49.7 epg. On Farm 22, the closantel-treated group had a >95% FECR at all sampling points. Prior to the closantel treatment at 21 dpt, all TCBZ-treated groups had a <95% FECR, ranging from -15.3% to 60.3%. At 35 dpt, a FECR of 99.6% was seen in the TCBZ+closantel-treated group on Farm 9. The FECR of the TCBZ+closantel-treated group on Farm 9 at 56 dpt was 99.6% and 98.8% in the same group on Farm 22.

Table 5.2 FEC, FECR and proportion of animals positive by FEC, on Farm 9 and Farm 22, respectively, treated with TCBZ or closantel, at 0, 7, 14, 21, 35 and 56 dpt. * indicates post-closantel treatment of the TCBZ-treated group. • indicates a significant difference between treatment groups on a farm (P < 0.0001)

	FEC (epg)				Faecal egg count reduction (FECR)				Proportion of animals FEC positive			
	Farm 9		Farm 22		Farm 9		Farm 22		Farm 9		Farm 22	
dpt	TCBZ	Closantel	TCBZ	Closantel	TCBZ	Closantel	TCBZ	Closantel	TCBZ	Closantel	TCBZ	Closantel
0	50	26.2	22.8	14.9	-	-	-	-	100%	100%	100%	100%
7	44.3•	0.6•	17.6•	0.6•	11.40%	97.20%	23%	96.20%	100%	40%	100%	27%
14	19.6•	4.3•	26.5•	0.2•	60.30%	83.70%	-15.30%	98.50%	100%	13%	100%	15%
21	39.9•	0.3•	21.5•	0.1•	20.10%	99%	6.30%	99.50%	100%	7%	100%	8%
35*	0.2	0.1	-	-	99.60%	99.20%	-	-	21%	20%	-	-
56*	0.1	0	0.2	0.1	99.60%	100%	98.80%	98.20%	21%	0%	24%	23%

Table 5.3 cELISA, CR and proportion of animals positive by cELISA, on Farm 9 and Farm 22, treated with TCBZ or closantel, at 0, 7, 14, 21, 35 and 56 dpt. * indicates post-closantel treatment of the TCBZ-treated group. • indicates a significant difference between treatment groups on a farm (P < 0.0001)

	cELISA (OD)				Coproantigen reduction (CR)				Proportion of animals cELISA positive			
	Farm 9		Farm 22		Farm 9		Farm 22		Farm 9		Farm 22	
dpt	TCBZ	Closantel	TCBZ	Closantel	TCBZ	Closantel	TCBZ	Closantel	TCBZ	Closantel	TCBZ	Closantel
0	2.492	2.262	1.198	1.128	-	-	-	-	100%	100%	100%	100%
7	1.086•	0.016•	1.423•	0.018•	56.40%	99.30%	-18.80%	98.40%	100%	0%	100%	0%
14	1.447•	0.116•	1.085•	0.023•	41.90%	94.90%	9.40%	98%	100%	6.70%	100%	0%
21	1.575•	0.11•	1.779•	0.011•	36.80%	95.10%	-48.50%	99%	100%	13.30%	100%	0%
35*	0.035	-0.003	-	-	98.60%	100.10%	-	-	0%	6.70%	-	-
56*	0.027	0.026	0.036	0.064	98.90%	98.90%	97%	94.30%	0%	0%	0%	7.70%

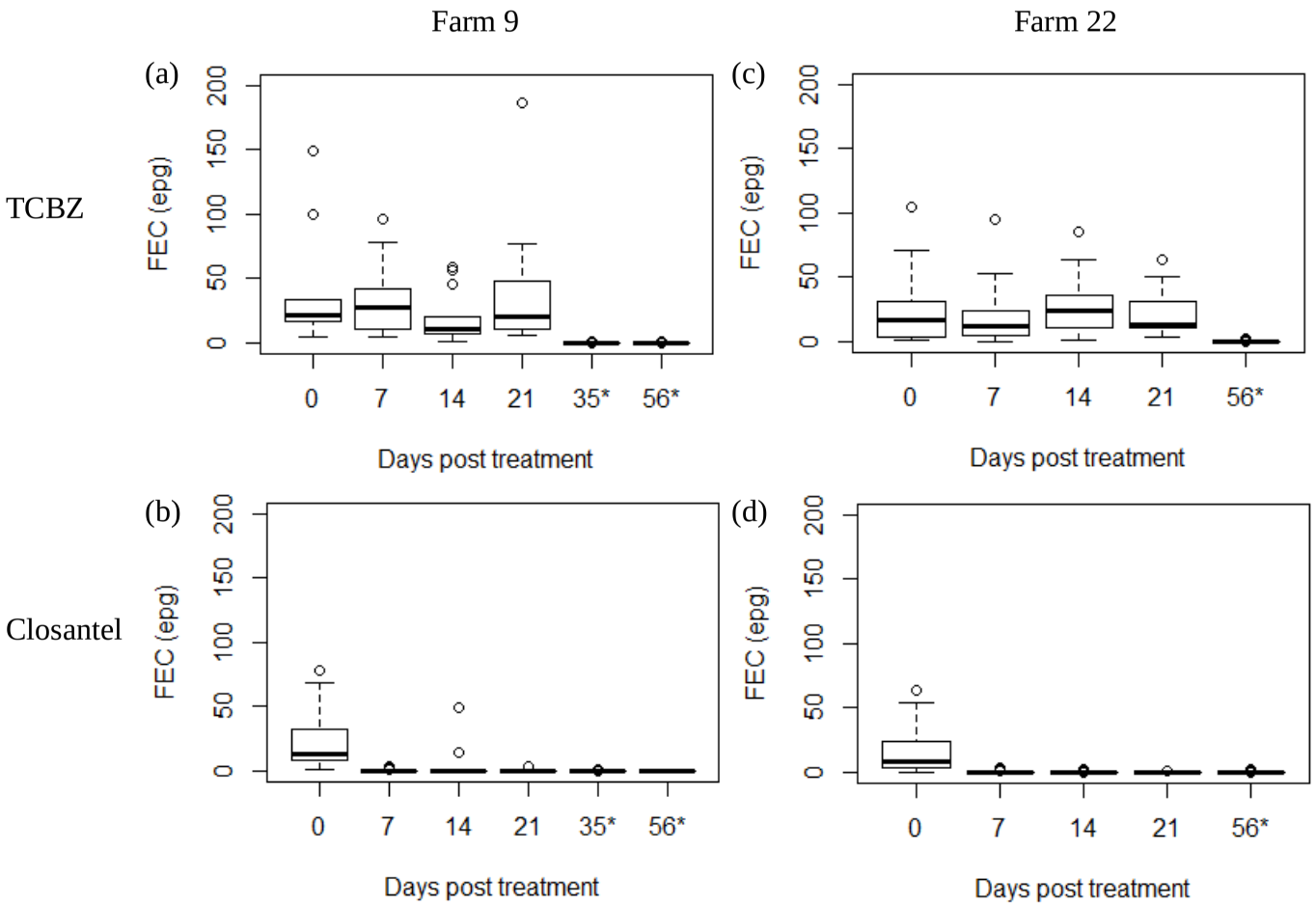

Figure 5.5 Boxplots showing eggs per gram (epg) for Farm 9 (a) TCBZ and (b) closantel, and Farm 22 (c) TCBZ and (d) closantel, treatment groups at 0, 7, 14, 21, 35 and 56 dpt. * indicates post-closantel treatment of the TCBZ group.

5.4.1.4 cELISA and CRT

All animals included in the analysis of post-treatment samples tested positive by cELISA at 0 dpt. Prior to treatment, cELISA OD did not differ significantly between TCBZ- and closantel-treated groups on either farm (P = 0.75 and P = 0.57 on Farm 9 and 22, respectively). On Farm 9, the OD ranged from 0.213 to 3.908 (mean = 2.492, median = 2.599) and from 0.203 to 3.642 (mean = 2.262, median = 2.988) in the TCBZ- and closantel-treated groups, respectively. On Farm 22, the OD ranged from 0.206 to 2.111 (mean = 1.198, median = 1.268) and from 0.15 to 2.451 (mean = 1.128, median = 1.112) in the TCBZ-treated and closantel-treated groups, respectively. The cELISA results of the TCBZ-treated and closantel-treated ewes on both farms over the duration of the study are shown in Figure 5.6 and Table 5.3.

One closantel-treated ewe tested positive on Farm 9 at 14 dpt (OD = 1.527), this ewe also had a positive FEC (49.7 epg). Two animals were cELISA positive in the closantel-treated group on Farm 9 at 21 dpt (ODs = 1.134 and 0.199), these were different animals to the one which had been positive at 14 dpt and neither animal had a positive FEC. One ewe in the closantel-treated group of Farm 9 at 35 dpt was positive by cELISA, with an OD of 0.279 and a negative FEC. This was again a different ewe to the ones which tested positive at 14 or 21 dpt. The closantel-treated group of Farm 22 at 56 dpt had 2 animals with positive ODs (0.15 and 0.349).

The results of the CRT and the proportion of animals testing cELISA positive at each sampling point on both farms can be seen in Table 5.3. A <95% CR was seen in both TCBZ-treated groups at 7, 14 and 21 dpt, ranging from 38.8% to 56.4% and from -48.5% to 9.4% on Farm 9 and Farm 22, respectively. Following closantel treatment of the groups which were initially dosed with TCBZ, a >95% CR was seen on both farms. For the closantel-treated groups, the CR was >95% on 7 of 9 occasions and just between 94% and 95% on the remaining 2 occasions (Table 5.3). This CR ranged from 95.1 to 100.1% and from 98 to 99% on Farm 9 and Farm 22, respectively. At 14 dpt on Farm 9, a reduction in mean OD of 94.9% was seen in the

closantel-treated group. On Farm 22, a 94.3% CR was seen at 56 dpt in the closantel-treated group.

5.4.1.5 FECRT and CRT agreement

A near perfect agreement was seen across all sampling points between treatment outcome according to the FECRT and according to the CRT, when using a ≥95% CR as the criterion for successful treatment in the CRT (Kappa = 0.89) (Table 5.4). The one instance of disagreement between the two tests was seen in the closantel-treated group on Farm 22 at 56 dpt. By FECRT at this sampling point, the treatment had been successful, but a 94.3% CR indicated a marginal treatment failure. If a ≥90% CR is used as the criterion for successful treatment, a near perfect agreement is again seen (Kappa = 0.88), with one disagreement. In this case, the 14 dpt sampling of the closantel-treated group on Farm 9 would indicate treatment failure as judged by the FECRT but successful treatment according to the CRT. If the FECRT is taken to be the gold standard, the CRT, with a cut-off of 95%, has a sensitivity of 90.9% and a specificity of 100% (PPV = 100%, NPV = 87.5%) when determining treatment success. If the FECRT is taken to be the gold standard and the CRT cut off is 90%, the CRT has a sensitivity of 100% and a specificity of 85.7% (PPV = 91.7%, NPV = 100%) when determining treatment success.

Table 5.4 Number of sampling points with either successful treatment or treatment failure, according the FECRT and CRT, respectively. A CR of ≥95% was required for a treatment to be deemed successful by the CRT. The Kappa value is shown.

Kappa = 0.89		CRT		
		Success	Failure	Total
FECRT	Success	10	1	11
	Failure	0	7	7
	Total	10	8	18

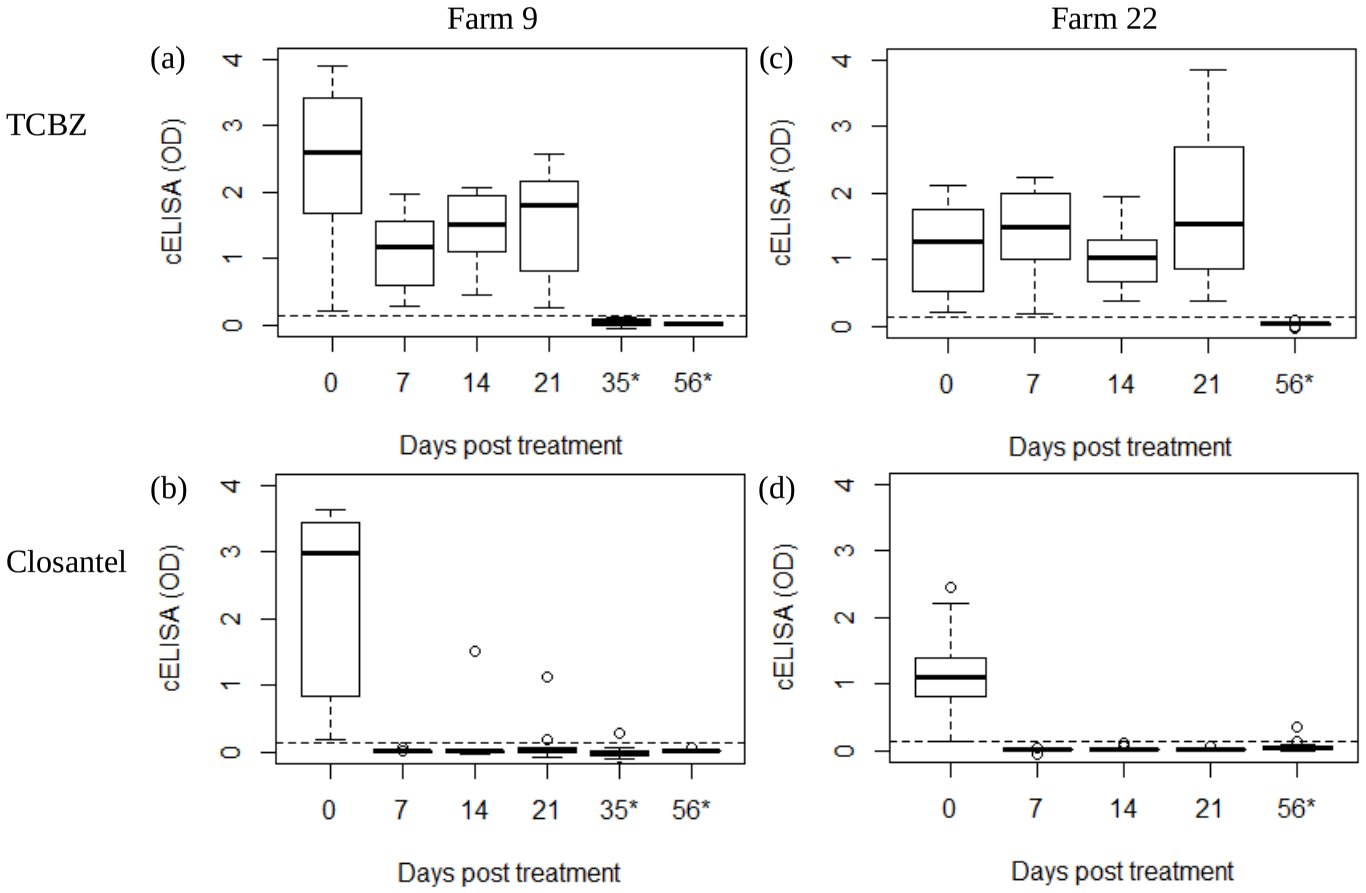

Figure 5.6 Boxplots of cELISA OD for the Farm 9 (a) TCBZ and (b) closantel and the Farm 22 (c) TCBZ and (d) closantel treatment groups at 0, 7, 14, 21, 35 and 56 dpt. * indicates post-closantel treatment of the TCBZ group. ELISA ODs are considered positive if ≥0.15.

5.4.2 Composite sampling evaluation

5.4.2.1 Study uptake and farmer compliance

Sampling packs were sent to 26 farmers (1 pack each) and 5 veterinarians (3 packs each). Of the 41 packs sent out in total, 25 were returned with pre-treatment samples. Six of the returned packs only contained samples for one group. One farmer submitted 9 samples for one group, rather than the requested 10. This gave a total of 439 pre-treatment samples from 44 groups. This was reduced to 358 samples from 36 groups post-treatment, due to non-submission by 6 farmers, 3 of whom had only submitted samples from 1 pre-treatment group and 1 who returned post-treatment samples from only 1 of their 2 pre-treatment groups. Two farmers, who submitted post-treatment samples from just one group, only returned 9 samples per group instead of 10. Groups containing <9 samples were excluded from the study. The participating farms were located in England, Scotland and Wales (Figure 5.7).

Despite the instructions given, there was variation between farmers in sample collection methodology. Five farmers collected samples from 10 individual animals rather than penning 12 and collecting 10 samples from the floor. Ten farmers submitted more than 10 samples for a group. In these instances, the first 10 from the group were counted as being the group and any others counted as excess and separate from the group. Excess samples were not included in composite formation, nor were they used to establish group infection status. Two farmers submitted an insufficient number of samples for a group (<9 samples), resulting in these groups subsequently having to be excluded from the study. Although the second sampling was requested at 21 dpt, with a date for sampling being communicated with the pre-treatment FEC results, the timing of the second sampling varied, ranging from 14-35 dpt (mean = 23.3 dpt, median = 22.5 dpt, n = 36).

Figure 5.7 Map of the UK showing location of farms which participated in this study.

5.4.2.2 Farmer selection of animals

Not all farmers gave information on the sheep selected for the study, and of those that did, not all gave full information. From the information given, sheep of different ages, sexes and parities were among the animals sampled. Various breeds of sheep were reported as being sampled in this study. Cross-bred sheep included mules, Texel cross, Lleyn cross, Cheviot cross, Lleyn x Charollais, Lleyn x Suffolk, Beltex x Cheviot and Cheviot x Blackface. Pure-bred sheep sampled included Bluefaced Leicester, Texel, Poll Dorset, Blackface, Herdwick, Shetland, Romney, Cheviot, Beltex and Hebridean. One farmer attempted to classify sheep body condition score, submitting sample pots with either ‘fat’, ‘fit’ or ‘thin’ written on them.

5.4.2.3 Detection of liver fluke infection

Evidence of liver fluke infection was detected on 22 out of the 25 farms. The number of groups positive by either average or composite FEC or cELISA testing is shown in Table 5.5. The percentage of samples per group which tested positive for liver fluke

by FEC and cELISA are shown in Figure 5.8, for the group with 9 samples the percentages were rounded to the nearest 10. The group average FECs ranged from 0 to 139.8 epg (mean = 11.1 epg, median = 1.5 epg), with the lowest positive FEC being 0.03 epg. Composite pre-treatment FECs ranged from 0 to 102.5 epg (mean = 9.6 epg, median = 1.3 epg), with the lowest positive FEC being 0.2 epg. Rumen fluke eggs were detected in samples from 2 groups of sheep on 1 farm pre- and post-treatment by both average and composite FEC. In addition, on a second farm, rumen fluke eggs were detected post-treatment by average FEC only. The group average pre-treatment cELISA results ranged from -1.21% to 97.42% (mean = 22.38%, median = 8.94%), with the lowest positive cELISA being 7.71%. Composite pre-treatment cELISAs ranged from -0.53% to 106.91% (mean = 25.71%, median = 11.32%), with the lowest positive cELISA being 7.46%.

Table 5.5 Number of groups positive for *F. hepatica* infection by average or composite FEC and cELISA testing

	FEC	cELISA
Average	36	24
Composite	31	25

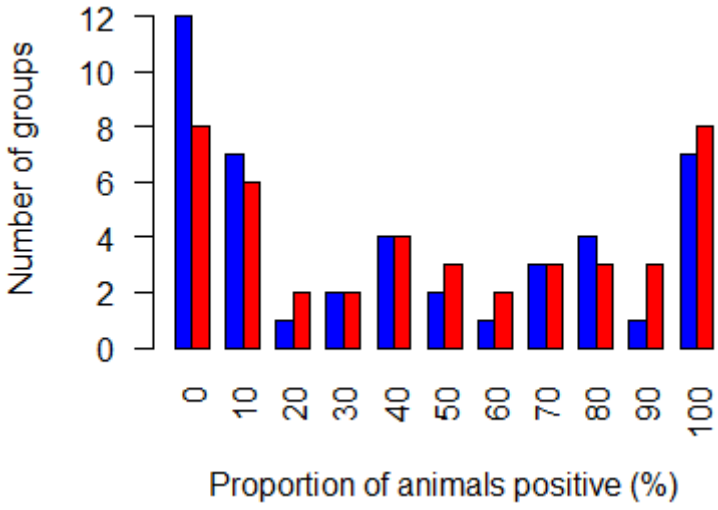

Figure 5.8 Proportions of individual samples positive for liver fluke per group by FEC (red) and cELISA (blue) pre-treatment.

5.4.2.3.1 Average vs. composite FEC

The arithmetic mean FEC (epg) was calculated for each group, pre- and post-treatment. This was compared to the composite FEC (epg) for the corresponding group (Figure 5.9). The agreement between the average and composite sampling, when determining the infection status of a group, was substantial pre-treatment (Kappa = 0.69) and moderate post-treatment (Kappa = 0.43) (Table 5.6). These low agreements by Kappa are in contrast to the high R^2 values shown in Figure 5.9. This is likely due to disagreements in low FEC samples. A difference of 0.1 or 0.5 epg between average and composite FECs will have little effect on the R^2 value but can result in a disagreement in treatment outcome, and affect the Kappa value, if one of the FECs happens to be 0 epg and the other 0.5 epg. In the 16 instances of disagreement between the two tests, all were positive by average FEC but negative by composite FEC (n = 5 pre-treatment, n = 11 post-treatment). Using the average FEC as the gold standard, the composite pre-treatment FEC had a sensitivity of 86.1% and a specificity of 100% (PPV = 100%, NPV = 61.5%) whilst the composite post-treatment FEC had a sensitivity of 47.6% and a specificity of 100% (PPV = 100%, NPV = 57.7%).

5.4.2.3.2 Average vs. composite cELISA

The arithmetic mean cELISA (PD) was calculated for each group, pre- and post-treatment. This was compared to the composite cELISA (PD) for the corresponding group (Figure 5.9). A group was considered positive for infection if the mean PD was greater than the batch cut-off of the cELISA plate used to test individual samples (as determined by the manufacturer). The agreement between the average and composite sampling, when determining the infection status of a group, was almost perfect pre-treatment (Kappa = 0.95) and post-treatment (Kappa = 0.80) (Table 5.6). In the 3 instances of disagreement between the two tests, all were negative by average cELISA but positive by composite cELISA (n = 1 pre-treatment, n = 2 post-treatment). If the average cELISA is taken to be the gold standard, the composite

pre-treatment cELISA had a sensitivity of 100% and a specificity of 95% (PPV = 96%, NPV = 100%) whilst the composite post-treatment cELISA had a sensitivity of 100% and a specificity of 93.6% (PPV = 71.4%, NPV = 100%).

5.4.2.3.3 Average FEC vs. average cELISA pre-treatment

Moderate agreement was seen between the average FEC and average cELISA pre-treatment (Kappa = 0.42) (Table 5.7), with all disagreements (n = 12, 27.3% of all groups) being FEC positive but cELISA negative. If FEC is taken to be the gold standard, the cELISA had a sensitivity of 66.7% and a specificity of 100% (PPV = 100%, NPV = 40%). If the cELISA is taken to be the gold standard, the FEC had a sensitivity of 100% and a specificity of 40% (PPV = 67.7%, NPV = 100).

5.4.2.3.4 Composite FEC vs. composite cELISA pre-treatment

The FEC and cELISA results from composite pre-treatment samples are shown in Figure 5.10. Substantial agreement was seen between the composite FEC and composite cELISA pre-treatment (Kappa = 0.71) (Table 5.7), with all disagreements (n = 6, 13.6%) being FEC positive but cELISA negative. If FEC is taken to be the gold standard, the cELISA had a sensitivity of 80.7% and a specificity of 100% (PPV = 100%, NPV = 68.4%). If the cELISA is taken to be the gold standard the FEC had a sensitivity of 100% and a specificity of 68.4% (PPV = 80.7%, NPV = 100).

5.4.2.3.5 Average FEC vs. average cELISA post-treatment

Fair agreement was seen between average FEC and cELISA post-treatment (Kappa = 0.21) (Table 5.7). Disagreement between tests was seen in 16 samples (44.4% of samples), which were FEC positive but cELISA negative. If the FEC is taken to be the gold standard, the cELISA had a sensitivity of 23.8% and a specificity of 100% (PPV = 100%, NPV = 48.4%). If the cELISA is taken to be the gold standard the FEC had a sensitivity of 100% and a specificity of 48.4% (PPV = 23.8%, NPV = 100%).

Table 5.6 Agreement between average and composite FEC and cELISA when used to determine infection status of a group pre- or post-treatment. The Kappa values are shown.

		FEC			cELISA		
Pre-treatment	Kappa= 0.69	Composite			Kappa = 0.95	Composite	
		Positive	Negative	Total	Positive	Negative	Total
Average	Positive	31	5	36	24	0	24
	Negative	0	8	8	1	19	20
	Total	31	13	44	25	19	44
Post-treatment	Kappa= 0.43	Composite			Kappa = 0.80	Composite	
		Positive	Negative	Total	Positive	Negative	Total
Average	Positive	10	11	21	5	0	5
	Negative	0	15	15	2	29	31
	Total	10	26	36	7	29	36

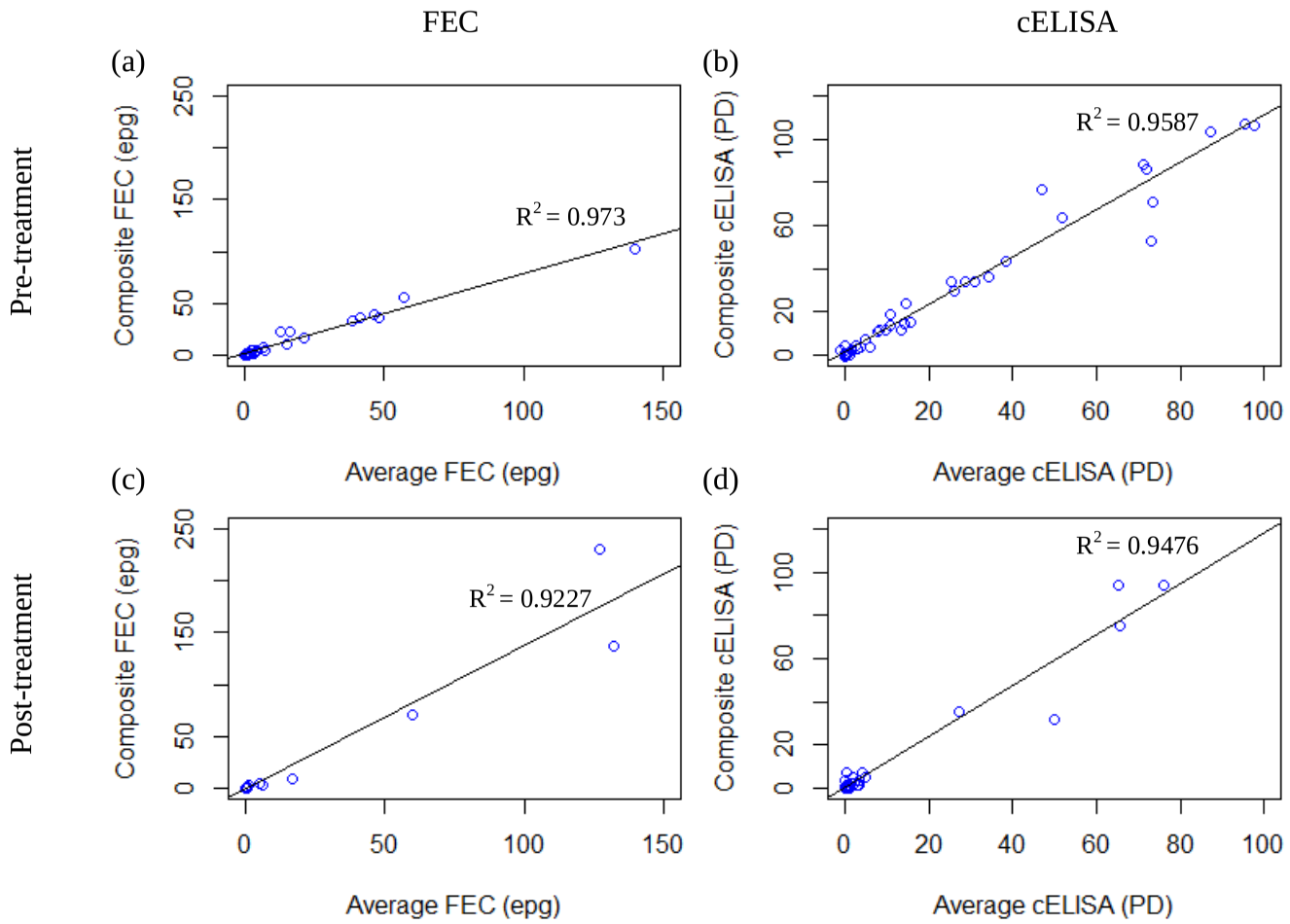

Figure 5.9 Scattergraphs showing the relationship between arithmetic mean and composite pre-treatment (a) FEC (epg) and (b) cELISA (PD), and between arithmetic mean and composite post-treatment (c) FEC (epg) and (b) cELISA (PD).

Table 5.7 Agreement between average FEC and cELISA and composite FEC and cELISA in pre- and post-treatment samples. The Kappa values are shown.

		Group average			Composite		
Pre-treatment	Kappa= 0.42	cELISA			Kappa= 0.71	cELISA		
		Positive	Negative	Total		Positive	Negative	Total
	FEC Positive	24	12	36	FEC Positive	25	6	31
	FEC Negative	0	8	8	FEC Negative	0	13	13
	Total	24	20	44	Total	25	19	44
Post-treatment	Kappa= 0.21	cELISA			Kappa= 0.62	cELISA		
		Positive	Negative	Total		Positive	Negative	Total
	FEC Positive	5	16	21	FEC Positive	6	4	10
	FEC Negative	0	15	15	FEC Negative	1	25	26
	Total	5	31	36	Total	7	29	36

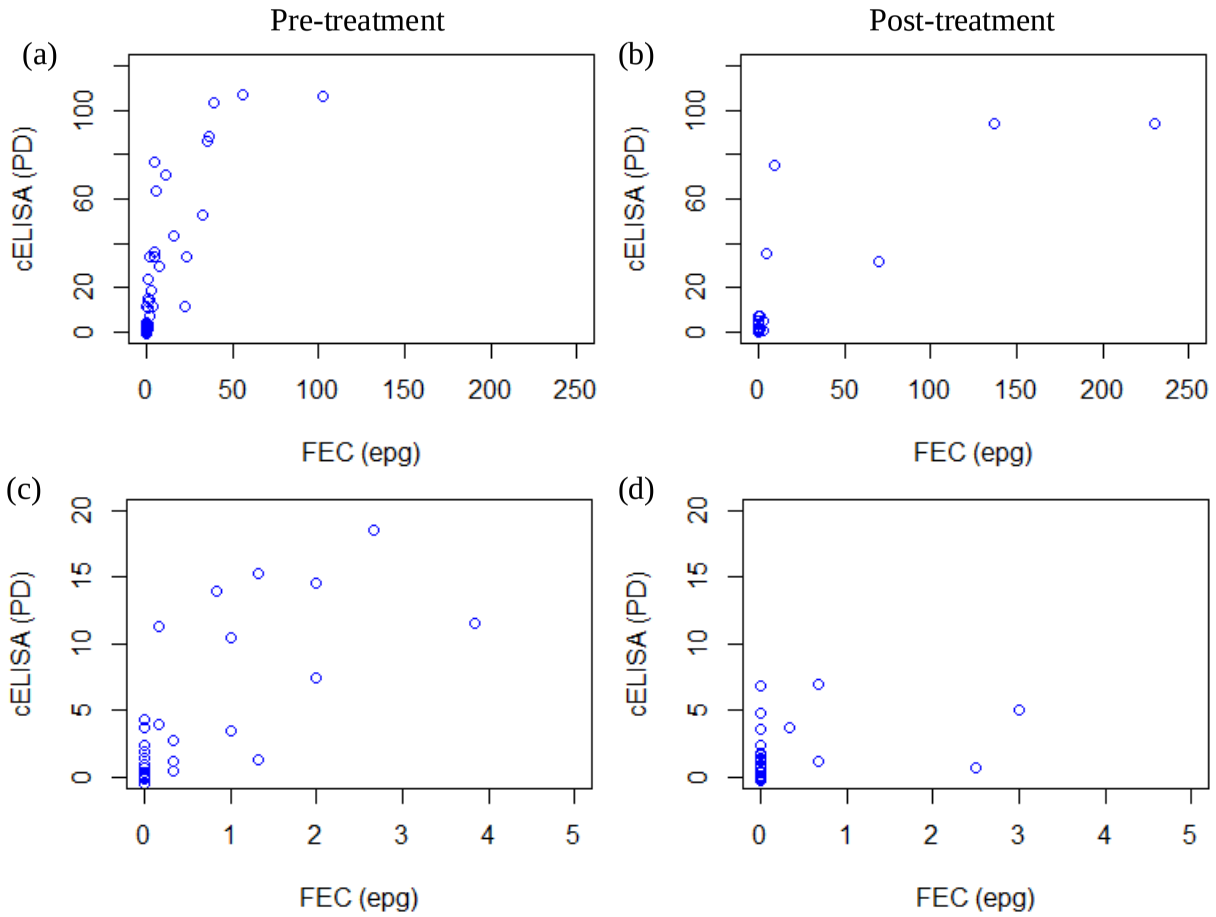

Figure 5.10 Composite FEC (epg) and cELISA (PD) results (a) pre- and (b) post-treatment with enlargement of the range of lower FEC and cELISA values (c) pre- and (d) post-treatment (epg = ≤ 20 and PD = ≤ 20)

5.4.2.3.6 Composite FEC vs. composite cELISA post-treatment

The FEC and cELISA composite results from post-treatment samples are shown in Figure 5.10. Moderate agreement was seen between composite FEC and cELISA post-treatment (Kappa = 0.62) (Table 5.7). Disagreement between tests was seen in 5 samples (13.8% of samples), 4 of which were FEC positive but cELISA negative. The composite sample from group 30 had a low positive cELISA result (PD = 6.82%, positive cut off = 6.07%) but had a negative FEC result. This was the only sample in average and composite sampling, pre- or post-treatment, to have a positive cELISA result, but a negative FEC result. If the FEC is taken to be the gold standard, the cELISA had a sensitivity of 60% and a specificity of 96.2% (PPV = 85.7%, NPV = 86.2%). If the cELISA is taken to be the gold standard the FEC had a sensitivity of 85.7% and a specificity of 86.2% (PPV = 60%, NPV = 96.2%).

5.4.2.4 Treatment of liver fluke and determination of treatment outcome

Of the 25 farmers taking part in this study, 68% reported that they used a method of weighing animals to determine treatment dosage, with two farmers submitting an indication of sheep weight (individual or a range of weights). Methods of weighing included the use of a weigh crate on some or all animals, as well as the use of bathroom scales in one instance. 12% of farmers reported that they estimated the weight of sheep by eye or by lifting, whilst 20% of farmers did not give a method of dosage calculation. The farms which did not indicate that a method was used to calculate the dosages are marked in Appendix 4.6 and 4.7.

TCBZ was the treatment of choice, used in 22 of the 44 groups, this was followed by closantel treatment used in 17 groups and albendazole used in 2 groups. Treatment choice was not disclosed by the farmers for three groups. Treatment outcomes were calculated for the 36 groups with post-treatment samples by both FECRT (based on mean and composite epg) and CRT (based on mean and composite PD). The percentage of samples positive by FEC and cELISA per group post-treatment is shown in Figure 5.11. The median FEC before treatment was 0 epg whilst the mean

was 11.1 epg. Positive post-treatment FECs ranged from 0.2 to 344.8 epg, with a mean of 9.72 epg and median of 0 epg once negative samples are also included.

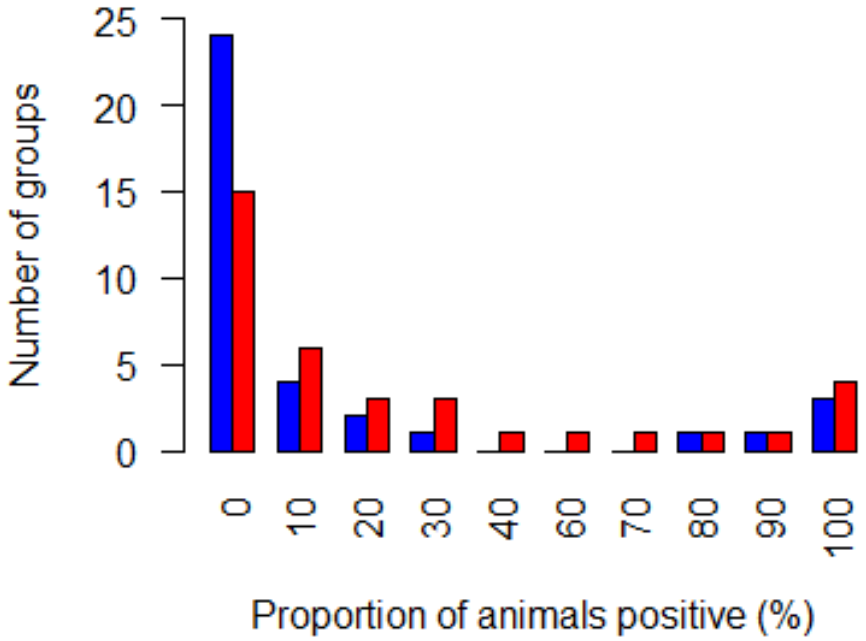

Figure 5.11 Histogram showing the percentage of individual samples positive for liver fluke per group by FEC (red) and cELISA (blue) post-treatment.

5.4.2.4.1 Treatment outcome as determined by FEC

Seven of the 36 post-treatment groups had no evidence of liver fluke infection pre-treatment, based on average or composite FEC, whilst one group had a positive pre-treatment average FEC (0.03 epg) but pre-treatment composite FEC of 0 epg. Details of the FECR in all groups can be seen in Appendix 4.

To determine the agreement between tests, only groups that were positive by average and composite FEC pre-treatment were included (n = 28), the FECR of these groups can be seen in Figure 5.12. There was substantial agreement (Kappa = 0.62) on the FECRT outcome between the two testing methods (average vs. composite) (Table 5.8). Agreement for treatment outcome was seen in 23 out of 28 groups (79%). In contrast to the results for determination of infection status, there was no clear superiority of one test over the other in terms of determining outcome.

Table 5.8 Agreement between composite and average treatment outcome by FECRT. The Kappa value is shown.

Kappa= 0.62		Composite		
		Success	Failure	Total
Average	Success	15	2	17
	Failure	3	8	11
	Total	18	10	28

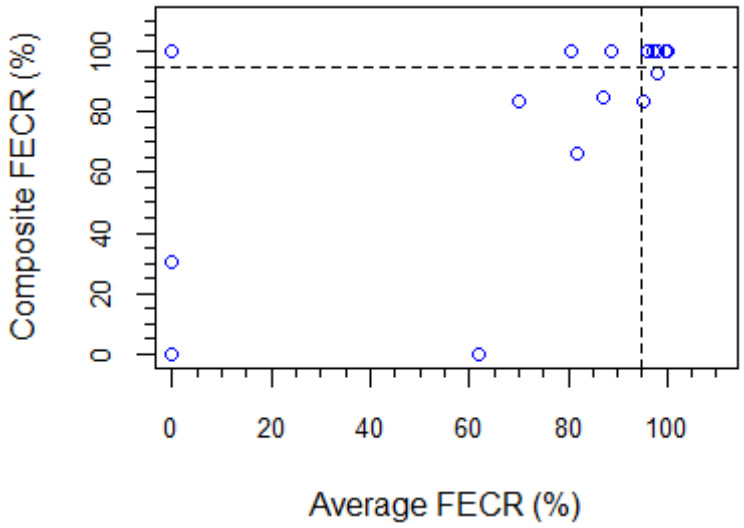

Figure 5.12 Scattergraph showing the FECR (%) by group average and composite samples. Negative FECRs are shown as 0% reduction. Only groups which were positive pre-treatment by both average and composite FEC are shown. --- indicates a 95% reduction. The number of data points appears reduced as there are 11 at 100,100 and 3 at 0,0

There was a disagreement in treatment outcome between the two sampling methods in 5 groups, the details of which can be seen in Table 5.9. Of the groups where average FECR showed that a treatment had failed but the composite FECR showed it to be successful, the average pre-treatment FECs were all <4 epg, whilst the average post-treatment FECs were <1 epg. The same was true for one of the groups with success based on average FECR but not based on composite FECR. In the final group, the composite pre-treatment FEC was 35.5 epg and the composite post-treatment FEC was 2.5 epg. In this last group, a cut-off of 90% to define successful treatment would have resulted in agreement between average and composite FECRT

results, but this was not true for the groups with low FEC prior to treatment (Table 5.10). Using the FECRT based on a group average value as the gold standard, the FECRT based on composite samples had a sensitivity of 88.2% and a specificity of 72.7% (PPV = 83.3%, NPV = 80%).

Table 5.9 Pre- and post-treatment FEC (epg), cELISA (PD), FECR and CR of average and composite results that did not result in treatment outcome agreement between sampling methods.

		Average			Composite		
Test	Group	Pre-treatment	Post-treatment	Reduction (%)	Pre-treatment	Post-treatment	Reduction (%)
FEC	3	41.4	0.9	97.87	35.5	2.5	92.96
FEC	14	3.8	0.7	80.69	2.7	0	100
FEC	19	0.7	0.1	88.89	1	0	100
FEC	35	0.1	0.2	-25	0.3	0	100
FEC	41	2.1	0.1	95.24	2	0.3	83.5
cELISA	24	14.4	-0.11	100.73	24.04	3.62	84.96

5.4.2.4.2 Treatment outcome as determined by cELISA

Fourteen of the 36 groups showed no sign of liver fluke infection pre-treatment by either composite or group cELISA. One further group tested positive pre-treatment by composite cELISA, but none of the individual samples within that group had tested positive. These 15 groups were omitted when determining the agreement between the sampling methods with regards to treatment outcome. Details of CRT outcome for all groups can be seen in Appendix 4. By the Kappa test, there was almost perfect agreement on CRT outcome between the two methods in the remaining 21 groups (Kappa = 0.90) (Table 5.10), the CRs of which can be seen in Figure 5.13. In the case of the one disagreement between average and composite CR, the composite CR was 84.96% but the average CR was >100%, however, the composite post-treatment cELISA tested negative (PD = 3.62%). If the CRT based on a group average value is taken as the gold standard, the CRT based on composite samples had a sensitivity of 90% and a specificity of 100% for determining treatment success (PPV = 100%, NPV = 91.7%).

Table 5.10 Agreement between composite and group treatment outcomes by cELISA. The Kappa value is shown.

Kappa = 0.90		Composite		
		Success	Failure	Total
Average	Success	9	1	10
	Failure	0	11	11
	Total	9	12	21

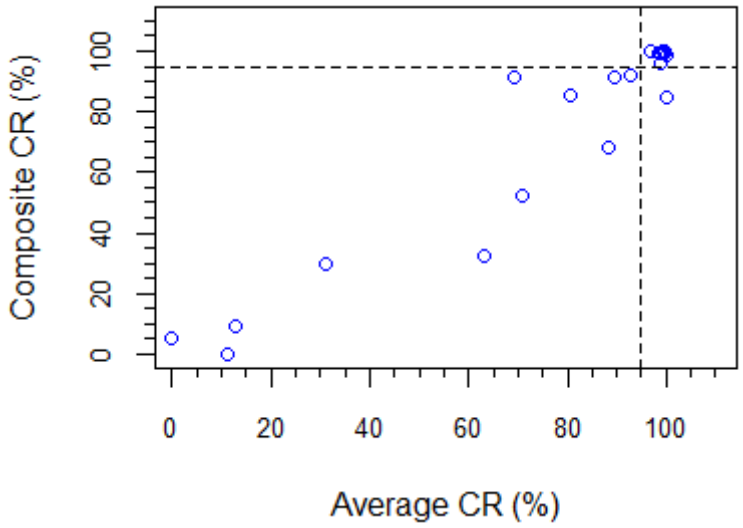

Figure 5.13 Scattergraph showing the CR (%) by group average and composite samples. Negative CRs are shown as 0% reduction. Only groups which were positive pre-treatment by both average and composite cELISA are shown. --- indicates 95% reduction.

5.4.2.4.3 Composite FECRT vs. composite CRT

Of the 36 groups for which farmers returned post-treatment samples, 22 were suitable for both FECRT and CRT. That is, these groups had both FEC and cELISA positive composite results pre-treatment. A moderate agreement was seen between composite FECRT and composite CRT, with 6 disagreements (Kappa = 0.47) (Table 5.11). Of these 6 disagreements, 5 were instances of treatment success by FECRT but treatment failure by CRT. All 5 of these groups were instances where the post-treatment PD was lower than the cELISA positive cut-off (6.07%), but the CR was <95%. The final case of treatment outcome disagreement occurred in group 3, where

the FECRT deemed treatment unsuccessful with a 93% FECR, but CRT deemed treatment successful with a 99.2% CR. If the FECRT is taken to be the gold standard the CRT has a sensitivity of 61.5% and a specificity of 88.9% (PPV = 88.9%, NPV = 61.5%).

Table 5.11 Agreement between composite FECRT and composite CRT outcomes. The Kappa value is shown.

Kappa = 0.47		CRT		
		Success	Failure	Total
FECRT	Success	8	5	13
	Failure	1	8	9
	Total	9	13	22

If the criterion for treatment success by CRT is amended to be a CR of ≥95% or a negative cELISA result post-treatment, a better agreement is seen (Kappa = 0.70) (Table 5.12). Disagreements are only seen where the composite FECRT deemed the treatment to have failed but the CRT deemed the treatment to be successful. Of the 3 disagreements, two were instances where the pre-treatment FEC was <3 epg. The final disagreement was in group 3, which is described above. If the cut-off for FECRT were set at 90%, this disagreement would be resolved. If the FECRT, using the 95% cut-off, is taken to be the gold standard the amended CRT has a sensitivity of 100% and a specificity of 66.7% (PPV = 81.3%, NPV = 100%).

Table 5.12 Agreement between composite FECRT and amended composite CRT outcomes. The Kappa value is shown.

Kappa = 0.70		CRT		
		Success	Failure	Total
FECRT	Success	13	0	13
	Failure	3	6	9
	Total	16	6	22

5.5 Discussion

5.5.1 Farmer compliance and practicalities

This chapter had three main aims (1) to evaluate the CRT in sheep naturally exposed to *F. hepatica*, as part of a treatment trial; (2) to evaluate the use of a composite cELISA for detecting *F. hepatica* infection status and determining treatment outcome in UK sheep flocks, in the form of a pilot study using group average as the gold standard; and (3) to provide a preliminary assessment of farmer compliance with the proposed composite sampling protocol for diagnosis and treatment evaluation.

In the evaluation of the CRT, although the sampling team performed all samplings, only 29 out of the 68 animals (42.6%) on Farm 9 were successfully sampled each week. This is in contrast to a >98% successful sampling rate at Farm 22. Both teams were led by Danielle Gordon-Gibbs, with pre-labelled sampling pots and an animal number check list for each sampling at each farm. The main difference between farms was the lengths to which the farmers had gone in order to facilitate the study. On Farm 22, animals were housed for the duration of the study, allowing for all to be rounded up for sampling. Farm 22 also had the facilities for animals which were 'empty' (i.e. no faeces could be retrieved from the rectum) to be penned until all other animals had been sampled. These animals were then re-sampled for faeces, at which point a sample could be retrieved. The one animal from Farm 22 which could not be included in the study was excluded as a result of a lost ear tag, rendering the animal unidentifiable. This also occurred on Farm 9, but in more animals.

On Farm 9, due to the impracticalities of penning 'empty' animals, insufficient faecal samples were often taken. The composite sampling protocol aims to overcome this problem by collecting 10 faecal samples from the floor of a pen containing 12 animals (Daniel et al., 2012). In addition, in the composite sampling study, a 'fill-to' line was also marked on each sampling pot to ensure that sufficient sample was collected. Despite this, some farmers collected samples from 10 specific animals and some were more creative in the choice of packaging of faecal samples; foregoing the supplied marked pots in favour of food bags, medicine pots and gloves. This reliance

on specific animals and alternative sample containers may have contributed to the submission of insufficient samples.

Although 80% of farmers in the composite sampling evaluation reported that animal weight was used to determine treatment dosage, this does not guarantee that animals were correctly dosed. The methods used by farmers most frequently indicated that all animals were dosed to the heaviest, and that the weight of the heaviest animal was determined using inaccurate methods such as estimating by eye, lifting animals or use of bathroom scales. Indeed, only 2 farmers gave data of the weights of animals, one which gave a range and a second which gave individual animal weights. This of course allows for potential under dosing of animals in this study, which is an inherent risk when sampling teams are not used. However, as the purpose of this particular study was to compare FEC and cELISA data, rather than to investigate instances of possible flukicide resistance, the data is still valid for this purpose.

Sample timings were very much controlled in the evaluation of the CRT. In the composite sampling evaluation, a post-treatment sampling date was communicated to farmers. Despite this, sampling times did vary between 14 and 35 dpt. This variation is no doubt related to the most convenient time for the farmer to gather his/her animals. As the study specified post-treatment sampling at 21 dpt, it is not possible to know when farmers would have re-sampled if no guidance had been given. A convenient sampling date would have been an interesting addition to the sampling questionnaire. It is also possible that the specification of a 21 dpt sampling was off-putting to farmers and could have contributed to the reduction in participating groups post-treatment.

Of course, allowing farmers to return post-treatment samples at any point is not always practical. One farmer returned post-treatment samples over 56 dpt; these were not included in the evaluation of the composite sampling. As flukicides typically have no residual or persistent therapeutic effect, a new infection can be picked up from the time the animal is returned to pasture. Diagnostic test results obtained at a 21 dpt sampling point would not be affected by re-infection as cELISA can only detect from 4 wpi whilst FEC can detect from 9 wpi (Chapter 3). This means that at

56 dpt (8 wpi), animals may be positive despite earlier successful treatment if they subsequently acquired a new infection.

An interesting observation in the composite sampling evaluation study is the number of groups which were treated but had no signs of fluke infection. This occurred in 7 of the 36 groups for which farmers returned post-treatment samples. Of the 8 groups for which post-treatment samples were not returned, one had no evidence of fluke infection by average FEC, 6 had average FECs of <3 epg whilst the last group had an average of 16.32 epg. The cELISA and composite data were not returned to farmers. It is a common belief that farmers treat for fluke without evidence due to the perception that it is cheaper than testing animals and then treating combined with the knowledge that a FEC cannot detect early infection (Besier and Love, 2012). Indeed, sample testing can be very expensive. A farmer's time is also valuable. In order to have evidence of infection, animals must be brought in to be sampled. The farmer must then wait for results, likely having to return the animals to grazing or incur further feed costs, and then gather the animals a second time for treatment. This extra animal handling is often unattractive to farmers but cannot be avoided unless a pen-side test is developed.

Indeed, in the study to evaluate the composite sampling option, free testing was offered to 41 farmers, 26 of whom had volunteered for the study. Only 25 farmers returned samples, indicating that cost of testing was not the only factor preventing them from seeking evidence of fluke infection. Although non-evidence-based treatment may at first appear financially attractive to farmers, it may be detrimental to animal health if other illnesses are not considered and, as such, result in increased costs for treatment of other conditions or even loss of stock down the line.

Results with respect to farmer behaviour support the need for a test that is more sensitive than FEC in detection of pre-patent infections, e.g. cELISA as reported in the literature, the need for simple protocols and cheap testing options, such as composite testing, as well as the development of convenient testing procedures, such as pen-side testing.

5.5.2 CRT

Biologically, early in infection (>4 wpi), it would be expected that FEC would be negative (pre-patent infection), but cELISA would be positive (Mezo et al., 2004). Once an infection had become patent, it would be expected that there would be no disagreement between the two tests, as long as an effective treatment had not been implemented. If treatment was successful, it would be expected that the FEC may remain positive, due to the release of sequestered eggs from the gall bladder being released, albeit with a FEC which is much lower than pre-treatment, whilst the cELISA would test negative.

The predicted agreement between FEC and cELISA at different stages of infection is based on the biology of infection and the principle of the diagnostic assay, whereby cELISA can detect coproantigen from immature as well as mature fluke, whilst FEC can only detect mature fluke that excrete eggs. This should result in a higher sensitivity of cELISA compared to FEC. This does appear to be the case in early infections in experimental challenge situations, where single high doses of infective metacercariae are given, but it does not appear to be the case in natural infections, where a trickle infection over time is more common. This difference in cELISA performance, dependent on whether an infection is natural or experimental, has been shown in Chapters 3 and 4, as well as in published reports on the performance of the cELISA in individual sheep and CRT (Flanagan et al., 2011a; Flanagan et al., 2011b; Gordon et al., 2012b; Mezo et al., 2004).

The predicted post-treatment agreements described above are based upon a successful treatment. If the treatment was unsuccessful, and the infection was patent, perfect agreement would be expected. This was seen on Farm 22, in the TCBZ-treated group, but not on Farm 9, where some issues arose in regards to animal identification.

The FECRT was developed for gastrointestinal nematode (GIN) infections, and the guidelines state that the group mean pre-treatment FEC should be ≥150 epg (Coles et

al., 1992). By this criterion, neither farm would be suitable for a FECRT to be carried out. It is worth noting that in *F. hepatica* infections FECs >150 epg are uncommon, whilst a GIN FEC of <150 epg would be considered to be low. As such a new minimum FEC should be proposed in the development of a *F. hepatica* FECRT, this would take into account the lower FECs seen in *F. hepatica* infections than in GIN infections. In this study low mean FECs were seen on both farms, which follow on from the low individual FECs in natural infections commonly seen in Chapter 4. Although a ≥95% FECR is commonly used to determine successful treatment, it is a somewhat arbitrary cut-off. Indeed, in nematode infections, the 95% is used to indicate how efficacious the treatment has been, as egg shedding ceases once a treatment has been successful. Due to the sequestering of eggs that occurs in trematode infections, it can only be used to indicate a reduction in egg shedding, which is not directly linked to the number of parasites remaining in the liver, at least until the sequestered eggs have been released. Other cut-offs have been used, such as a 90% reduction (Brockwell et al., 2013; Levecke et al., 2012; McKenna, 1994). Indeed the Australian Pesticides and Veterinary Medicine Authority recommends the use of a >90% cut off for the FECRT when used to evaluate flukicides in ruminants (APVMA, 2001).

The biology of *F. hepatica* infection results in eggs being sequestered in the gall bladder, and shed in small number for weeks after a successful treatment has been given (Valero et al., 2002). This was seen in the closantel-treated groups of the CRT evaluation on both farms, with the number of animals shedding eggs decreasing as the study progressed. However, at 35 dpt and 56 dpt, the proportion of closantel-treated ewes shedding eggs increased. This may be due to immature fluke which were not targeted by closantel now maturing and producing eggs, or due to the sporadic shedding of eggs from the gall bladder.

If, at the time of treatment, immature fluke are present that are not killed by the treatment product, or a sub-effective dose was given, diagnostic results at a 21 dpt sampling point or later could be affected by the maturation of fluke which were present but not killed at the time of treatment. In this situation, the CRT should still

be able to give an accurate indication of treatment outcome, whereas a FECRT would indicate successful treatment despite the presence of pre-patent fluke.

On Farm 9 at 14 dpt, an 83.7% FECR was seen in the closantel-treated group. This would indicate treatment failure and is a worrying result at face value. When the group is observed at the individual level it becomes apparent that this low FECR is the result of a single animal with a FEC of 49.7 epg. This animal also had a similarly high cELISA OD at the same sampling point, but tested negative by cELISA and had a very low FEC at 7, 21, 35 and 56 dpt. The negative results at all post-treatment samplings, other than 0 and 14 dpt, make it unlikely that the animal was under-dosed through poor calibration or partial ingestion of the dose. A mix-up of samples within the laboratory is also unlikely as no animals in the TCBZ-treated group had a low FEC or negative cELISA result at this sampling point and no other fluke studies were ongoing at the time. Animal identification on Farm 9 had been difficult and it is highly likely that this 14 dpt sample was taken from a different ewe, with a similar identification number, and the lowered FECR is a result of animal misidentification rather than closantel treatment failure. One other point to note is the low number of animals used in analysis from the closantel-treated group of Farm 9 (n = 15). If the mean FEC from all closantel-treated animals sampled at 0 and 14 dpt on Farm 9 are used in the FECRT the reduction is higher at ~91%.

The Farm 9, closantel-treated, animal which was strongly positive by FEC and cELISA at 14 dpt is also likely responsible for the reported closantel treatment failure by the CRT with a CR of 94.9%. However, this animal was not the only one which had fluctuating cELISA results. This also occurred in different animals on Farm 9 at 21, 35 and 56 dpt. Whilst it is possible that these positives at 35 and 56 dpt are due to the maturation of immature fluke which had not been targeted by closantel, it is unlikely in the case of the 21 dpt sampling due to the fact that the animals then tested negative at a later date. This phenomenon has been reported before and it was suggested that these ‘positive blips’ could be due to the breakdown of successfully killed fluke releasing coproantigen (Flanagan et al., 2011a). However, this does not seem likely at 21 dpt. It is worth noting that no fluctuations were seen on Farm 22, where animals were housed and animal identification was not

problematic, until 56 dpt in the closantel-treated group, which may well have been the maturation of immature fluke which are not targeted by closantel.

Flanagan et al. (2011b) has suggested a CRT which has an 'all or nothing' criterion. That is, if any animal is cELISA positive at the post-treatment sampling the treatment is deemed to have failed. This is based upon the claim that a cELISA positive result can only be due to live fluke being present in the animal, although in the original paper detailing the cELISA, it was reported that coproantigens were released for 1-3 weeks post-treatment (Bio-X, 2010; Flanagan et al., 2011b; Mezo et al., 2004). Despite this criterion, in the paper where the CRT was suggested and in a subsequent paper by the same authors, one of the six animals tested positive at both 7 and 14 dpt in an experimental challenge study (Flanagan et al., 2011a; 2011b). This is also seen in the original cELISA report by Mezo et al. (2004) where one of the six animals continued shedding coproantigens until 3 wpt and was reported by Flanagan et al. (2011b) as being most likely due to the release of coproantigens from dying fluke. The use of an 'all or nothing' criterion in light of these 'anomalous' results in a sample size of 6 animals is worrying indeed.

For the CRT presented in this study, the same criterion was used as for the FECRT. This cut-off has been arbitrarily chosen and will need further evaluation. In the CRT evaluation, on two occasions the closantel-treated group would have been deemed to have had treatment failure, based upon the ≥95% cut-off (94.9% and 94.3%). A ≥90% reduction criterion for the CRT would have given better agreement with the FECRT. However, if the criterion for a successful treatment is lowered, it may reduce the specificity of the test, allowing treatment failures to be wrongly identified as successful treatments. The CRT will need further evaluation in situations where the treatment outcome is known in order to truly understand the dynamics of coproantigen shedding post-treatment and determine a suitable CR cut-off.

Another suggestion for the improvement of the CRT would be to use an actual reduction criterion such as ≥95% but also have treatment automatically deemed successful if the post-treatment cELISA has a negative result. In both cases of apparent closantel treatment failure mentioned above, the mean OD would return a

negative cELISA result. These new criteria of ≥95% CR or an OD/PD value which returns a negative cELISA result would have indicated successful treatment. The downside of such criteria would be that if a large number of animals are sampled, i.e. >10 animals, and a low number have a very low positive cELISA result, it may be diluted down so that the mean OD/PD is a negative cELISA result. These criteria would be suitable for the average or composite CRT presented in this chapter.

From the evaluation of the CRT on Farm 9 and Farm 22, it can be seen that the CRT can give a good indication of treatment outcome from 7 dpt but the use of an 'all-or-nothing' criterion or the use of a ≥95% CR in isolation may return inaccurate results.

5.5.3 Composite sampling

The evaluation of a composite sampling strategy for cELISA testing followed the same protocol for the collection of samples as developed by Daniel et al. (2012), using 10 samples from a group of 12 animals.

At both the pre- and post-treatment samplings, disagreements between the average and the composite FEC result were always in the same direction i.e. where the average was positive but the composite sample was negative. This was not surprising considering the low mean FEC seen in this study. It is already known that fluke eggs are not distributed evenly in faeces (Hall, 1982). Samples were mixed with a spatula, with all pellets broken up, prior to sub-sampling for FEC in order to try to overcome this potential problem. Without the addition of water, it is not possible to ensure that the faeces used in tests have come from a truly homogenous mixture. Thus, taking 1 g from each sample limits the possibility of selecting an egg in samples with low epg. This results in the potential for a lowered sensitivity in composite testing and, indeed, this is what was seen in this study.

In the paper which proposed the composite sampling strategy, Daniel et al. (2012) performed FECs on the entire 50 g composite sample and calculated the number of eggs per 5 g. This counting of a larger volume of faeces will increase the sensitivity of the FEC but may not be practical for routine laboratory use. In addition, this

composite eggs per 5 g was compared against the average when counting between 20 and 40 g of faeces from 10 individual animals. This is a smaller volume of faeces per animal than is viewed for the composite, which may explain the improved performance of the composite FEC in the original paper. Lastly, composite FECs presented by Daniel et al. (2012) were higher than those presented in this study, ranging from 0 to 585.8 epg (mean = 58.3, median = 9.6).

The agreement between average and composite FECs was better pre-treatment than post-treatment in this study and this is again likely to be due to the very low epg seen in the post-treatment samples, a result of successful treatments. Further investigation may yield a better agreement in those groups where treatment failure occurred.

In the cELISA samples, it is interesting to note that the opposite was true. That is, the disagreements in cELISA test results between the average and the composite samples all occurred where average samples were negative but composite samples were positive. In this situation, it is likely to be a form of dilution in the average samples as a result of the way the test results are reported. In FEC, if a single sample is positive then the mean epg will be positive, resulting in a positive group result. However, if a single sample is cELISA positive, but the other 9 are cELISA negative, with a negative PD value, it is likely that the group mean cELISA PD will indicate a negative result, giving the group a negative infection status, especially if that single positive sample has a low PD.

This dilution of a single positive animal is not seen in a composite sample as only one result is reported. As such, it would appear that the composite cELISA is more sensitive than the average cELISA if only the mean PD is taken into account. If a smaller group is used to form the composite sample this dilution effect may not be an issue. Indeed in cattle samples Brockwell et al. (2013) found that 1 positive cattle sample could be detected by cELISA in a composite with 4 negative samples. If the infection status of each animal is taken into account when determining group infection status, the sensitivity of the average cELISA may increase. It is worth noting that less disagreement was seen between the average and composite cELISA results than between the average and composite FEC results. This is likely due to

coproantigens being more evenly distributed within the faeces, or being more abundant in the faeces than eggs.

Farm 7, used in the composite sampling evaluation, is worth an individual mention. Neither group on this farm tested positive by cELISA, nor were any individual samples cELISA positive. In contrast, low positive FECs were seen in both groups by average and composite testing. The cELISA was repeated, with the same supernatant and with a freshly made supernatant, and the samples did have a second FEC carried out. The initial results were confirmed. Although rumen fluke eggs had been seen in samples from some farms, they were not present in the samples from Farm 7. It is not clear why the cELISA was unable to detect *F. hepatica* infection on this farm. One explanation could be that a treatment had been given to animals prior to the study starting, however, if this was the case, it was not disclosed by the farmer. This could possibly represent a population- or strain-specific lack of reactivity with the MM3 MAb in the cELISA kit. To determine if it was a true characteristic of the strain of *F. hepatica* present on Farm 7, further work would need to be carried out, including FEC and cELISA testing of animals other than those included in this study. Unfortunately, there was no chance to return to this farm for further investigation.

In the composite CRT evaluation, low mean and composite FECs are seen, making many groups unsuitable for a FECRT by the criteria adopted for nematodes (Coles et al., 2006). This was also seen for the two farms used in the CRT evaluation study. The composite CRT, using a ≥95% CR for successful treatment, has the same specificity issue as the average CRT i.e. groups with no evidence of fluke infection post-treatment had <95% CR and, as such, were deemed to have had treatment failure. This is again due to the fact that for cELISA, a result that indicates no fluke can have a positive value (>0), whereas a FEC can be no lower than 0. If a pre-treatment FEC is positive and a post-treatment FEC is negative (0 epg), the FECR will automatically be 100%, but this is not the case in cELISA as a negative result, indicating no fluke infection, can have a positive PD value. This is illustrated by the composite samples in group 24. This group had a pre-treatment PD of 24%, which is not unusually low, but had a post-treatment PD of 3.6%. Whilst the post-treatment PD indicated that no fluke were present, the CR was only 84.96%.

If the criteria of a ≥95% CR, together with a negative cELISA result, are used to determine treatment success, a near perfect agreement between average and composite CRT is seen. The only disagreement is in group 15, where the treatment is successful according to average CRT but has failed according to composite CRT. The number of successfully treated groups also rises from 9 to 15. A good agreement is also seen between the amended composite CRT and the composite FECRT with only 3 disagreements where composite FECRT indicates treatment failure but composite CRT indicates treatment success. In two of these cases the pre-treatment FEC is low, <5 epg. When there are low pre-treatment FECs, the release of sequestered eggs can result in low FECRs in the case of successful treatment. The last disagreement had a reasonable pre-treatment FEC (35.5 epg) but only achieved a 93% FECR, the average FECRT did indicate treatment success. It is also worth noting that the 95% FECR cut-off is based on nematode infections and that use of 90% cut-off has been suggested to give a truer indication of treatment outcome (APVMA, 2001; Brockwell et al., 2013; Levecke et al., 2012; McKenna, 1994). The 95% FECRT cut-off may require optimisation for *F. hepatica* FECRT.

The usefulness of the composite CRT may be limited by the lower sensitivity of a composite cELISA in comparison to composite FEC. Twenty-eight groups could be analysed by FECRT, that is they were positive by average and composite FEC pre-treatment. Only 21 groups were positive by average and composite cELISA pre-treatment and thus could be analysed by CRT. Of the 7 groups which were analysed by FECRT but not CRT, 6 had composite FECs of ≤1.33 epg, the remaining group had no evidence of fluke by average cELISA and the average FEC was 2 epg.

5.5.4 Conclusion

In conclusion, from initial evaluation, the CRT is a promising method to allow for the early determination of treatment outcome in naturally infected flocks of sheep. However, optimisation of test interpretation criteria to define treatment success is needed. Whilst composite sampling may appear attractive, to increase the uptake of evidence-based treatment decisions by farmers, care should be taken as to what

situations it is used in. In farms with a low prevalence of infection, or low fluke burdens, the composite FEC may not perform well. Composite CRT is a promising alternative to composite FECRT but may be limited by poor sensitivity of the cELISA in low burden situations. Once an infection has been detected pre-treatment by composite cELISA, the composite CRT performs very well in comparison to composite FECRT. Further optimisation of the criteria for a positive cELISA result would also be useful. Lastly, despite efforts by the scientific community to make diagnostic tests for *F. hepatica* more rapid, sensitive, specific, affordable and convenient for farmers, effort is still needed on the part of farmers. If farmers will not submit samples for testing, even if the testing is free, it will not matter how useful the tests are. More communication with the farming community, knowledge exchange to advertise the advances that have been made and to understand the needs of farmers are needed to ensure that evidence-based treatment can become common practice.

Chapter 6: Non-invasive diagnosis using DNA-based methods

6.1 Abstract

The currently available diagnostic tests to detect *Fasciola hepatica* infection all have their limitations. Faecal egg counting (FEC) can only detect a patent infection and samples may be falsely labelled positive due to the presence of rumen fluke eggs or the release of sequestered eggs following a successful treatment. Detection of GLDH and GGT enzyme concentrations are not specific to liver fluke and there is lack of clarity on normal levels. Anti-*Fasciola* antibody ELISA (AbELISA) testing cannot distinguish between a current or historic infection. The coproantigen ELISA (cELISA) appears to detect early or low levels of in infection in experimentally challenged animals only, but not in naturally exposed animals. A rapid and reliable test for detecting *F. hepatica* infections is needed. A faeces-based DNA detection method may fill this niche. This chapter aims to (1) assess the analytical specificity of existing PCR and LAMP assays for *F. hepatica*, (2) if required, develop a *F. hepatica*-specific faeces-based LAMP assay, and (3) evaluate the clinical sensitivity of PCR and LAMP based assays using samples obtained from a challenge study (0 to 17 weeks post-challenge (wpc)), and compare these with FEC and cELISA results from the same samples.

To assess the analytical specificity of primer sets, a number of liver fluke, rumen fluke and gastrointestinal nematode DNA samples were used. The published ITS-2+ and IGS LAMP primer sets were found to lack analytical specificity because they amplified *C. daubneyi* as well as *F. hepatica* DNA, necessitating the development of new LAMP primers. The Cox_1 and the Fh ITS-2 primer sets were found to amplify *F. hepatica* DNA but not rumen fluke DNA. DNA extracts from 17 ovine faecal samples were used to develop a LAMP assay and to implement PCR assays. PCR amplification of DNA extracted from faeces was inconsistent regardless of the primer set used. This inconsistency was seen to a lesser extent when DNA was amplified using the LAMP assay. The LAMP assay performed best when an incubation time of 2 hours at 61°C was used, with a 2 minute 80°C stop. When evaluated with samples from a challenge study, the LAMP assay had the earliest

detection (3 wpc), followed by cELISA (7 wpc), FEC (10 wpc) and PCR (13 or 14 wpc depending on the choice of primer set). However, the number of animals testing positive by the LAMP assay fluctuated between sampling points. In conclusion, a faecal *F. hepatica* LAMP assay has been developed which can detect infection from 3 wpc and does not cross-react with rumen fluke DNA. Further work to characterise the analytical specificity and to improve the sensitivity of this assay is needed.

6.2 Introduction

Acute and sub-acute fasciolosis, caused by the immature stages of *Fasciola hepatica*, both have a significant clinical impact on sheep, often resulting in sudden death. A rapid, simple diagnosis in the live animal can be difficult due to the absence of fluke eggs in faeces. A robust and reliable diagnostic test for acute/sub-acute fasciolosis is an urgent industry need, especially for sheep farmers, and has become a research priority. An early diagnosis may be made when using a serum anti-*Fasciola* antibody ELISA (AbELISA) or a coproantigen ELISA (cELISA), but both these tests can be time consuming and expensive, and, in the case of AbELISA, obtaining samples is invasive, requiring a veterinarian to take the sample.

The faecal egg count (FEC) is unable to diagnose a pre-patent infection, by definition, but is routinely used to diagnose chronic fasciolosis. However, intermittent shedding of eggs and the recent emergence of rumen fluke, which have morphologically similar eggs to *F. hepatica*, reduces the reliability of FEC (Gordon et al., 2013; Valero et al., 2011).

The cELISA has been evaluated within this thesis and was found to perform well in experimental challenge situations, but poorly in naturally exposed animals, in terms of detecting the onset of infection in individual animals and in detecting existing infection at both the individual and group level (Chapters 3, 4 and 5). As such, it is not a reliable diagnostic test in naturally exposed animals.

DNA-based assays have the potential to offer specific and sensitive diagnosis of *F. hepatica* infection. PCR is known to be inhibited by substances within faecal

samples, making amplification difficult (Wilson, 1997). Despite this, PCR performed on DNA extracted from faecal samples has been used to identify *F. hepatica* infection from 2 weeks post-challenge (wpc) in sheep (Martínez-Pérez et al., 2012; Robles-Pérez et al., 2013). This approach does indicate that liver fluke DNA can be detected in host faeces before eggs appear and, therefore, has obvious and attractive potential from a diagnostic standpoint. Even so, the cost, requirement of expensive laboratory equipment and lengthy DNA extraction and amplification procedures make it impractical for routine diagnosis.

Loop-mediated isothermal amplification (LAMP) is a new technique, which specifically amplifies DNA under isothermal conditions, rather than the thermal cycling required for PCR. LAMP assays are typically set up in a single tube, and can produce results in as little as 45 minutes, and these can be read by eye without the need for sophisticated equipment (Notomi et al., 2000). The use of 3 sets of primers allows for very specific amplification of even low concentrations of DNA. LAMP is also less sensitive to inhibitory substances typically found in faecal samples than PCR (Mukhopadhyay et al., 2012). Due to the nature of LAMP, the turbidity of the reaction mix, resulting from successful amplification, can give a visual indication of the amount of DNA amplified; this can also be visualised using coloured or fluorescent dyes.

There is also the potential for LAMP to be developed to a pen-side test. In order for LAMP to be used as a pen-side (or bed-side) test it would require simplification and standardisation of DNA extraction and assay set-up, including the lyophilisation of the LAMP reagents, and a suitable detection method which could be translated onto a lateral-flow device or similar (Njiru, 2012). The company which produces the LAMP reagents, MAST Group Ltd, is currently developing a version of the kit which uses lyophilised reagents and that may yield results in as little as 10 minutes (I. McElarney, personal communication to P. Skuce, 2014). Lateral flow devices have been used to detect parasite, bacterial and viral DNA amplified by LAMP assays (Kiatpathomchai et al., 2008; Njiru, 2011; Rigano et al., 2010).

A LAMP protocol has recently been published, which successfully differentiates *F. hepatica* from *F. gigantica* using DNA extracted from adult parasite and egg samples (Ai et al., 2010b). A faeces-based LAMP assay, which uses a simple freeze/thaw DNA extraction protocol, has been developed for canine parvovirus, but has not yet been evaluated for use with trematode infections (Mukhopadhyay et al., 2012).

This chapter aims to evaluate, and if required develop, faeces-based PCR and LAMP assays to detect liver fluke infection, assessing both the analytical sensitivity and specificity, using a range of trematodes and gastrointestinal parasites, and the clinical sensitivity, using material obtained through a challenge study.

6.3 Materials and Methods

6.3.1 Primer evaluation

6.3.1.1 Parasite material

Control *F. hepatica* genomic DNA was obtained from a single parasite retrieved from the liver of animal A1074 (Chapter 3). Sequencing of the ITS-2 region confirmed species identity. *F. gigantica* control DNA was obtained from a single parasite kindly donated by Dr Alan Trudgett, Queen's University, Belfast. The parasite originated from Chennai, Tamil Nadu, India. Species identification was based on sequencing of the ITS-2 region. *Dicrocoelium dendriticum* control DNA was obtained from several parasites collected on the Isle of Coll in the Outer Hebrides, these were a generous gift of Mr. Graham Baird, SAC C VS, Perth. Species identification was based on sequencing mitochondrial DNA.

Calicophoron daubneyi (rumen fluke) control DNA was obtained from a single parasite which had been stored in an unknown concentration of ethanol. It had been retrieved from a 47 month-old Aberdeen Angus cow in 2009 and was kindly donated by Helen Carty, SAC C VS, Ayr. *Paramphistomum cervi/leydeni* control DNA was obtained from a pool of 6 parasites retrieved from a Slovakian red deer and kindly donated by Dr Marian Varady, Parasitological Institute, Slovak Academy of Sciences, Kosice. Nucleotide sequencing of the PCR amplicon in plasmids purified

from three transformed bacterial colonies revealed two *P. cervi* whilst one was identified as *P. leydeni*.

Preparation of material for sequencing was performed by Danielle Gordon-Gibbs, Stuart Dawes and Gillian Mitchell (MRI) as described in Gordon et al. (2013).

Gastrointestinal nematode (GIN) DNA samples were supplied by Lynsey Melville (MRI) and comprised of *Haemonchus contortus, Trichostrongylus vitrinus, T. axei, T. colubriformis, Teladorsagia circumcincta, Cooperia curticei, Chabertia ovina* and *Nematodirus battus*. All samples were ethanol fixed and morphologically speciated by staff at Moredun Research Institute, UK (MRI).

The aim of using this reference collection was to establish the analytical specificity of the primers for *F. hepatica*, i.e. to confirm that they would amplify DNA from *F. hepatica* only and not from other liver fluke species, rumen fluke species or GIN species that might be present in faecal samples of animals at risk of liver fluke.

To assess the analytical sensitivity of PCR, i.e. the range of amplifiable DNA template concentrations, a dilution series was created from the *F. hepatica* control DNA described above. The undiluted DNA was at a concentration of 200 ng/µl, and the dilution series ran from 10^{1} ng/µl to 10^{-7} ng/µl.

6.3.1.2 DNA extraction

DNA was extracted from the parasites described in Section 6.3.1.1 using the DNEasy® Blood and Tissue kit (Qiagen) as per the manufacturer's guidelines. Full details of the protocol can be seen in Section 2.5.1 and Appendix 1. Extracted DNA was tested on a NanoDrop 1000 Spectrophotometer (Thermo Scientific) to determine quantity and purity of extracted DNA.

6.3.1.3 DNA amplification

6.3.1.3.1 Primers

Previously published primers were used in this chapter. These included a PCR primer set based upon *F. hepatica* mitochondrial DNA; Cox_1 (Martínez-Pérez et al., 2012), a PCR primer set based upon the trematode ITS-2 region; ITS-2+ (Itagaki et al., 2003), and a *F. hepatica* LAMP primer set, IGS LAMP (Ai et al., 2010b). Three LAMP primer sets were also designed for this chapter by the MAST Group Ltd, using their proprietary Primer Explorer software; two of these primer sets were based upon the ITS-2 region of *F. hepatica* and of *C. daubneyi;* Fh ITS-2 and Cd ITS-2, respectively. The full sequences of all primers used in this chapter can be seen in Table 6.1.

Table 6.1 Primer sequences for detection of liver fluke (Cox_1, ITS-2+, IGS LAMP, Fh ITS-2) or rumen fluke (Cd ITS-2, Cd MAST) by means of PCR or LAMP assay.

Identifier	Target species	Target region	Assay	Primer name	Primer sequence (5' - 3')
Cox1 (Martínez-Pérez et al., 2012)	*F. hepatica*	mtDNA	PCR	Cox_1F	GTTGGCATATTGCGGCTTAG
				Cox_1R	AGGGATCTGCACCTCAACTC
ITS-2+ (Itagaki et al., 2003)	*F. hepatica*	ITS-2	PCR	ITS-2 F	TGTGTCGATGAAGAGCGCAG
				ITS-2 R	TGGTTAGTTTCTTTTCCTCCGC
IGS LAMP (Ai et al., 2010)	*F. hepatica*	IGS	LAMP	IGS F3	CATTACCGACTCAGCTTGCA
				IGS B3	ACCAAACGTTCGGTTAAGGT
				IGS FIP	GCCGAATCAACCAGCCCTGAAAATGACGGTCCGGTATAGGTC
				IGS BIP	AGCGGATTCCAACTTCCATGGCACGCGACGCTCATGAGAT
				IGS FLP	GATGGCGCTGGAGCGTCGGA
				IGS BLP	CACCGTCCTGCTGTCTGG
Fh ITS-2	*F. hepatica*	ITS-2	LAMP	Fh ITS-2 F3	TCCGCTTAGTGATATGCTTAAG
				Fh ITS-2 B3	GGTTGGTACTCAGTTGTCAG
				Fh ITS-2 FIP	TGATGCTGAACTTGGTCATGTGTCGAGGTCAGGAAGACAGA
				Fh ITS-2 BIP	CCAATGACAAAGTGACAGTGACGCTAGTCGGCACACTTATGATT
				Fh ITS-2 FLP	CATATAGCGACGGTACCCTTC
				Fh ITS-2 BLP	AACGTGCCTGGTATGGAATT
Cd ITS-2	*C. daubneyi*	ITS-2	LAMP	Cd ITS-2 F3	TGAGGTGCCAGATCTATGG
				Cd ITS-2 B3	CTTAAGTTCAGCGGGTATTCA
				Cd ITS-2 FIP	GCAGAGCGTGCTACCATTACACTTGCTGGTAGCACAGAC
				Cd ITS-2 BIP	TTGAGATGCTATTGCTGTCCGTCCCGAGGTCAGGTAACAGA
				Cd ITS-2 FLP	GTTAACTGAGCCACGACTCT
				Cd ITS-2 BLP	CAATCATGATCACCTACTGTGGT
Cd MAST	*C. daubneyi*		LAMP	Cd M F3	AAAGAAACTAACCAGGATTCCC
				Cd M B3	AAGGTCTGCTCAGGAAGAT
				Cd M FIP	CTAACCTGAACACCACATTGCCTATTAGTAACGGCGAGTGAAC
				Cd M BIP	CGCGAAGATGCTGCTCCAGCCTTTCACCCTCATTGG
				Cd M FLP	AATGACCACAGGCTTCGG
				Cd M BLP	ATGAGTAAGGTTACTCGGACATG

6.3.1.3.2 PCR

PCR was carried out as described in Section 2.6.1. In all PCRs, a 10 reaction mastermix was used, comprising 25 µl 10x reaction buffer (Invitrogen), 25 µl 10x forward primer, 25 µl 10x reverse primer, 7.5 µl 50mM $MgCl_2$ (Invitrogen), 2.5 µl dNTPs (Invitrogen), 2 µl Platinum® *Taq* polymerase (Invitrogen) and 138 µl nuclease-free (NF) H_2O (Sigma). Each reaction contained 2.5 µl of template DNA. PCR run conditions can be seen in Table 6.2. PCR products were run on agarose gels and viewed on a transilluminator (Section 2.6.1). Specificity was initially tested by PCR using the F3 and B3 primers of the respective LAMP primer set as this primer pair determines the specificity of the LAMP reaction.

Table 6.2 PCR run conditions for each primer set

		Cycling					
Primer set	Denaturation	Denaturation	Annealing	Extension	Extension	Hold	Number of cycles
Cox_1	10 mins at 95˚C	30 secs at 95˚C	30 secs at 63˚C	45 secs at 72˚C	10 mins at 72˚C	4˚C	40
ITS-2+	10 mins at 94˚C	1 min at 94˚C	90 secs at 53˚C	1 min at 72˚C	10 mins at 72˚C	4˚C	40
IGS LAMP	10 mins at 94˚C	40 secs at 94˚C	40 secs at 52˚C	40 secs at 72˚C	5 mins at 72˚C	4˚C	35
Fh ITS-2	10 mins at 95˚C	30 secs at 95˚C	30 secs at 56˚C	1 min at 72˚C	10 mins at 72˚C	4˚C	25
Cd ITS-2	10 mins at 95˚C	30 secs at 95˚C	30 secs at 56˚C	1 min at 72˚C	10 mins at 72˚C	4˚C	25
Cd MAST	10 mins at 95˚C	30 secs at 95˚C	30 secs at 55˚C	1 min at 72˚C	10 mins at 72˚C	4˚C	25

6.3.1.3.3 LAMP

LAMP reactions were carried out as described in Section 2.6.2. The volumes and concentrations of reagents used in the mastermix and primer mix were the same for each run, regardless of primer set. Each 25 µl reaction mix contained 12.5 µl of reaction mix, 1 µl Loopamp® fluorescent detection reagent, 1 µl *Bst* polymerase, 3.5 µl NF H_2O, 2 µl primer mix and 5 µl DNA. A primer mix contained 0.4 µl FIP, 0.4 µl BIP, 0.2 µl FLP, 0.2 µl BLP, 0.05 µl F3, 0.05 µl B3 and 0.7 µl NF H_2O per reaction. Primer sequences are shown in Table 6.1. Both the IGS LAMP and the Fh ITS-2 primer sets were used with an incubation temperature of 61˚C and a final 2 minute incubation at 80˚C. Finished reactions were viewed on a transilluminator.

6.3.2 PCR and LAMP assay development

6.3.2.1 Samples

As part of challenge study 1 (Chapter 3), adult parasites were retrieved from the livers of animals and allowed to lay eggs overnight in Earls Balance Salt Solution at 37˚C. Eggs were then washed in distilled H_2O prior to being stored in RNAlater® (Qiagen) at room temperature. Full details of this procedure can be seen in Section 2.4.4. These fluke eggs were used for PCR and LAMP assay development.

Faecal samples used in this chapter originated from 17 sheep from 4 different farms. Details of individual samples can be seen in Table 6.3. Samples were stored at 4˚C until they could be homogenised and weighed prior to freezing at -20˚C.

Samples F2994 to F3000 were composite samples. Each sample was made up of 1g of homogenised faeces taken from 5 individual sheep samples. The composite sample was then homogenised prior to use in any DNA extraction, FEC or cELISA methods.

Table 6.3 Details of faecal samples used for DNA extraction, origin, FEC (epg), cELISA (PD) and DNA extraction method. * indicates sample is a composite, ^ indicates rumen fluke eggs were also present, bold indicates a positive test result for FEC or cELISA.

Sample	Farm	FEC (epg)	cELISA (PD)	DNA extraction method
F2897	10	**4**	**17.26**	DNEasy® Blood and Tissue
F2904	10	**37.17**	**58.32**	DNEasy® Blood and Tissue
F2994*^	44	**95**	**121.71**	QIAmp® DNA Stool Mini
F2995*	44	**112.67**	**68.45**	QIAmp® DNA Stool Mini
F2996*	44	**143.83**	**57.6**	QIAmp® DNA Stool Mini
F2997*^	44	**103.5**	**115.52**	QIAmp® DNA Stool Mini
F2998*	44	**150.5**	**29.87**	QIAmp® DNA Stool Mini
F2999*	44	**249.83**	**60.67**	QIAmp® DNA Stool Mini
F3000*	44	**318.5**	**109.69**	QIAmp® DNA Stool Mini
F3205	44	**208**	**104.57**	Freeze/thaw
F3207	44	**121**	**10.11**	Freeze/thaw
F3208	44	**82**	**80.75**	Freeze/thaw
F3543	45	**17.83**	0.59	Freeze/thaw
F3578	23	0	1.12	QIAmp® FAST DNA Stool Mini
F3582	45	**0.67**	-2.79	QIAmp® FAST DNA Stool Mini
F3586	10	**3.83**	2.79	QIAmp® FAST DNA Stool Mini
F3590	10	**34.5**	-6.7	QIAmp® FAST DNA Stool Mini

6.3.2.2 DNA extraction

DNA was extracted from *F. hepatica* eggs using DNEasy® Blood and Tissue kit (Qiagen), as described in Section 2.5.1. Parasite egg samples were subject to one of 5 pre-DNA extraction treatments in order to obtain DNA from within the egg; (1) microwave heating, (2) vortexing, (3) freeze/thawing, (4) grinding in liquid nitrogen or (5) no pre-treatment. In each case, 200 µl aliquots of eggs collected from adult *F. hepatica* parasites (Section 6.3.1.1) were used. In all cases other than treatment 4, only 1 aliquot of eggs was used.

For method 1, the sample was placed in a 2 ml screw cap microcentrifuge tube with a pierced lid, and heated at full power for 1 minute in a 600W (category E) microwave. In method 2, glass beads were added to the sample at a 1:1 volume ratio in a 2 ml screw cap microcentrifuge tube and vortexed for 20 minutes. The freeze/thaw method consisted of freezing the sample at -80˚C for 10 minutes followed by incubation at 60˚C for 10 minutes, repeated three times. For method 4, samples were frozen in liquid nitrogen, ground using a pestle and mortar, thawed and re-suspended in NF H_2O (Sigma).

Four different DNA extraction methods were used on faecal samples in this study. Details of which sample was used in which method are presented in Table 6.3. DNA was extracted from some faecal samples using either the QIAmp® DNA Stool Mini, or the QIAmp® FAST DNA Stool Mini Qiagen DNA extraction kits, as per the manufacturer's guidelines, or using a freeze-thaw protocol. Full details of the protocols can be found in Section 2.5 and Appendix 1. The QIAmp® DNA Stool Mini kit was discontinued during the final testing of the *F. hepatica* faecal LAMP, so the QIAmp® FAST DNA Stool Mini kit was used as a replacement. For samples F2897 and F2904, 3 g of homogenised faeces was used in a FEC procedure (Section 2.2.1.2) up until the point of the addition of methylene blue. At this point the sediment was collected and 200 µl used in the DNEasy® Blood and Tissue kit (Qiagen), as described in Section 2.5.1.

The freeze/thaw DNA extraction protocol was based upon that described by Mukhopadhyay et al. (2012). Briefly, 0.25 g of homogenised faeces was mixed with either 250 µl, 500 µl or 1000 µl of NF H_2O (Sigma). This mixture was heated to 96˚C for 10 minutes then immediately placed on ice for 1 minute prior to centrifugation at 12,000 g for 10 minutes. The supernatant was removed and used in a DNA amplification method, PCR and/or LAMP, either neat or diluted 1:10 in NF H_2O (Sigma).

6.3.2.3 DNA amplification

DNA extracts from eggs and from faecal samples described in Section 6.3.2.1 were used in a PCR or LAMP reaction as described in Section 2.6. Only the ITS-2+, IGS LAMP and Fh ITS-2 primer sets were used for this aspect of the study.

6.3.3 PCR and LAMP evaluation for diagnostics

6.3.3.1 Samples

Faecal samples originated from 6 one-year-old castrated male sheep which had been challenged with *F. hepatica* metacercariae of the triclabendazole resistant Moredun isolate as part of a vaccine trial (Chapter 3). Sample collection occurred between 0 to 18 weeks post challenge (wpc), and 0.25 g of homogenised faeces from each animal, at each sampling, was frozen for use in this study. Details of the liver burden at slaughter as well as the FEC and cELISA results at each sampling can be seen for each animal in Appendix 5.

6.3.3.2 DNA extraction and amplification

DNA was extracted using the QIAmp® DNA Stool Mini kit (Qiagen) for the 18 wpc samples and the QIAmp® FAST DNA Stool Mini kit (Qiagen) for all other samples. The freeze/thaw method based upon that described by Mukhopadhyay et al. (2012),

with faeces diluted in 500 µl NF H_2O (Sigma) was also used on a second 0.25 g sub-sample for the 18 wpc samples.

Extracted DNA samples were used in two PCR assays, one using the Cox_1 primer set and the other using the ITS-2+ primer set, and in a LAMP assay using the Fh ITS-2 primer set.

6.3.4 Statistical analysis

In the testing of faecal samples the agreement between FEC and both PCR and LAMP were evaluated using Cohen's unweighted Kappa test (1960) via the "confusionMatrix()" function (Kuhn, 2014). The sensitivity of both PCR and LAMP was also calculated using FEC as the gold standard.

6.4 Results

6.4.1 Primer evaluation

6.4.1.1 Evaluation of analytical specificity

A summary of analytical specificity of the PCR and LAMP assays can be seen in Table 6.4. Due to time constraints not all primer sets were tested against all available DNA samples. Additional specificity testing was conducted by Gillian Mitchell (MRI). Details of all parasite DNA samples can be seen in Section 6.3.1.1.

Table 6.4 Analytical specificity of primer sets. ✓ indicates DNA was amplified, × indicates no DNA was amplified, red symbols indicate samples tested by Gillian Mitchell (MRI) ND = no data available, GIN = gastrointestinal nematodes – see Section 6.3.1.1 for full list, individual species DNA was tested.

	Parasite material					
Primer set	*F. hepatica*	*F. gigantica*	*D. dendriticum*	*C. daubneyi*	*P. cervi/P.leydeni*	GIN
Cox_1	✓	×	×	×	×	×
ITS-2+	✓	✓	✓	✓	✓	×
IGS LAMP	✓	✓	✓	✓	✓	×
Fh ITS-2	✓	✓	✓	×	×	×
Cd ITS-2	✓	ND	ND	✓	ND	ND
Cd MAST	×	×	×	✓	✓	×

The Cox_1 primer set (Martínez-Pérez et al., 2012) was found to amplify *F. hepatica* DNA but not *C. daubneyi* DNA, and was the only *F. hepatica*-specific primer set in this study. The trematode ITS-2 primer set (ITS-2+) (Itagaki et al., 2003) was found to amplify DNA extracted from adult parasites of both *F. hepatica* and rumen fluke species, with bands of distinctly different molecular weight being produced (Figure 6.1).

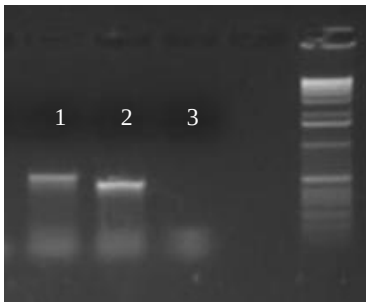

Figure 6.1 Specificity testing of ITS-2+ primer set. Gel image of *F. hepatica* and rumen fluke species DNA amplified using the ITS-2+ primer set. Well 1 = control *F. hepatica* DNA, well 2 = *C. daubneyi* DNA, well 3 = NF H_2O. 1Kb Plus DNA ladder (Invitrogen)

The F3/B3 primer set of the IGS LAMP primer set (Ai et al., 2010b) were found to amplify DNA extracted from adult parasites of both *F. hepatica* and rumen fluke species, the bands could not be differentiated by molecular weight.

Newly designed F3/B3 primer sets were then tested for specificity for *F. hepatica* and rumen fluke species DNA. The Fh ITS-2 F3/B3 primer set was found to amplify *F. hepatica* DNA but not rumen fluke species DNA when used in PCR, under standard conditions, or LAMP amplification with a 1 hour incubation at 61˚C (Figure 6.2). This primer set was also found to amplify *F. gigantica* and *D. dendriticum* DNA when used in the 2 hour 61˚C LAMP assay (data not shown), i.e. it is not specific to *F. hepatica* alone. DNA from 8 GIN species was tested individually and no amplification was seen (data not shown). The Cd ITS-2 F3/B3 primer set was also found to be non-specific, and amplified both liver fluke and rumen fluke DNA, albeit producing bands of different molecular weight. The Cd MAST F3/B3 primer set was found to be specific for rumen fluke species, with amplification of both *C. daubneyi* and *P. cervi*¸ but not *F. hepatica*.

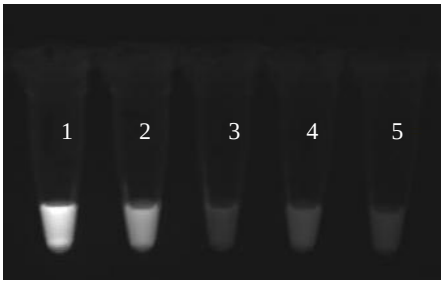

Figure 6.2 LAMP reaction using Fh ITS-2 primers. 1 = MAST Isoplex® DNA kit control, 2 = *F. hepatica* DNA, 3 = *C. daubneyi* DNA, 4 = *P. cervi* DNA, 5 = NF H_2O. White contents indicate positive results, grey contents indicate negative results.

6.4.1.2 Evaluation of analytical sensitivity

A *F. hepatica* DNA dilution series described in Section 6.3.1.1 was used in a PCR assay with the ITS-2+ primer set. No amplification was seen in the 200 or 100 ng/µl concentrations but amplification was seen from 10 to 0.001 ng/µl (Figure 6.3).

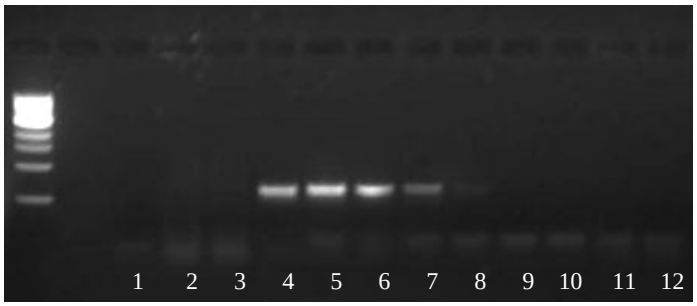

Figure 6.3 Gel image of *F. hepatica* DNA amplified using the ITS-2+ primer set. Well 1 = NF H_2O, well 2 = 200 ng/µl *F. hepatica* DNA, wells 3 to 12 = 10^2 to 10^{-7} ng/µl dilution series of *F. hepatica* DNA. 100bp DNA ladder (Invitrogen)

6.4.2 PCR and LAMP assay development

A summary of the samples used (Table 6.3) and the associated outcome of PCR and LAMP amplification can be seen in Appendix 5.

6.4.2.1 DNA from eggs

DNA was extracted from eggs which had been collected from the gall bladders of *F. hepatica*-infected sheep. DNA extraction was carried out as described in Section 6.3.2.2 using 5 pre-DNA extraction treatments, namely, microwaving, vortexing with glass beads, freeze/thawing, freezing in liquid nitrogen and grinding, and no treatment. The quantity of DNA recovered was highest in the freeze/thawing treatment (240 ng/µl) and lowest in the liquid nitrogen grinding treatment (13 ng/µl). In each case, PCR amplification was successful using the ITS-2+ primer set, irrespective of extraction method (Figure 6.4).

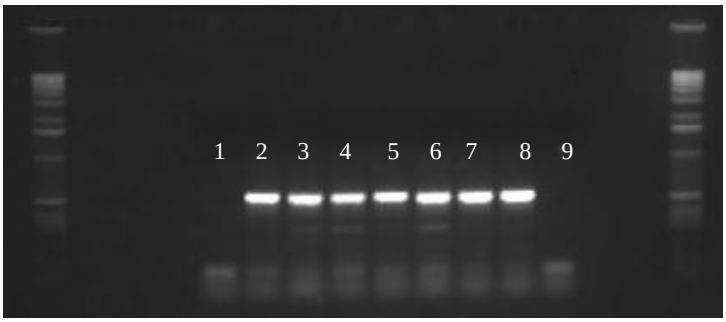

Figure 6.4 Gel image of PCR products of *F. hepatica* egg DNA after different DNA extraction methods. Wells 1 and 9 = NF H_2O, well 2 = *F. hepatica* control DNA, well 3 = no pre-treatment, well 4 = microwaving, well 5 = vortexing with glass beads, well 6 = freeze/thawing, wells 7 and 8 = liquid nitrogen and grinding. 1Kb Plus DNA ladder (Invitrogen)

DNA was extracted from the eggs and faecal sediment of subsamples of samples F2897 and F2904, as described in Section 6.3.2.2, using the DNEasy® Blood and Tissue kit. Whilst no amplification was observed after PCR using the ITS-2+ primer set on those DNA extracts, DNA was amplified from the F2904 sample using the IGS LAMP assay (data not shown); demonstrating that *F. hepatica* DNA was present in the sample.

6.4.2.2 DNA from faeces

Three 0.25 g sub-samples of ovine faeces with a FEC of 17.83 epg (F3543) were used for freeze/thaw DNA extraction, diluted in either (a) 250 µl, (b) 500 µl, or (c) 1000 µl (Section 6.3.2.2). The resulting DNA was diluted 1:10 with NF H_2O (Sigma) and used for amplification by PCR with the ITS-2+ primer set and by LAMP with the Fh ITS-2 primer set. No amplification occurred by PCR (data not shown), but amplification by LAMP was seen in the sample extracted by method (b) when incubated at 61˚C for 1 hour 45 minutes (Figure 6.5).

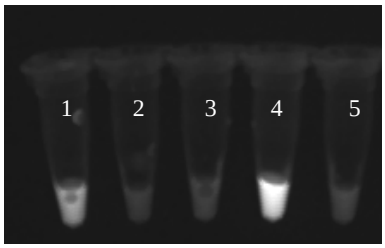

Figure 6.5 Results of LAMP with the Fh ITS-2 primer set used on DNA extracted from F3543 using 3 different DNA extraction techniques. 1 = MAST Isoplex® DNA kit control, 2 = NF H_2O, 3 to 5 = methods (a) to (c) of freeze/thaw DNA extraction. White contents indicate positive results, grey contents indicate negative results.

DNA was extracted from 0.2 g of a known negative faecal sample (F3578), two low FEC faecal samples (F3582 = 0.67 epg and F3586 = 3.83 epg) and a medium FEC faecal sample (F3590 = 34.5 epg), using the QIAmp® FAST DNA Stool Mini kit, at 95˚C. Amplification was seen in F3582 and F3590 by LAMP with the Fh ITS-2 primer set (data not shown).

DNA was extracted from 0.2 g subsamples of 7 composites (F2994-F3000), described in Section 6.3.2.1, using the QIAmp® DNA Stool Mini Kit as described in Section 6.3.2.2. Two of the DNA extracts, F2995 and F2998, were amplified by PCR using the ITS-2+ primer set (Figure 6.6). Two bands were amplified from the F2998 DNA extract, indicating both *F. hepatica* and rumen fluke DNA. In addition, the DNA samples were used in a PCR using the Cox_1 primer set; only DNA from sample F2995 was successfully amplified (data not shown).

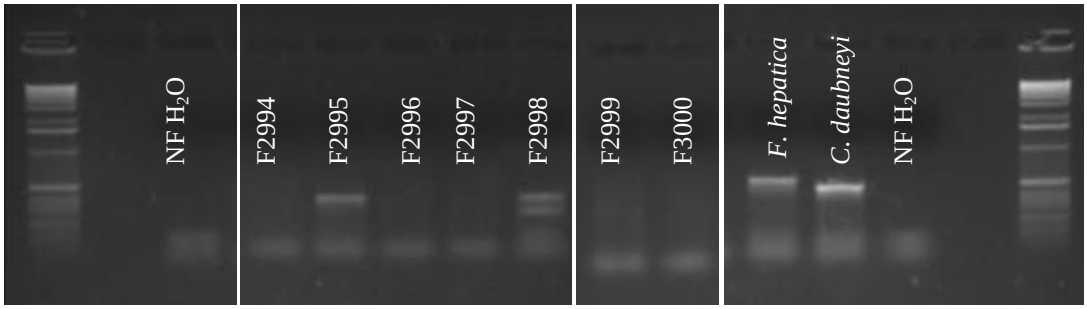

Figure 6.6 Gel image of PCR amplification of DNA from faecal composite samples F2994-F3000 using the ITS-2+ primer set. DNA extracted using QIAmp® DNA Stool Mini kit with incubation at 95˚C. NF H_2O = negative control, *F. hepatica* and *C. daubneyi* DNA used as positive controls. 1Kb Plus DNA ladder (Invitrogen)

These samples were also used in a 2 hour LAMP reaction using the Fh ITS-2 primer set. All samples, except F2994 and F2998, were positive by LAMP (Figure 6.7). Sample F2998 had been positive by PCR amplification with the ITS-2+ primer set.

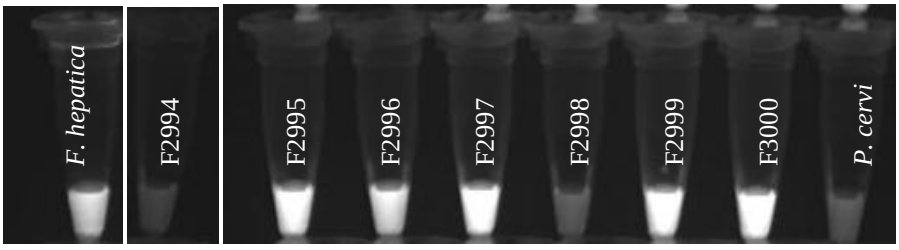

Figure 6.7 LAMP amplification of composite faecal sample (F2994 – F3000) DNA extracted using QIAmp® DNA Stool Mini kit with incubation at 95˚C. *F. hepatica* and *P. cervi* DNA used as positive and negative controls, respectively. White contents indicate positive results, grey contents indicate negative results.

6.4.2.3 LAMP Incubation times

DNA was extracted, using a freeze/thaw method, from four 0.25 g faecal samples mixed with 500 µl NF H_2O (Sigma). These four samples, F3205, F3207, F3208, F3210, had faecal egg counts which ranged from 50 to 208 epg. The resulting DNA extracts were used either as they were or diluted 1:10 with NF H_2O (Sigma) for amplification by LAMP using the Fh ITS-2 primer set. After 1 hour at 61˚C, only the *F. hepatica* control had amplified, incubation at 61˚C for a further hour resulted in

two further positive results for DNA extracts that had been diluted, i.e. from F3205 (208 epg) and F3208 (82 epg) (Figure 6.8). The diluted DNA extract from the sample of F3210 (50 epg) was not tested by LAMP.

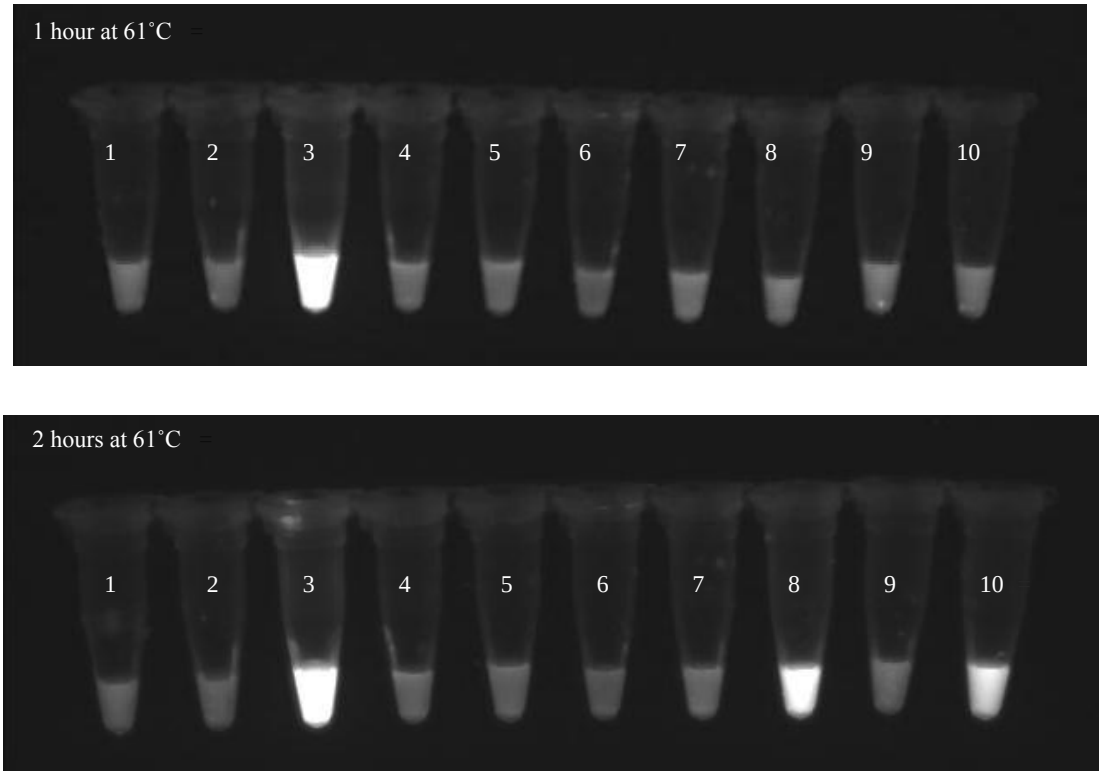

Figure 6.8 LAMP results of freeze/thaw faecal DNA extraction after 1 or 2 hours incubation at 61˚C. 1 = NF H_2O, 2 = MAST Isoplex® DNA kit control, 3 = *F. hepatica* positive control, 4-7 = undiluted faecal DNA extracts, 8 = F3205 diluted DNA extract 1:10, 9 = F3207 diluted DNA extract 1:10, 10 = F3208 diluted DNA extract. 1:10. White contents indicate positive results, grey contents indicate negative results.

6.4.2.4 Test sensitivity

If FEC is taken to be the gold standard and all results are included, regardless of primer set, then in the limited number of samples tested, PCR had a sensitivity of 20% (Table 6.5), whilst LAMP had a sensitivity of 68.8% (Table 6.6).

Table 6.5 Agreement between FEC and PCR testing of faecal samples

Kappa = 0.04		PCR		
		Positive	Negative	Total
FEC	Positive	2	8	10
	Negative	0	1	1
	Total	2	9	11

Table 6.6 Agreement between FEC and LAMP testing of faecal samples

Kappa = 0.21		LAMP		
		Positive	Negative	Total
FEC	Positive	11	5	16
	Negative	0	1	1
	Total	11	6	17

6.4.3 PCR and LAMP evaluation for diagnostics

The number of samples positive at each wpc, by FEC, cELISA and each faecal DNA amplification method, can be seen in Figure 6.9. Amplification of DNA was rarely seen following PCR with either primer set, apart from a spike in PCR positives at 13 to 15 wpc, with the Cox_1 PCR performing consistently better than the ITS-2+ PCR. Amplification following LAMP fluctuated, with a series of LAMP positives in early samples (3 to 6 wpc), followed by a noticeable dip at 7 to 8 wpc and a steady rise to a peak of 100% of animals positive by 12 wpc. The individual results for each animal are presented in Appendix 5. Results in Figure 6.9 are based on DNA extracts obtained with the QIAmp® (FAST) DNA Stool Mini kit. No amplification was seen with any of the DNA-based methods when using freeze-thaw derived DNA extracts from samples obtained at 18 wpc.

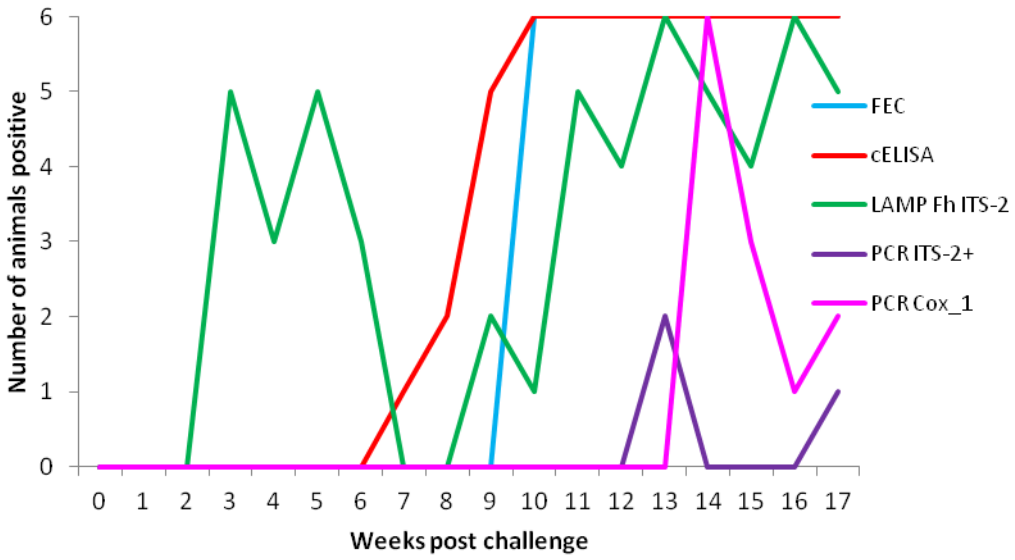

Figure 6.9 Number of animals positive by faecal-based tests following experimental challenge with *F. hepatica*.

6.5 Discussion

This chapter aimed to evaluate, and if required develop, faeces-based PCR and LAMP assays to detect liver fluke infection, with emphasis on the analytical specificity of the respective primers and the clinical sensitivity of the tests by way of a challenge study.

As only a small number of samples was tested, and repeat testing was not performed, this chapter represents a proof-of-concept analysis rather than a rigorous evaluation of primers, DNA extraction methods, PCR and LAMP assays.

6.5.1 Primer evaluation

When selecting primers it is important to ensure that they are suited to both the DNA amplification method being used and to the geographical area from which the samples originate. In this case, *F. hepatica* is the predominant liver fluke in UK with *D. dendriticum* only found in specific geographic locations and no natural *F.*

gigantica infections. Hence, primers for *F. hepatica* that cross-react with *F. gigantica* might be of diagnostic use in the UK, whereas they would be of limited diagnostic used in geographic areas where both *Fasciola* species exist together. Co-infections with gastrointestinal nematodes and with the rumen fluke, *C. daubneyi*, are often seen in the UK. Thus, for use in the UK, it is important that primers for detection of *F. hepatica* do not cross-react with *C. daubneyi*. *P. cervi*, a rumen fluke species previously thought to occur in the UK and Ireland, has not been identified by DNA-based methods from UK samples (Gordon et al., 2013; Willmott, 1950)(unpublished data). Therefore, all primers were tested against *C. daubneyi* but not against *P. cervi.* Given that *P. leydeni* has recently been reported from deer in Ireland (O'Toole et al., 2014) and that the geographical distribution of rumen fluke species is often based on morphological studies, which have been contradicted by DNA-based identification, further evaluation of primer specificity relative to a range of rumen fluke species would be useful.

The two PCR primer sets in this chapter were tested for specificity to *F. hepatica* and *C. daubneyi*, respectively. Those published by Martínez-Pérez et al. (2012) (Cox_1) were found to not amplify *C. daubneyi* DNA. The primer set published by Itagaki et al. (2003) (ITS-2+) amplified both *F. hepatica* and *C. daubneyi* but, as the PCR product for each fluke species was of a different molecular weight, they could be differentiated when the results were viewed on an agarose gel. These primers are not suitable for methods that do not include detection of amplicon size, e.g. real-time PCR or LAMP, which is a major drawback if the assay is to be developed into a pen-side diagnostic with quick turn-around.

Whilst the new *F. hepatica* LAMP primers presented in this chapter (Fh ITS-2) do not amplify rumen fluke DNA, they do amplify other liver fluke DNA i.e. *F. gigantica* and *D. dendriticum*. Despite this, the primer set is suitable for samples originating from UK as *F. gigantica* is not present and *D. dendriticum* is rarely seen. Further work on demonstrating specificity of *F. hepatica* primers or on development of specific primers will be needed to ensure that they are suitable for the geographic location and host species they are to be used in i.e. use of these primers on DNA

extracted from bovine faecal samples will require further specificity testing against GIN species found in bovines.

6.5.2 PCR and LAMP assay development

It is generally assumed that fluke eggs must be disrupted in order for DNA to be extracted from within them (Ai et al., 2010a; McNally et al., 2013). Therefore, the amplification of DNA from eggs which had not undergone a pre-DNA extraction treatment was somewhat unexpected. These eggs had been laid by adult parasites and washed in distilled water during the storage process. It is possible that free, i.e. soluble, DNA was still present following the washes and resulted in successful amplification.

PCR and LAMP assays were used to amplify DNA extracted from FEC positive faeces, which had been diluted and allowed to sediment for 3 minutes. In these instances, no pre-DNA extraction treatment was performed on the sample to crack eggs, and the sample was diluted in water prior to sedimentation. PCR was unable to amplify DNA from either sample, although LAMP amplified DNA from the sample with 31.17 epg. It is likely that the second sample, which had a FEC of 4 epg, had too few eggs or not enough free DNA present for amplification to occur. Because the aim of this chapter was to provide proof-of-concept for the utility of PCR or LAMP as diagnostic methods for liver fluke, full optimisation of DNA extraction methods and full analysis of analytical sensitivity were not undertaken.

DNA amplification by LAMP but not PCR was also seen when DNA, extracted using a freeze/thaw method from faeces, was used in PCR and LAMP assays. In this case, the faecal sample had a FEC of 17.83 epg. When using the QIAmp® DNA Stool Mini Kit, PCR was able to amplify DNA from 2 of 7 samples (FECs of 112.67 and 150.5 epg), although these had neither the highest nor lowest FEC or cELISA results. When the same DNA extracts were used in a 2 hour LAMP assay, DNA amplification occurred in one of the samples amplified by PCR and in 4 other samples (5 of 7 in total), with FECs ranging from 103.5 to 318.5 epg. Again these

samples included neither the highest nor the lowest FEC or cELISA results. DNA from sample F2998 was amplified by PCR but not LAMP this is an unexpected result. It is unlikely to be due to the DNA extraction process, as the same DNA extract was used. If the mastermix was not sufficiently mixed prior to pipetting aliquots for each sample it could have affected the reaction in this tube. This is unlikely as positive and negative controls were normal and samples which had not been amplified by PCR were amplified by LAMP. The lack of amplification of this sample by LAMP is likely a false negative result.

When tested against DNA extracted from low FEC samples, LAMP amplified DNA from samples with 0.67 and 34.5 epg, but not 3.83 epg. These results indicate that successful DNA amplification by PCR or LAMP assays may not be dependent on the level of FEC or cELISA. As there is a positive relationship between both FEC epg and cELISA PD and the burden of fluke in the liver, this may suggest that there is no direct correlation between DNA amplification and fluke burden using either PCR or LAMP. Testing of a larger number of samples would be useful to determine if this is truly the case.

DNA extracted from a faecal sample from an uninfected animal was not amplified by a 2 hour LAMP assay. Whilst this may indicate that genuinely negative faecal samples will test negative by LAMP, it was only one sample, tested once and may be the result of the low sensitivity shown by LAMP. Again, testing of a larger number of samples would be useful.

It is possible that rather than fluke burden it is the DNA extraction process which is affecting the amplification of fluke DNA by PCR. When extracting DNA from egg positive faeces, Ai et al. (2010a) vortexed 5-10 g of faeces with glass beads to disrupt egg shells prior to DNA extraction with the Wizard® SV genomic DNA purification system (Promega, WI). Both Martínez-Pérez et al. (2012) and Robles-Pérez et al. (2013) did not use any pre-DNA extraction treatments but used an alternative DNA extraction kit (SpeedTools Tissue DNA Extraction kit, Biotools, Spain), which was not available for use in this study. To improve the quality of extracted DNA, Robles-Pérez et al. (2013) added an ethanol precipitation step following DNA extraction. In

both cases, DNA amplification by PCR using the Cox_1 primers was successful. Other than the use of different buffers, the SpeedTools kit differs from the QIAmp® kit in that it does not have the initial incubation at 95˚C and discards the supernatant formed from the initial centrifugation step, using the pellet for DNA extraction instead.

For the extraction of DNA from faecal samples containing nematode eggs, McNally et al. (2013) used a different approach to those described so far. Faecal samples were stored in ethanol. To prepare the sample, ethanol precipitation and grinding was followed by the use of glass beads to disrupt egg shells and the addition of polyvinylpolypyrrolidone to remove inhibitory substances prior to DNA extraction using magnetic capture (MagMax Total Nucleic Acid extraction kit, Ambion, USA).

The use of a pre-DNA extraction treatment (i.e. disrupting eggs with glass beads) or a post-DNA extraction treatment (i.e. ethanol precipitation) or the use of an alternate DNA extraction method, may have increased the sensitivity of the PCR assays. Further work to optimise a DNA extraction protocol would be of value, potentially including the use of a pre-DNA extraction treatment.

In the case of the LAMP assay, incubation time was found to affect the amplification of DNA, with more samples being successfully amplified with longer (2 hours) incubation times. Although, even with a 2 hour incubation, only 2 of 3 samples were amplified (82 and 208 epg) and a high FEC sample (121 epg) was not amplified. One of the attractive features of the LAMP assay was the rapid amplification it is capable of (45 minutes), which is lost if the incubation time is extended to 2 hours. With modifications to the DNA extraction process, such as those mentioned above, it may be possible to reduce this incubation time.

6.5.3 PCR and LAMP evaluation

The FECs of the 6 animals in the challenge study were moderate, with eggs first being detected 10 wpc and ranging from 13.83 to 48 epg at 17 wpc. Coproantigens were detected later than expected, first being seen at 7 wpc, with all animals positive

by 10 wpc. The cELISA PD values were also moderate, ranging from 31.88% to 71.62%. This is likely due to the low challenge the animals received (~90 infective cysts), and the resulting low fluke burdens seen (22 - 44).

The lack of DNA amplification by LAMP on 18 wpc samples extracted using the freeze/thaw method was disappointing. Amplification had been successful using the method during the assay development in 3 of the 4 samples tested, with egg counts from 17.83 epg. The addition of pre- and post-DNA extraction treatments, as mentioned above, may improve this method. It is also worth noting that these samples had been stored at -20˚C for several months prior to DNA extraction, whereas the samples used in the assay development were fresh. This may have resulted in degradation of the DNA. Storage in ethanol, as performed by McNally et al. (2013), who stored samples for a year prior to DNA extraction, may result in better preservation of the sample.

In the PCR assays, although only a small proportion of samples were amplified, more samples were amplified using the Cox_1 primer set than the ITS-2+ primer set (12/108 vs. 3/108). It is unclear why this occurred but it may be due to the Cox_1 primer set amplifying mitochondrial DNA (mtDNA) whilst the ITS-2+ primer set amplifies nuclear DNA. It is possible that mtDNA is more abundant or more easily extracted than nuclear DNA. Both primer sets failed to amplify DNA in early samples, the ITS-2+ primer set had earlier detection (13 wpc), but this was only in two samples, with a third sample testing positive at 17 wpc. The two 13 wpc ITS-2+ positive samples were not amplified with the Cox_1 primer set. Although the Cox_1 primer set had slightly later detection (14 wpc), amplification was seen in more samples. However, although DNA amplification was successful from 14 wpc, it did not remain so for the duration of the study. Only faecal samples from one animal (A1333) had DNA which was consistently amplified (14 – 17 wpc). That the Cox_1 primers were unable to detect early infection is contrary to the published account, stating that DNA can be amplified from faecal samples taken at 2 wpc (Martínez-Pérez et al., 2012; Robles-Pérez et al., 2013). Once again, it is possible that this is due to the difference in DNA extraction kit used in the published and current studies.

Other research groups have also reported difficulties with PCR-based detection of *F. hepatica* DNA in faeces (D. Williams, personal communication to R. Zadoks, 2014).

The LAMP assay was able to amplify DNA extracted from 3 wpc faecal samples. This very early detection is a promising indication of the potential of a LAMP assay and was seen in 5 of the 6 samples. Despite this the number of samples that tested positive for infection by LAMP fluctuated until, at 7 and 8 wpc, no samples tested positive. At 9 wpc, the number of samples testing positive began to rise again and consistent amplification was seen in samples from 3 animals.

It is possible that the fluctuations in numbers of samples positive each week could be reduced with a modified DNA extraction method, but it is clear that the LAMP assay is able to amplify DNA in samples where the PCR assays cannot. The literature highlights the increased sensitivity of LAMP compared to that of PCR, reporting its ability to detect even low numbers of DNA copies (Notomi et al., 2000). It may be that whilst PCR assays would benefit from a more refined DNA extraction method than used in this study, that LAMP assays do not require this. It is worth noting that each sample only had one DNA extraction made and that DNA extract was only used in one LAMP assay. As such, if DNA is present in the sample, but it is low sensitivity of the LAMP assay causing the fluctuations, testing samples in duplicate or triplicate would highlight this.

Sample storage may also affect test results, due to degradation of DNA. All faeces were collected, homogenised, sub-sampled and frozen in the same way, with samples being frozen on the day of collection. However, when the time came for DNA extraction, the 0 wpc samples had been frozen for 18 weeks longer than the 17 wpc samples.

In this study, and in the previously published studies by Martínez-Pérez et al. (2012) and Robles-Pérez et al. (2013), which saw successful DNA amplification from 2 wpc, the source of the detected DNA is not known. As such, it is difficult to fully explain the kinetics of the release of DNA. It is unlikely at 2 or 3 wpc that the DNA being detected was from non-viable metacercarial cysts, these would likely have

been passed out with faeces in the first week post-challenge. At 3 wpc, the parasites have excysted, immature fluke are within the liver and have begun shedding tegument and excretions (Reddington et al., 1984). From this shed tegument and excretions excretory-secretory antigens can be detected in ovine serum from 1 wpc via an ELISA, but these antigens are not detectable in faeces via cELISA until 5 wpc (Duménigo et al., 2000; Mezo et al., 2004). It is possible that, being more sensitive than cELISA, the PCR and LAMP assays are amplifying DNA from the shed tegument and excretions within the faeces. Eggs may also be the source of DNA in successful amplification once an infection has become patent.

The cause of the drop in the proportion of animals testing positive by LAMP at 7 and 8 wpc is not clear. This may be due to fluctuations in the quality of the DNA extracts as mentioned above, or may be due to an aspect of the biology of *F. hepatica*. It is at around this time that fluke migrate into the bile ducts; this transition may play a role in the release of DNA into faeces. However, as excretory-secretory antigens were detected at 7 and 8 wpc by cELISA, LAMP should thus be able to amplify DNA from the shed tegument and excretions at these times. It is possible that storage of these samples is the cause of the lack of amplification. Samples were stored for a year and it is possible that the box these samples were stored in was closer to the door, or taken out to access other samples, thus resulting in a degradation of sample. As has been mentioned previously, this chapter is a proof-of-concept and testing of a larger number of samples would be beneficial in understanding both the potential and limitations of the faecal LAMP assay.

6.5.4 Conclusion

This study provides proof-of-concept that LAMP can be used to detect *Fasciola hepatica* infections in sheep during the pre-patent and patent stages of infection using faecal samples. These promising results warrant further effort into development of species-specific primers, optimisation of DNA-extraction methods, and evaluation of clinical and epidemiological sensitivity and specificity of the assay in sheep and other host species.

Chapter 7: Discussion

This thesis aimed to evaluate currently available and novel diagnostic tests for *F. hepatica* in sheep. To achieve this the coproantigen ELISA (cELISA), along with the faecal egg count (FEC), and where feasible the anti-*Fasciola* antibody ELISA (AbELISA) and the use of glutamate dehydrogenase (GLDH) and gamma-glutamyl transferase (GGT) concentrations, were evaluated in (1) an experimental challenge model (Chapter 3), (2) individual naturally exposed sheep, in early infection as well as in pre- and post-treatment situations (Chapter 4), (3) groups of naturally exposed sheep, including composite samples, in pre- and post-treatment situations and evaluating the FEC reduction test (FECRT) and the coproantigen reduction test (CRT) (Chapter 5), and lastly existing PCR assays and a newly developed loop-mediated isothermal amplification (LAMP) assay were evaluated against cELISA and FEC for the detection of *F. hepatica* infection in experimentally challenged sheep (Chapter 6).

7.1 Serum-based diagnosis

The AbELISA and the use of GLDH and GGT concentrations to indicate liver and bile duct damage are commonly used serum-based diagnostic tests for the detection of *F. hepatica* infection in sheep. The data presented in Chapter 4 of this thesis calls into question the use of GLDH and GGT concentrations to indicate liver fluke infection using current SAC C VS criteria. When used in lambs both GLDH and GGT concentrations were above SAC C VS "normal" ranges at the start of the study, when the lambs were 2 months old and unlikely to be infected with the adult fluke needed to stimulate an increase in GGT concentrations. It is possible that the young age of these animals contributed to the high concentrations of GLDH and GGT seen. If this is the case then work would be needed to evaluate the age at which GLDH and GGT concentrations are suitable as indicators of liver fluke infection. It is not clear how the "normal" range for these enzyme concentrations have been determined by SAC C VS. No information on "normal" ranges for these enzymes could be found in the literature. Instead, each published study consulted that uses GLDH and GGT concentrations to indicate liver fluke infection do so by comparing the concentrations

of the infected group to those of a control and requiring a significant increase from the control in order for liver fluke infection to be indicated (Ferre et al., 1996; Ferre et al., 1997; Raadsma et al., 2007). The control is either pre-infection concentrations of these same sheep or concentrations from an uninfected group of sheep of a similar age, parity, breed and sex, kept under the same conditions. Indeed, the control concentrations of these enzymes vary between studies, as do the characteristics of the animals used, with concentrations in adult control animals being higher than the "normal" range set by SAC C VS. When the GLDH and GGT concentrations from the first sampling of the lambs in this study were used as a control, the enzyme concentrations gave a better indication of infection, with GLDH and GGT concentrations significantly higher than the control concentrations in October and September, respectively. To use a single "normal" range for GLDH and GGT concentrations to indicate liver fluke infection, regardless of animal age, appears misguided and is likely to result in misdiagnosis of animals. If a pre-infection or control group concentration of GLDH or GGT is not available then these tests should not be used in isolation to diagnose liver fluke infection.

In the studies presented in this thesis involving adult sheep (Chapter 3), the AbELISA performed as would be expected based upon the reports in the literature (Zimmerman et al., 1982). Positive AbELISA titres were seen from 3 weeks post-challenge (wpc) in experimentally challenged adult sheep. The titres of the animals remained consistently positive throughout the studies. No studies within this thesis used the AbELISA in sheep which had been successfully treated for liver fluke infection. It is known however that anti-*Fasciola* antibodies persist following successful treatment (Ibarra et al., 1998; Sánchez et al., 2001). In naturally exposed lambs, early positive AbELISA titres were followed by a period of negative AbELISA titres and finally positive AbELISA titres, which remained so until the end of the study and death of the animals (Chapter 4). These early positive AbELISA titres are likely to be due to anti-*Fasciola* maternal antibodies in these young lambs, 2 months old at the time of the first positive. Indeed, when the AbELISA results of all lambs in the study are looked at, a decreasing AbELISA titre was seen between June and August in all but 2 animals. The dynamics of anti-*Fasciola* maternal

antibodies in lambs does not appear to be fully understood; at the time of writing no published reports could be found. In calves, anti-*Fasciola* maternal antibodies persisted for between 7 and 12 weeks, depending on the number of infective metacercarial cysts the mother was exposed to prior to birth (Mezo et al., 2010). In the absence of data from lambs, it would be inadvisable for the AbELISA to be used on samples from sheep younger than 12 weeks old.

Both the AbELISA and measuring GLDH and GGT concentrations rely on obtaining serum samples. In the UK, this can only be done by a veterinarian for routine diagnostic purposes. This leads to an increase in the costs of the tests, which may limit uptake by sheep farmers. However, it is worth noting that these tests can give very early indication of *F. hepatica* infection, which may reduce potential financial losses through early detection and treatment, and that the AbELISA is still a valuable test if used in an animal's first grazing season. Until other tests can reliably indicate pre-patent *F. hepatica* infections the AbELISA and potentially, if the criteria for interpretation is optimised, the use of GLDH and GGT concentrations, will remain a valuable tool for farmers and veterinarians in the control of *F. hepatica*.

7.2 Faeces-based diagnosis

As for the AbELISA, the FEC performed as was expected in the studies within Chapters 3, 4 and 5 of this thesis (Chowaniec and Darski, 1970; Valero et al., 2006). In the experimental challenges described in Chapter 3, FECs became positive no earlier than 9 wpc and in both naturally exposed and experimentally challenged animals FECs fluctuated between sampling points (Chapters 3, 4 and 5). The shedding of sequestered eggs following successful treatment was seen in Chapter 5, where low FECs were seen in a number of animals following successful closantel treatment. Rumen fluke eggs were seen in samples alongside *F. hepatica* eggs in Chapters 4 and 5. Care must be taken when performing FECs to ensure that *F. hepatica* and rumen fluke eggs can be distinguished. This includes not only training to differentiate between the two fluke eggs when seen side-by-side or in isolation, but also time and care to ensure the microscope is correctly set up to help

differentiate between the eggs. Poor lighting can make distinguishing the eggs of these two trematodes near impossible, especially when only one type of trematode egg is present.

The FEC was also used on composite samples in Chapter 5, which were collected using a modified version of the protocol proposed by Daniel et al. (2012). Infection was detected from fewer composite samples than when FEC was performed on individual samples and an average calculated. This is understandable in low FEC samples. If a single sample has a FEC of 1 epg whilst all other samples in the group are FEC negative it is unlikely that that the one egg will be included in the 3 g of composite sample viewed for FEC. However, when the average of that group is taken the FEC will be 0.1 epg resulting in a positive group average FEC. Although the lower sensitivity of composite FEC compared to individual FEC is understandable it is contrary to the findings of Daniel et al. (2012). This is likely due to the larger composite sample used in the original study, 50 g, compared to the 10 g composite sample prepared in this study. In addition, in the original study the whole 50 g was used in a FEC, whereas only 3 g of the composite was used in this study. Lastly, the FECs of the original study were higher than those seen within this thesis.

These factors are important. The composite FEC was performed with the smaller volume and in a standard FEC procedure in order to evaluate how it would likely be used. In several studies within this thesis the collection of insufficient faecal samples was an issue, despite ‘fill-to’ lines being used or the volume of faeces required being communicated. Often samples which were sent in had less than 4 g available, only just enough to perform a single FEC and cELISA. Obtaining a 5 g sample from 10 animals may not be practical. Even if a sufficient volume of faeces were collected it is not practical to perform FECs on a 50 g sample using the method employed in this thesis. Large volumes of water and containers would be required, sedimentation would be difficult and a longer period of sedimentation may be needed to ensure that all eggs sediment. Counting would need to be performed in batches to prevent plates from drying out on the microscope. The issue of the difference in burdens is also one that must be taken into consideration. Low burdens were seen on most farms which took part in the studies within this thesis (Chapters 4 and 5). The prevalence of

infection on farms did not follow a normal distribution, with either very few or nearly all animals being positive. Whilst the composite FEC using 10 g samples may perform well when used on samples from farms with high prevalence and moderate burden, it does not when used on samples from farms with low prevalence or low burden.

The BIO K201 cELISA appeared to be a very promising diagnostic test for the detection of pre-patent *F. hepatica* infection based upon its use in experimental challenge situations (Flanagan et al., 2011a; 2011b; Mezo et al., 2004). Indeed this was seen in the experimental challenge studies described in Chapter 3. However, when used in 2 month old lambs grazing pasture known to support the liver fluke life cycle, early detection was not seen (Chapter 4). The cELISA became positive in the same month that FEC did. Advertising from the company that produces the cELISA, (Bio-X Diagnostics, Belgium) and the published reports of the test's performance in experimental studies would indicate that the cELISA will detect early infections (Bio-X, 2010; Flanagan et al., 2011a; 2011b). Contrary to this, in the original report of the MM3 cELISA, in low burden infections the cELISA did not become positive until 7 wpc (Mezo et al., 2004). This reflects what was seen in the naturally exposed lambs, where burden was no more than 10 fluke.

It is also worth noting that the dynamics of an infection arising from an experimental challenge differs from those seen in a naturally acquired infection. Other than in the case of acute fasciolosis, naturally acquired infections often occur in the form of a trickle challenge, with metacercariae being ingested over a period of time ranging from hours to months. This is in contrast to an experimental challenge where 150 to 200 metacercariae are ingested all at once. Indeed in the experimental challenge studies of Chapter 3 all externally visible liver damage occurred on the left lobe of the liver, a contrast to the widespread damage which is often seen in the livers of naturally infected animals. This difference in the route of infection (experimentally challenged vs. naturally acquired) and in parasite burden (high in the case of experimental challenge but often low in natural infections) must be taken into account before tests are offered to farmers and veterinarians.

Although the cELISA was shown to not have the advantage of detection pre-patent infection in low burden situations in Chapter 4, it was still expected to be able to detect patent infections, except in the case of low positive FECs following successful treatment (Mezo et al., 2004; Valero et al., 2002). This detection of patent infections was not seen when the cELISA was evaluated using individual and group samples from adult sheep which may have been naturally exposed to *F. hepatica* (Chapters 4 and 5). The lowered sensitivity of the cELISA (85.1%) in comparison to the FEC may be due to the low burdens seen in both studies evaluating the cELISA in adult sheep (Chapters 4 and 5). Although livers were only recovered for a small minority of these animals FECs indicated low infections, with FECs of <10 epg in cELISA negative samples.

A study by Novobilský et al. (2012), carried out at the same time as the study on Farm 9 and Farm 22 (Chapter 5), aimed to evaluate the CRT in naturally exposed sheep. Three groups of 8 sheep were used but whilst 7 animals in each group were FEC positive, only 4 animals were positive by cELISA. This is another example of poor sensitivity by the cELISA compared to FEC in naturally exposed animals. As the FECs were not disclosed in the publication and livers were not recovered it is not possible to determine if fluke burden could be responsible for the low cELISA sensitivity. It is also worth noting that on two farms with high FECs the cELISA detected more infections than FEC did (Farm 9 and Farm 22, Chapter 5), again indicating that low burden may be the cause of the lowered sensitivity of the cELISA in comparison to FEC seen in studies involving naturally exposed sheep within this thesis (Chapters 4 and 5) and in the literature (Novobilský et al., 2012).

The lack of detection by cELISA in patent low burden infections is in contrast to the original description of the MM3 cELISA, where the presence of a single fluke was detected in sheep (Mezo et al., 2004). Changes to the cELISA between original publication and commercialisation may explain this change in sensitivity (Bio-X, 2010; Mezo et al., 2004).

Current guidelines from Bio-X Diagnostics (Belgium) use the same cut-off for the cELISA, regardless of the species the sample originated from. An extensive study by

Palmer et al. (2014) had the benefit of access to known negative samples. Together with known positive samples (positive by FEC), custom cut-offs were created for the cELISA depending on the species the samples originated from. By using these custom cut-offs the sensitivity of the cELISA was increased from 88%, which is similar to the sensitivity seen in the studies within this thesis, to 100% when used on ovine samples. This indicates that, despite being presented as a standardised commercial assay, further optimisation of the cELISA kit, including the use of species specific cut-offs, could improve its performance when used on samples from animals naturally exposed to *F. hepatica*.

When the cELISA was used in a composite sampling strategy in Chapter 5, modified from that described by Daniel et al. (2012), it was found to be more sensitive than when used on individuals and an average PD calculated. The increased composite cELISA sensitivity may not be seen if the results of the individual cELISAs of the samples making up the composite sample are taken into account. This is due to the fact that in a low prevalence flock the cELISA positive result of a single animal can be "diluted" when viewed with 9 other cELISA negative animals i.e. the mean PD will not indicate a positive cELISA result.

The issue of low burdens, as described by FEC, has been mentioned several times in this discussion. It is worth noting firstly that FEC is not always indicative of burden. The fluctuations in FEC epg between sampling points makes calculating true correlations difficult (Valero et al., 2002). One published report which did look at correlations with liver burden found no relationship in cattle and an inverse relationship in sheep; that is an increasing adult burden lead to a lower FEC epg (Valero et al., 2006). Secondly, if the low FECs are representative of a low fluke burden in the liver, is it truly an issue that the cELISA and composite FEC fail to detect these infections? Are these low infections clinically relevant? If they are not clinically relevant, i.e. not causing the animal distress and not significantly affecting production qualities (wool yield, weight gain, milk yield and fecundity), then not treating would reduce costs for farmers and help preserve the efficacy of treatments. The relationship between fluke burden and production losses has been explored in cattle and indicates that low burden infections have less of an effect on animals

(Charlier et al., 2014). The idea of only treating animals which are affected by disease has been proposed in gastrointestinal nematode (GIN) infections as targeted selective treatment (TST)(Kenyon and Jackson, 2012).

7.3 Diagnosis using DNA amplification methods

The commercially available diagnostic tests for the detection of *F. hepatica* are limited in their use depending on the age of animal, potential co-infections, prevalence and intensity of infection and the criteria used to interpret them. More work is needed before the use of DNA-based diagnosis for *F. hepatica* could replace or be used alongside any of the existing diagnostic tests. Despite this the promise of a test which is able to detect pre-patent *F. hepatica* infection, is highly sensitive and specific and could be rapid and cost-effective is highly attractive.

In Chapter 6 of this thesis PCR was performed on DNA extracted from faeces using previously published primers. In previous publications these primers and PCR assay were reported to detect infection from 2 wpc (Martínez-Pérez et al., 2012; Robles-Pérez et al., 2013). This was not seen in the study in Chapter 6, no DNA amplification occurred until 13 wpc, and the infection status of animals according to this PCR assay fluctuated. There are two possible explanations for the lack of DNA amplification. Firstly, the use of a different kit for the extraction of DNA from faecal samples may have affected the quality and/or quantity of DNA extracted from faeces. The kit used by Martínez-Pérez et al. (2012) and Robles-Pérez et al. (2013) (SpeedTools Tissue DNA Extraction kit, Biotools, Spain) was not available for use in this study. It is possible that the SpeedTools Tissue DNA Extraction kit (Biotools, Spain) is more effective at extracting DNA from faecal samples. The second explanation is the length of time samples were stored for. In the study presented in Chapter 6 of this thesis samples were stored at -20˚C for ~1 year. This extended storage, without the use of a preservative, may have degraded DNA samples. More work is needed to evaluate and optimise the DNA extraction process from faecal samples, including investigation into the effect of different storage conditions on samples.

Although a LAMP assay exists for the detection of *F. hepatica* it was found in Chapter 6 of this thesis that this assay also amplified the DNA of *Calicophoron daubneyi*, a rumen fluke commonly found in the UK (Ai et al., 2010b; Gordon et al., 2013). New primers were developed which amplify liver fluke DNA (*F. hepatica*, *F. gigantica* and *Dicrocoelium dendriticum*) but not rumen fluke DNA (*C. daubneyi* and *Paramphistomum cervi*/*P. leydeni*), and are thus suitable for use in the UK. When these primers were used in a 2 hour 61˚C LAMP assay, with DNA extracted from ovine faeces, amplification was seen at 3 wpc. This is a very promising result, particularly in light of the poor performance of PCR when used on the same DNA extracts. If the DNA extraction and sample storage procedures are optimised it may be possible to shorten the LAMP assay. Development for use on a real-time platform, such as has been done for the *Haemonchus contortus* LAMP assay, would allow for the quantification of DNA and potentially indicate burden in the animal (Melville et al., 2014). In addition, MAST Group Ltd, who produce the LAMP reagents, are developing a lyophilised version of the kit which would be more thermostable than the current reagents. LAMP products have also been visualised using a lateral flow device for parasite, bacterial and viral DNA extracts (Kiatpathomchai et al., 2008; Njiru, 2011; Rigano et al., 2010). Together, these two developments would make a pen-side *F. hepatica* LAMP a realistic possibility.

7.4 Determining treatment outcome

Currently there is no practical, standardised test to determine treatment outcome on working farms. In light of the increasing reports of TCBZ treatment failure, and confirmed cases of TCBZ resistance, such tests are of increasing importance (Álvarez-Sánchez et al., 2006; Gordon et al., 2012a; Lane, 1998; Mitchell et al., 1998; Moll et al., 2000; Olaechea et al., 2011; Ortiz et al., 2013; Overend and Bowen, 1995; SAC, 1998; Thomas, 2000). To perform a controlled efficacy trial the farmer would need to be willing to not treat a portion of his flock, leaving them at risk of suffering from *F. hepatica* infection, and to slaughter another portion of his flock whilst they are still in the meat withdrawal stage and thus not suitable for selling into the food market.

The FECRT is frequently used on farms to indicate treatment outcome. This has not been standardised for *F. hepatica* infections and this lack of standardisation presents issues. When used for sheep infected with gastrointestinal nematodes, the FECRT is not recommended for use if the FEC is <150 epg. For the studies within Chapters 4 and 5 of this thesis over 800 pre-treatment FECs were performed on samples from naturally exposed sheep. Of these, only 8 had FECs ≥150 epg and only 26 had FECs ≥100 epg. The criteria of samples having to have a FEC of ≥150 epg simply will not work for many British *F. hepatica* infections. As such it is ignored more often than not. This is far from ideal. Using the FECRT without a minimum FEC criterion can lead to misleading results. Within this thesis, in cases of very low pre-treatment FEC the FECRT proved unreliable when compared to the cELISA (Chapter 5). This is due to the release of small numbers of sequestered eggs from the gall bladder following successful treatment. If the pre-treatment FEC is less than 1 epg then even if a single sequestered egg is seen in the post-treatment FEC the treatment will be deemed to have failed. Work is needed to identify what the minimum FEC for the use of the FECRT in *F. hepatica* infections should be.

In GIN infections the timing of the post-treatment sampling varies depending on the class of anthelmintic used. Again this has not been standardised for *F. hepatica* infections and as such post-treatment sampling tends to occur between 14 and 21 dpt, generally depending on when is suitable for the farmer and veterinarian. Only one anthelmintic for *F. hepatica* is able to target all stages of the liver fluke and thus the anthelmintic given should be taken into account when determining the timing of the post-treatment sampling. For example, treatment with closantel can target *F. hepatica* from 7 weeks post-infection (wpi) but eggs can appear in faeces from 9 wpi, as such, at 3 wpt, eggs may be seen in the faeces from fluke which were ≤7 weeks old at the time of treatment and have now developed into egg producing adults.

Lastly, as eggs are stored in the gall bladder and the number of eggs is not always indicative of the number of fluke in the liver, a FECRT can only give information on the reduction of eggs, not the reduction of parasites. This was seen in bovines with a discrepancy in treatment efficacy being reported depending on if a FECRT or CET was used (Malone et al., 1982). Indeed, even determining the reduction of eggs is

unreliable due to the sporadic shedding of eggs and fluctuations that can be seen within two samples from the same animal. The 95% cut-off for the FECRT is used in *F. hepatica* simply because it is used in the FECRT for sheep infected with GINs. Work is needed to determine an appropriate cut-off for the FECRT in sheep infected with *F. hepatica*.

A CRT has been proposed to determine treatment outcome in sheep infected with *F. hepatica* (Flanagan et al., 2011a; 2011b). This requires all animals to be negative following successful treatment and is based upon the premise that the cELISA is specific to live fluke. In both of the studies where this CRT was presented, 1 of the 6 animals remained cELISA positive for 1 to 3 weeks following successful treatment (Flanagan et al., 2011a; 2011b). This suggests that 'all or nothing' criterion is not suitable. Indeed, if the publication the cELISA was originally presented in is consulted, it is stated that coproantigens can be detected for 3 weeks following successful treatment (Mezo et al., 2004).

In Chapter 5 of this thesis an alternative CRT was proposed, which was based upon the FECRT and required a ≥95% CR in order for the treatment to be deemed successful. This new criteria was also found to be unsuitable. This was due to the way in which cELISA results are reported. A sample could test negative by cELISA post-treatment yet the PD value be <95% than that of the pre-treatment PD value, resulting in the CRT indicating treatment failure even though there is no evidence of *F. hepatica* infection post-treatment. A third proposal was made of a CRT which incorporates a reduction in cELISA PD but also takes into account the post-treatment infection status of the animal or group according to the cELISA. As such, in order for a treatment to be deemed successful the CR must be ≥95% and the post-treatment cELISA result must indicate no *F. hepatica* infection. This third version of the CRT, when used on composite samples, had good agreement with the FECRT. As the CRT cut-off of ≥95% has been selected based on its use in the GIN FECRT, more work is needed to optimise the CRT.

7.5 Conclusion

In conclusion (1) the current use of "normal" ranges of GLDH and GGT concentrations is unsuitable and should be revised to avoid misleading results, (2) care should be taken when using the AbELISA in very young sheep due to the presence of anti-*Fasciola* maternal antibodies, (3) practitioners should be aware of the possible presence of both *F. hepatica* eggs following successful treatment and rumen fluke eggs pre- or post-treatment and the implications these can have on FEC, (4) in low burden situations the cELISA may not be able to detect pre-patent or patent *F. hepatica* infections, (5) the use of 10 g composite samples has promise for cELISA testing but is not suitable for either FEC or cELISA in low burden situations, (6) further work is needed to standardise the FECRT, whilst a new set of criteria for the CRT has been proposed and lastly (7) a faeces-based LAMP has been developed for the detection of *F. hepatica* from 3 wpc, with potential for development into a pen-side assay that may increase the uptake of evidence-based treatment by farmers, although more work is required to optimise this assay.

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Appendices

Appendix 1

1.1 Modified BIO K201 coproantigen ELISA protocol

Before using the kit, the concentrated dilution buffer (5x) and washing buffer (20x) must be diluted to a 1x concentration. Bring all reagents to 21°C +/- 3°C before use.

Homogenise faecal sample using a metal spatula and take a 0.5 g (± 0.03 g) subsample.

Add 2 ml of dilution buffer to the subsample and vortex for 10 seconds.

Centrifuge for 10 minutes at 1,000 g, collect the supernatant into a 1.5 ml Eppendorf.

Add 100 µl aliquots of the supernatant to two wells, a test well and control well; test wells are on rows A, C, E and G, while control wells are on rows B, D, F and H. Reconstitute the freeze-dried positive control with distilled water and add to two wells in the same manner.

Incubate the plate at 21°C (±3°C) for 2 hours on a plate agitator, cover with film.

Rinse plate with washing solution as follows. Flip plate sharply over a sink and fill wells with washing solution using a plastic wash bottle, avoiding the formation of bubbles. Empty the plate by flipping again. Rinse twice more. Remove excess wash solution by drumming the plate upside down against paper towel.

Dilute the biotin-linked anti-*Fasciola hepatica* conjugate fiftyfold in the dilution buffer. Mix gently avoiding the formation of bubbles.

Add 100 µl of diluted biotin-linked anti-*Fasciola hepatica* conjugate to each well

Incubate at 21°C (±3°C) for 1 hour on a plate agitator, cover with film. Rinse plate 3 times as described above.

Dilute the avidin-peroxidase conjugate fifty-fold in the dilution buffer. Mix gently, avoiding the formation of bubbles.

Add 100 µl of diluted avidin-peroxidase conjugate to each well.

Incubate at 21°C (±3°C) for 1 hour on a plate agitator, cover with film. Rinse plate 3 times as described above.

Add 100 µl of chromogen tetramethylbenzidine (TMB) solution to each well. The chromogen should be colourless when added to wells; if a blue colour is present the solution has been contaminated.

Incubate at 21°C (±3°C) for 10 minutes. Do not cover. A blue colour will appear in some samples.

Add 50 µl of stop solution to each well. The blue colour will become yellow.

Read the optical densities of the wells using a plate reader and a 450 nm filter.

1.2 Liver fluke extraction

If eggs are required from the gall bladder, it must be clipped off prior to removal from the liver and processed as described for the liver fluke egg recovery protocol. The liver may then be processed as described.

If eggs are not required, cut open the gall bladder and place any fluke present in cold tap water.

Cut the liver along the main bile duct and apply pressure to aid in the removal of fluke.

Slice the remaining liver into 1 inch wide slices from the central point outwards and apply pressure as above. Any fluke present, including partial fluke, should be placed with fluke from the gall bladder.

Fill a bucket with lukewarm water (~25˚C) and apply pressure to a liver slice whilst under the water. Set the slice aside in a tray or on a chopping board and repeat for the remaining liver slices.

Examine the slices for fluke.

Transfer the water to a second bucket via a 300 µm sieve. Pour this water through the sieve a second time prior to disposal.

Wash the contents of the sieve onto a chopping board and examine for the presence of whole or partial fluke.

Fill a bucket with lukewarm water (~25˚C).

Place one to three pieces of disrupted liver in a square of muslin (35 cm x 35 cm) and apply pressure whilst under the water to fully disrupt the tissue.

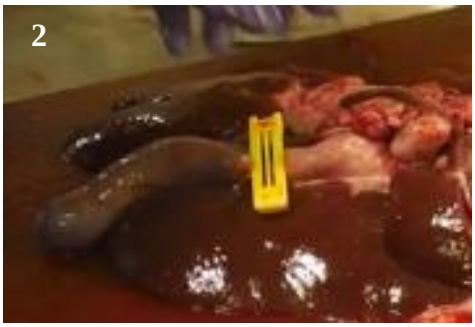
Examine the disrupted tissue and muslin square for whole and partial fluke. Repeat until all pieces of liver have been processed.

Pass the water through a 300 μm sieve to recover whole or partial fluke as above.

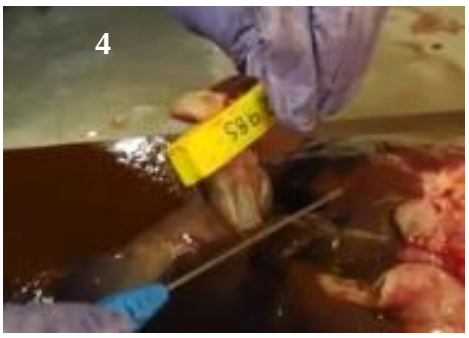
Count and measure all recovered fluke prior to storing in 100% ethanol.

1.3 Liver Fluke Egg Recovery

Make an incision between the main bile duct and the liver (image 1). Place a clip around the main bile duct (image 2).



Cut the bile duct above this clip (image 3) and carefully remove the remaining bile duct and gall bladder from the surface of the liver using a knife, working downwards, towards the gall bladder (image 4).



Remove the clip and tip the gall bladder over a 150 µm sieve, eggs and fluke present will be caught on the sieve surface, the bile that passes through can be discarded.

Cut the bile duct open and rinse it with cold tap water over the sieve using a plastic wash bottle.

Remove any fluke and rinse with tap water over the sieve. Place fluke with those recovered from the liver.

Wash eggs with water to remove bile.

Wash eggs from sieve into a beaker.

Allow eggs to sediment and remove excess water. Wash sediment into a universal tube for storage.

Allow eggs to sediment and remove excess water.

For DNA extraction re-suspend eggs in 100% ethanol and store at room temperature.

For hatching re-suspend eggs in distilled water and store at 4˚C.

1.4 Modified DNeasy® Blood And Tissue Kit – Animal Tissues (Spin-Column Protocol) protocol

Cut up to 0.25 g of parasite tissue and place in a 1.5 ml Eppendorf tube and add 180 µl Buffer ATL.

Add 20 µl proteinase K. Vortex and incubate in a water bath at 56˚C overnight to completely lyse tissue.

Dry sample tubes and vortex for 15 seconds. Add 200 µl Buffer AL, vortex, add 200 µl 100% ethanol and vortex.

Pipette mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at 6,000 g for 1 minute. Discard flow through and collection tube. If the volume of the mixture exceeds the volume of the spin column repeat this step with the excess mixture.

Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl Buffer AW1, and centrifuge for 1 minute at 6,000 g. Discard flow through and spin column.

Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl Buffer AW2, and centrifuge for 4.5 minutes at 15,000 g. Discard flow through and spin column.

Place the DNeasy Mini spin column in a clean 1.5 ml centrifuge tube and pipette 100 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 minute and then centrifuge at 6,000 g for 1 minute to elute the DNA.

1.5 Modified QIAmp® DNA Stool Mini Kit (Stool Pathogen Detection) protocol

Weigh 200 mg (±30 mg) stool into a 1.5 ml Eppendorf tube and place on ice if performing DNA extraction immediately. If not store at -20˚C for future use. Do not allow frozen samples to thaw until Buffer ASL has been added.

Add 1.2 ml Buffer ASL to each sample. Vortex for 2 minutes to homogenise.

Heat at 70˚C for 5 minutes in a water bath (can be increased to 95˚C). Dry sample tubes then briefly centrifuge to remove condensation.

Vortex for 15 seconds and centrifuge at 15,000 g for 1.5 minutes.

Pipette 1.2 ml of supernatant into a new 2ml tube.

Add 1 InhibitEX® Tablet to each tube and vortex immediately and continuously for 2 minutes. Incubate at room temperature for 1 minute.

Centrifuge at 15,000 g for 4.5 minutes.

Pipette supernatant into a new 1.5 ml tube. Centrifuge at 15,000 g for 4.5 minutes.

Pipette 15 µl of proteinase K into new 1.5 ml tube.

Pipette 200 µl of supernatant into the tube containing proteinase K.

Add 200 µl of Buffer AL and vortex for 15 seconds.

Incubate at 70˚C for 10 minutes in a water bath. Dry sample tubes and briefly centrifuge to remove condensation.

Add 200 µl of ethanol (100%) and vortex.

Transfer lysate to spin column without moistening rim. Centrifuge at 15,000 g for 1.5 minutes. Place column in new collection tube.

Add 500 µl Buffer AW1. Centrifuge at 15,000 g for 1.5 minutes. Place column in new collection tube.

Add 500 µl Buffer AW2. Centrifuge at 15,000 g for 4.5 minutes.

Place column in new collection tube and centrifuge at 15,000 g for 4.5 minutes.

Transfer spin column to 1.5 ml tube. Pipette 100 µl Buffer AE. Incubate at room temperature for 1 minute, then centrifuge at 15,000 g for 1.5 minutes.

1.6 Modified QIAmp® Fast DNA Stool Mini Kit (Stool Pathogen Detection) protocol

Weigh 200 mg (±30 mg) stool into a 1.5 ml Eppendorf tube and place on ice if performing DNA extraction immediately, if not store at -20˚C for future use. Do not allow frozen samples to thaw until the InhibitEX Buffer has been added.

Add 1ml of InhibitEX Buffer to each sample. Vortex until homogenised.

Heat at 95˚C for 5 minutes in a water bath. Dry sample tubes and vortex for 15 seconds.

Centrifuge at 15,000 g for 1.5 minutes.

Pipette 15 µl of proteinase K into new 1.5 ml tube.

Pipette 200 µl of supernatant into the tube containing proteinase K. Do not transfer any solid material.

Add 200 µl of Buffer AL and vortex for 15 seconds.

Incubate at 70˚C for 10 minutes in a water bath. Dry sample tubes and briefly centrifuge to remove condensation.

Add 200 µl of ethanol (100%) and vortex.

Transfer 600 µl lysate to spin column without moistening rim. Centrifuge at 15,000 g for 1.5 minutes. Place column in new collection tube.

Add 500 µl Buffer AW1. Centrifuge at 15,000 g for 1.5 minutes. Place column in new collection tube.

Add 500 µl Buffer AW2. Centrifuge at 15,000 g for 4.5 minutes.

Place column in new collection tube and centrifuge at 15,000 g for 4.5 minutes.

Transfer spin column to 1.5 ml tube. Pipette 100 µl Buffer ATE. Incubate at room temperature for 1 minute, then centrifuge at 15,000 g for 1.5 minutes.

Appendix 2

2.1 FECR results of TCBZ-R isolate identification

Animal	wpt	epg	FECR (%)
A0155	-1	76.67	-
	0	27.17	-
	1	20	26.38
	2	140.83	-418.42
	3	87.33	-221.48
	8	52.5	-93.26
	0	65.67	-
	1	66.5	-1.27
A0187	-1	103.67	-
	0	62.67	-
	1	21	66.49
	2	34	45.74
	3	76.33	-21.81
	8	44.83	-41.27
	0	103	-
	1	32	68.93

FEC (epg) and FECR (%) of ewes naturally infected with a potential TCBZ-R isolate of *F. hepatica*, at each week post treatment (wpt) with TCBZ. Per animal, two treatments are shown. The TCBZ-R isolate harvested from these animals was subsequently used for propagation through snails and challenge studies.

2.2 Liver burdens of animals infected with TCBZ-R fluke

Animal	Liver burden	Experiment
A0155	46	Original isolation
A0187	75	Original isolation
A1074	80	Challenge experiment 1
A1075	43	Challenge experiment 1
A1076	22	Challenge experiment 1
A1077	41	Challenge experiment 1
A1078	28	Challenge experiment 1
A1079	52	Challenge experiment 1
A1330	44	Challenge experiment 2
A1331	22	Challenge experiment 2
A1333	41	Challenge experiment 2
A1337	36	Challenge experiment 2
A1339	42	Challenge experiment 2
A1342	29	Challenge experiment 2

Fluke burdens in the liver after treatment with triclabendazole of the donor ewes and the experimentally challenged wethers.

2.3 FEC results of challenge experiment 1

wpc	A1074	A1075	A1076	A1077	A1078	A1079
0	0	0	0	0	0	0
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	**1.83**	**0.5**	0	**0.67**	0	0
10	**16.5**	**3**	0	**0.33**	**3.83**	**1**
11	**40.67**	**10.67**	**1.83**	**7**	**23.67**	**19.67**
12*	**88**	**29.33**	**5**	**15**	**18.17**	**21**
13	**92.83**	**12**	**8.67**	**11.33**	**14.33**	**15**
14	**69**	**41.67**	**26.5**	**25.17**	**30**	**25.83**
15*	**171.5**	**57.5**	**14.33**	**22.83**	**26.17**	**28**
16	**82.83**	**34.33**	**28.5**	**48.33**	**24.67**	**37**
17	**119.83**	**71.83**	**28**	**51.33**	**24.83**	**37.17**

Results show epg. **Bold** indicates a positive result. * indicates a TCBZ treatment. wpc = weeks post challenge. Column headers represent animal identification.

2.4 cELISA results of challenge experiment 1

wpc	A1074	A1075	A1076	A1077	A1078	A1079
0	1.05	2.42	1.12	1.61	1.18	0.43
1	0.24	0.1	-1.43	0.38	0.72	1.72
2	2.06	2.2	1.2	1.53	0.91	0.53
3	**15.09**	**9.5**	-5.89	**18.59**	**15.44**	**6.18**
4	**17.48**	**10.14**	**10.55**	**13.23**	1.46	**12.47**
5	**8.57**	**7.4**	**8.92**	**17.13**	-6.06	5.36
6	2.1	3.09	5.59	1.68	1.95	**6.42**
7	**16.86**	**7.77**	5.74	**25.75**	**7.62**	**19.6**
8	**47.08**	**26.92**	**20.11**	**23.62**	**42.15**	**60.18**
9	**65.11**	**60.39**	**14.17**	**44.19**	**71.36**	**93.25**
10	**97.35**	**57.22**	**38.01**	**66.76**	**68.41**	**100.6**
11	**90.13**	**93.84**	**95.76**	**104.7**	**108.21**	**106.62**
12*	**117.62**	**74.04**	**88.74**	**100.07**	**106.29**	**109.8**
13	**93.45**	**106.85**	**89.02**	**83.75**	**77.08**	**105.7**
14	**99.85**	**65.01**	**100.61**	**81.82**	**91.37**	**104.13**
15*	**96.65**	**77.8**	**93.72**	**95.93**	**94**	**115.77**
16	**116.35**	**85.65**	**111.78**	**104**	**95**	**107.07**
17	**108.42**	**79.37**	**109.99**	**98.86**	**102.28**	**119.2**

Results show PD. **Bold** indicates a positive result (≥6.07%). * indicates a TCBZ treatment. wpc = weeks post challenge. Column headers represent animal identification.

2.5 AbELISA results of challenge experiment 1

wpc	A1074	A1075	A1076	A1077	A1078	A1079
0	0	0	0	0	0	0
1	1	0	0	0	1	0
2	0	0	3	0	3	0
3	20	**36**	**82**	**67**	**79**	17
4	**85**	**53**	**58**	**99**	**61**	**32**
5	**141**	**128**	**180**	**136**	**146**	**63**
6	**152**	**142**	**181**	**156**	**140**	**73**
7	**174**	**152**	**195**	**177**	**194**	**112**
8	**129**	**97**	**142**	**126**	**156**	**127**
9	**177**	**175**	**193**	**197**	**200**	**158**
10	**205**	**205**	**152**	**190**	**193**	**159**
11	**184**	**199**	**205**	**223**	**214**	**172**
12*	**178**	**188**	**183**	**240**	**223**	**180**
13	**150**	**149**	**154**	**164**	**164**	**144**
14	**203**	**192**	**212**	**223**	**234**	**194**
15*	**183**	**177**	**235**	**241**	**255**	**167**
16	**159**	**164**	**198**	**204**	**196**	**181**
17	**161**	**154**	**173**	**189**	**189**	**160**

Results show antibody titre. **Bold** indicates a positive result (>30%). * indicates a TCBZ treatment. wpc = weeks post challenge. Column headers represent animal identification.

2.6 FEC results of challenge experiment 2

wpc	A1330	A1331	A1333	A1337	A1339	A1342
0	0	0	0	0	0	0
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	**0.83**	**1.83**	**10**	**1**	**1**	**2.17**
11	**11.17**	**5**	**13.33**	**1.83**	**9**	**6.17**
12	**8.67**	**10.67**	**36.5**	**4.67**	**12.83**	**7.5**
13	**11.5**	**9.33**	**38.33**	**18.17**	**33.5**	**15.17**
14	**22.83**	**9.33**	**34.17**	**36.83**	**34.17**	**23**
15	**25.83**	**16.67**	**42.83**	**48.33**	**51.17**	**26.5**
16*	**42**	**22.67**	**53.33**	**28.67**	**51.5**	**31.67**
17	**37**	**13.83**	**47**	**31**	**48**	**35**
18	**87.67**	**39.5**	**29.5**	**36.83**	**110.33**	**38.5**

Results show epg. **Bold** indicates a positive result. * indicates a TCBZ treatment. wpc = weeks post challenge. Column headers represent animal identification.

2.7 cELISA results of challenge experiment 2

wpc	A1330	A1331	A1333	A1337	A1339	A1342
0	0.54	0.24	0.1	3.5	0.1	0.24
1	-0.05	1.4	0.49	-0.38	0.49	0.22
2	-0.18	0.12	0.12	0.42	0.06	-0.06
3	-0.07	1.51	0.36	0.87	0.22	0.58
4	0.07	0.58	0.29	0.43	0.94	1.08
5	-0.3	0.38	0.3	0.54	0.3	0.18
6	0.18	0.3	0.89	0.66	0.24	0.54
7	1.43	**9.25**	1.91	1.64	0.79	0.62
8	3.4	**21.88**	**29.48**	3.12	4.02	3.68
9	**13.79**	**19.32**	**32.31**	3.06	**12.13**	**7.68**
10	**39.08**	**39.94**	**56.58**	**18.68**	**52.28**	**36.71**
11	**13.42**	**22.44**	**31.67**	**23.96**	**40.24**	**29.56**
12	**49.66**	**76.63**	**81.44**	**82.71**	**86.09**	**66.21**
13	**60.23**	**72.45**	**52.56**	**62.72**	**53.04**	**51.24**
14	**51.18**	**64.7**	**52.98**	**74.77**	**68.75**	**66.17**
15	**39.03**	**37.88**	**39.9**	**5.31**	**52.33**	**64.04**
16*	**42.64**	**65.96**	**75.75**	**49.87**	**48.36**	**75.55**
17	**31.88**	**71.62**	**68.28**	**70.92**	**70.65**	**64.89**
18	**60.3**	**40.56**	**59.01**	**51.64**	**89.67**	**49.92**

Results show PD. **Bold** indicates a positive result (≥7.49%). * indicates a TCBZ treatment. wpc = weeks post challenge. Column headers represent animal identification.

2.8 AbELISA results of challenge experiment 2

wpc	A1330	A1331	A1333	A1337	A1339	A1342
0	3	2	0	0	1	17
1	0	2	0	1	2	0
2	1	1	2	1	1	0
3	26	**38**	**53**	5	**64**	**33**
4	**97**	**86**	**85**	**67**	**120**	**87**
5	**76**	**75**	**90**	**84**	**75**	**94**
6	**178**	**148**	**108**	**129**	**192**	**130**
7	**131**	**189**	**155**	**234**	**221**	**257**
8	**156**	**151**	**137**	**114**	**171**	**122**
9	**151**	**144**	**172**	**68**	**163**	**190**
10	**180**	**174**	**150**	**92**	**234**	**124**
11	**157**	**172**	**141**	**101**	**183**	**130**
12	**182**	**191**	**147**	**105**	**191**	**130**
13	**177**	**214**	**137**	**101**	**201**	**113**
14	**209**	**237**	**138**	**88**	**162**	**110**
15	**182**	**214**	**124**	**136**	**269**	**178**
16*	**211**	**257**	**157**	**122**	**213**	**131**
17	**173**	**196**	**128**	**132**	**171**	**92**
18	**190**	**230**	**154**	**101**	**178**	**102**

Results show antibody titres. **Bold** indicates a positive result (>30%). * indicates a TCBZ treatment. wpc = weeks post challenge. Column headers represent animal identification.

Appendix 3

3.1 GLDH levels of lambs naturally exposed to *F. hepatica*

	Sampling month					
Animal	Jun	Jul	Aug	Sep	Oct	Nov
A0249	**25**	**34**	**21**	**28**	**50**	**186**
A0250	**18**	**16**	**60**	**37**	**112**	**80**
A0251	**21**	10	**13**	10	**46**	**74**
A0252	**33**	**138**	**20**	**82**	**119**	**56**
A0253	**15**	**44**	**40**	**45**	**64**	**99**
A0254	9	**11**	**14**	**35**	**41**	**21**
A0255	**22**	**15**	**17**	**16**	**26**	**31**
A0256	**35**	**31**	**48**	**18**	**131**	**198**
A0257	**19**	**17**	**15**	**35**	**42**	**24**
A0258	**14**	10	**14**	**18**	**40**	**33**
A0259	**12**	**13**	**11**	**31**	**90**	**143**
A0260	**21**	**16**	**15**	**23**	**23**	**31**
A0261	**13**	**16**	**14**	**82**	**365**	**68**
A0262	**16**	**19**	**26**	**156**	**125**	**246**
A0263	**29**	**14**	**34**	**199**	**254**	**163**
A0264	**23**	**21**	**35**	**33**	**158**	**47**
A0265	**22**	**30**	**34**	**39**	**40**	**34**
A0266	**31**	9	**13**	**11**	**17**	**296**
A0267	**12**	10	**20**	**27**	**86**	**222**
A0268	**13**	10	6	9	**11**	**12**
A0269	9	**15**	**15**	**1**	**24**	**68**
A0270	**19**	**16**	**38**	**62**	**131**	**347**
A0271	**11**	**35**	**16**	**53**	**32**	**91**
A0272	**1**	**18**	**37**	**25**	**73**	**135**
A0273	**14**	**19**	**95**	**23**	**115**	**32**
A1556	**13**	9	7	**135**	**34**	**119**
A1557	**16**	**16**	**46**	-	-	-

Bold indicates a result outside of the SAC healthy reference range (2-10 IU/L)

3.2 GGT levels of lambs naturally exposed to *F. hepatica*

	Sampling month					
Animal	Jun	Jul	Aug	Sep	Oct	Nov
A0249	**53**	**48**	**48**	**43**	**38**	**50**
A0250	**55**	**51**	**50**	**45**	**50**	**86**
A0251	**64**	**51**	**43**	**46**	**47**	**141**
A0252	**60**	**56**	**49**	**51**	**58**	**72**
A0253	**54**	**46**	**47**	**46**	**54**	**65**
A0254	**62**	**58**	**55**	**70**	**94**	**69**
A0255	**11**	**54**	**45**	**68**	**18**	**47**
A0256	**56**	**53**	**81**	**65**	**68**	**67**
A0257	**57**	**50**	**46**	**46**	**48**	**70**
A0258	**54**	**53**	**52**	**44**	**46**	**71**
A0259	**77**	**10**	**47**	**52**	**66**	**44**
A0260	**63**	**53**	**54**	**45**	**43**	**115**
A0261	**62**	**41**	**38**	**40**	**76**	**40**
A0262	**37**	**47**	**47**	**69**	**145**	**228**
A0263	**61**	**54**	**48**	**52**	**166**	**177**
A0264	**41**	**39**	**44**	**57**	**91**	**50**
A0265	**51**	**48**	**44**	**39**	**54**	**78**
A0266	**64**	**56**	**48**	**50**	**56**	**363**
A0267	**53**	**55**	**48**	**51**	**52**	**86**
A0268	**48**	**47**	**49**	**47**	**48**	**48**
A0269	**54**	**54**	**45**	**46**	**62**	**113**
A0270	**51**	**53**	**38**	**50**	**71**	**209**
A0271	**76**	**65**	**59**	**60**	**150**	**99**
A0272	**122**	**119**	**51**	**43**	**49**	**143**
A0273	**54**	**46**	**47**	**44**	**17**	**80**
A1556	**79**	**72**	**55**	**140**	**58**	**66**
A1557	**52**	**55**	**54**	-	-	-

Bold indicates a result outside of the SAC healthy reference range (27-31 IU/L)

3.3 AbELISA titre of lambs naturally exposed to *F. hepatica*

	Sampling month					
Animal	Jun	Jul	Aug	Sep	Oct	Nov
A0249	10	3	1	**53**	**189**	**239**
A0250	-1	1	1	**39**	**236**	**235**
A0251	0	2	1	1	**175**	**259**
A0252	10	2	1	**230**	**334**	**286**
A0253	19	4	1	26	**143**	**125**
A0254	5	1	22	**86**	**142**	**197**
A0255	**106**	21	6	10	**38**	**124**
A0256	**65**	**197**	**135**	**172**	**235**	**260**
A0257	16	4	1	**169**	**230**	**223**
A0258	1	0	1	11	**212**	**248**
A0259	**79**	**70**	5	**132**	**190**	**245**
A0260	12	3	1	5	**86**	**193**
A0261	6	1	14	**31**	**92**	**68**
A0262	24	6	13	**226**	**273**	**256**
A0263	2	2	0	**32**	**117**	**122**
A0264	5	4	12	**122**	**216**	**190**
A0265	**51**	**152**	**89**	**148**	**185**	**183**
A0266	16	4	1	0	**113**	**146**
A0267	1	1	4	2	**118**	**205**
A0268	3	2	2	0	2	2
A0269	6	8	2	**174**	**284**	**313**
A0270	20	1	1	20	**198**	**259**
A0271	20	5	2	**121**	**182**	**139**
A0272	16	2	2	9	0	**216**
A0273	1	1	2	**39**	**233**	**213**
A1556	-5	**102**	22	**94**	**107**	**219**
A1557	-7	2	**37**	-	-	-

Bold indicates a positive AbELISA result (>30%)

3.4 FEC (epg) of lambs naturally exposed to *F. hepatica*

	Sampling month					
Animal	Jun	Jul	Aug	Sep	Oct	Nov
A0249	0	0	0	0	0	**0.33**
A0250	0	0	0	0	0	**1**
A0251	0	0	0	0	0	**0.33**
A0252	0	1	0	0	0	NS
A0253	0	0	0	0	0	**1**
A0254	0	0	0	0	NS	NS
A0255	0	0	0	0	**0.33**	**0.33**
A0256	0	**0.66**	0	0	**0.33**	**1**
A0257	0	0	0	0	NS	**1.33**
A0258	0	0	0	0	NS	**1**
A0259	0	0	0	0	0	0
A0260	0	0	0	0	0	NS
A0261	0	**0.33**	0	0		**2.33**
A0262	0	0	0	0	0	**0.66**
A0263	0	0	0	0	0	**0.33**
A0264	0	0	0	0	NS	**0.66**
A0265	0	0	**0.66**	0	0	NS
A0266	0	0	0	0	0	0
A0267	0	0	0	0	0	0
A0268	0	0	0	0	0	0
A0269	0	0	0	0	0	NS
A0270	0	0	0	0	0	0
A0271	0	0	0	0	0	0
A0272	0	0	0	0	0	**1.66**
A0273	0	0	0	0	0	**0.33**
A1556	0	0	0	0	NS	NS
A1557	0	0	0	-	-	-

Bold indicates a positive FEC result (0 epg), NS = no sample retrieved

3.5 cELISA OD of lambs naturally exposed to *F. hepatica*

	Sampling month					
Animal	Jun	Jul	Aug	Sep	Oct	Nov
A0249	0.003	0.005	0.002	0.003	0.021	**0.446**
A0250	-0.002	-0.001	0.002	0.003	0.006	**0.506**
A0251	0.002	0.003	0.012	-0.001	0.004	0.129
A0252	-0.001	0.005	-0.02	0.006	0.008	NS
A0253	0.01	-0.005	0.001	0.004	0	0.094
A0254	-0.051	-0.006	0.012	0.01	0.045	**0.298**
A0255	0.01	**0.254**	0.133	0.035	0.142	**0.273**
A0256	-0.002	0.003	0.086	0.08	**0.319**	0.03
A0257	0.001	0.015	0.006	0.01	0.053	-0.001
A0258	0.008	-0.001	0.004	0.01	0.009	**0.262**
A0259	0	0.006	0.008	0.003	0.018	**0.738**
A0260	0.003	-0.036	0.004	0.01	0.004	**0.262**
A0261	0.007	0.022	NS	0.004	**0.185**	-0.025
A0262	0.006	0.018	-0.016	0.008	**0.317**	**1.03**
A0263	0.001	0.024	-0.012	0.001	0.099	**0.573**
A0264	-0.002	0.011	0.01	0.029	0.01	**0.479**
A0265	0.007	0.02	0.075	0.001	0.058	**0.252**
A0266	0.002	0.002	-0.002	0	0.047	0.068
A0267	0.001	0.004	-0.002	-0.001	-0.008	0.028
A0268	0	0.012	0.007	-0.001	0.008	0.002
A0269	-0.004	0.001	0.004	-0.001	0.085	NS
A0270	0.007	0.02	0.001	0.001	0.009	**0.401**
A0271	NS	0.002	-0.003	0.001	0.067	**0.158**
A0272	NS	NS	0.006	0.001	0.015	**0.689**
A0273	0.004	0.019	-0.003	0	NS	**0.516**
A1556	0.016	0.043	0.122	0.025	NS	NS
A1557	-0.008	0.005	0.003	-	-	-

Bold indicates a positive cELISA OD result (>0.150), NS = no sample retrieved

3.6 Example of FEC and cELISA data from cross-sectional sample collection as recorded in Access database

AID	FID	Collected	Well_1	Well_2	OD	PD	CE_outcome	FEC_1	FEC_2	EPG	FEC_outcome	wpt	Farm
A0166	F0321	12-Jan-11	2.765	0.314	2.451	129.82	Positive	68	76	24	Positive	0	22
A0167	F0322	12-Jan-11	2.007	0.288	1.719	91.049	Positive	70	53	20.5	Positive	0	22
A0177	F0332	12-Jan-11	1.943	0.179	1.764	93.432	Positive	43	54	16.17	Positive	0	22
A0178	F0333	12-Jan-11	1.822	0.292	1.53	81.038	Positive	162	163	54.17	Positive	0	22
A0179	F0334	12-Jan-11	2.084	0.282	1.802	95.445	Positive	61	60	20.17	Positive	0	22
A0180	F0335	12-Jan-11	1.775	0.212	1.563	82.786	Positive	108	100	34.67	Positive	0	22
A0181	F0336	12-Jan-11	1.458	0.19	1.268	67.161	Positive	44	49	15.5	Positive	0	22
A0121	F0365	20-Jan-11	1.735	0.098	1.637	57.967	Positive	120	118	39.67	Positive	0	9
A0082	F0366	20-Jan-11	0.281	0.078	0.203	7.188	Positive	23	28	8.5	Positive	0	9
A0128	F0367	20-Jan-11	1.78	0.09	1.69	59.844	Positive	101	98	33.17	Positive	0	9
A0074	F0368	20-Jan-11	3.101	0.113	2.988	105.807	Positive	37	37	30.83	Positive	0	9
A0112	F0369	20-Jan-11	2.349	0.092	2.257	79.922	Positive	73	71	24	Positive	0	9
A0210	F0370	20-Jan-11	1.006	0.1	0.906	32.082	Positive	44	48	15.33	Positive	0	9
A0133	F0376	20-Jan-11	3.583	0.126	3.457	122.415	Positive	25	34	9.83	Positive	0	9
A0080	F0377	20-Jan-11	3.896	0.125	3.771	133.534	Positive	50	58	18	Positive	0	9
A0503	F1010	02-May-11	0.079	0.071	0.008	0.465	Negative	0	0	0	Negative	0	40
A0504	F1027	08-Jul-11	0.089	0.079	0.01	0.581	Negative	0	0	0	Negative	0	5
A0350	F1049	16-Aug-11	0.111	0.102	0.009	0.573	Negative	0	0	0	Negative	0	10
A0349	F1050	16-Aug-11	0.083	0.093	-0.01	-0.636	Negative	0	0	0	Negative	0	10
A0348	F1051	16-Aug-11	0.094	0.092	0.002	0.127	Negative	0	0	0	Negative	0	10
A0347	F1052	16-Aug-11	0.101	0.087	0.014	0.891	Negative	0	0	0	Negative	0	10

AID = Unique animal ID, FID = Unique faecal sample ID, Collected = date collected from animal, cELISA data = Well 1 optical density (OD), Well 2 OD, net OD, percentage difference (PD) and test outcome, FEC data = first count (FEC_1), second count (FEC_2), epg and test outcome, week post-treatment sampled and the Farm ID

3.7 cELISA, FEC and liver burden data used in Chapter 4

AID	FID	Well_1	Well_2	OD	PD	CE_outcome	FEC_1	FEC_2	EPG	FEC_outcome	GB	LB	LB_outcome
A0002	F0126	0.074	0.064	0.01	0.583	Negative	0	-	0	Negative	No	0	Negative
A0056	F0127	0.064	0.054	0.01	0.583	Negative	0	-	0	Negative	No	0	Negative
A0057	F0128	0.079	0.051	0.028	1.632	Negative	1	-	0.33	Positive	No	0	Negative
A0058	F0129	0.059	0.049	0.01	0.583	Negative	0	-	0	Negative	No	0	Negative
A0059	F0130	0.074	0.061	0.013	0.758	Negative	0	-	0	Negative	Yes	0	Negative
A0060	F0131	0.145	0.056	0.089	5.186	Negative	3	-	1	Positive	Yes	2	Positive
A0061	F0132	0.084	0.047	0.037	2.156	Negative	0	-	0	Negative	No	0	Negative
A0062	F0133	0.056	0.05	0.006	0.35	Negative	0	-	0	Negative	No	0	Negative
A0063	F0134	1.581	0.056	1.525	88.869	Positive	242	-	80.67	Positive	No	5	Positive
A0064	F0135	0.063	0.062	0.001	0.058	Negative	1	-	0.33	Positive	No	0	Negative
A0065	F0136	0.379	0.046	0.333	19.406	Positive	2	-	0.67	Positive	No	1	Positive
A0066	F0137	0.725	0.052	0.673	39.219	Positive	40	-	13.33	Positive	No	0	Negative
A0067	F0138	0.059	0.044	0.015	0.874	Negative	0	-	0	Negative	No	0	Negative
A0068	F0139	0.439	0.064	0.375	21.853	Positive	5	-	1.67	Positive	No	2	Positive
A0069	F0140	0.102	0.055	0.047	2.739	Negative	2	-	0.67	Positive	No	0	Negative
A0155	F1013	2.351	0.072	2.279	132.346	Positive	196	203	66.5	Positive	Yes	46	Positive
A0187	F1014	1.749	0.068	1.681	97.619	Positive	94	98	32	Positive	Yes	75	Positive
A0250	F0287	1.669	0.074	1.595	72.533	Positive	10	15	4.17	Positive	No	5	Positive
A0251	F0292	2.311	0.074	2.237	101.728	Positive	38	-	19.19	Positive	No	6	Positive
A0257	F0288	0.719	0.084	0.635	28.877	Positive	12	-	5.15	Positive	No	3	Positive
A0262	F0289	2.836	0.104	2.732	124.238	Positive	63	59	20.33	Positive	No	10	Positive
A0263	F0290	0.341	0.068	0.273	12.415	Positive	21	-	7	Positive	No	4	Positive
A0273	F0291	0.412	0.088	0.324	14.734	Positive	22	-	7.33	Positive	No	5	Positive
A0498	F0993	0.216	0.118	0.098	4.88	Negative	5	6	1.83	Positive	Yes	3	Positive
A0500	F0995	0.15	0.172	-0.022	-1.096	Negative	122	122	40.67	Positive	Yes	24	Positive

AID = Unique animal ID, FID = Unique faecal sample ID, cELISA data = Well 1 optical density (OD), Well 2 OD, net OD, percentage difference (PD) and test outcome, FEC data = first count (FEC_1), second count (FEC_2), epg and test outcome, GB = Gall bladder recovered, LB = fluke burden in liver, LB_outcome = Liver examination test outcome

Appendix 4

4.1 Composite evaluation pre-treatment letter to farmers

21 October 2014

Moredun
Research Institute

Pentlands Science Park
Bush Loan
Penicuik, near Edinburgh
EH26 0PZ
Scotland, UK
Tel: +44 (0)131 445 5111
Fax: +44 (0)131 445 6111
Philip.Skuce@moredun.ac.uk
www.moredun.ac.uk

<ADDRESS>

Re: Use of faecal samples for detection of liver fluke infection and treatment success in sheep

Dear <NAME>,

Thank you for your interest in participating in our study. With financial support from QMS, we aim to assess the usefulness of coproantigen ELISA testing on composite faecal samples for detection of liver fluke infection and flukicide treatment success. In addition, we want to evaluate the ease of the proposed sampling strategy. Finally, we will determine whether liver fluke treatment has been effective in your flock by comparison of faecal samples on the day of treatment with faecal samples obtained from the same animals 21 days after treatment.

In your sampling kit you will find

a) A laminated sheet with sampling instructions
b) 25 sample collection pots (two sets of 12 pots and one spare)
c) A return slip with a few questions
d) An envelope with freepost label for shipping of samples to Moredun

After receipt of your samples, we will process them at the Moredun Research Institute and we will contact you with the results. At this time, we will also ask you for feedback on the procedure (is it simple and fast enough?) and we will ship new pots for collection of the second set of samples.

We are trying to make this as simple and robust as possible and value your time and effort. If you have any questions regarding the study, please contact us by phone (0131-4455111) or e-mail (Philip.skuce@moredun.ac.uk or Ruth.zadoks@moredun.ac.uk).

Sincerely,

Philip Skuce, PhD Danielle Gordon, MSc Ruth Zadoks, DVM, PhD

4.2 Composite evaluation pre-treatment information slip

Use of faecal samples for detection of liver fluke infection and treatment success in sheep – information to be included with samples on the day of treatment

1. Your name and address:

2. Date of treatment and sample collection:

3. Treatment product:

4. Method used to determine weight of sheep:

5. Comments on ease of use of the sampling materials and the sampling method (optional):

After collection, please return the faecal samples and this information slip to:

Danielle Gordon
The Moredun Foundation
FREEPOST EH1686
Penicuik
EH26 0FG

If you have any questions regarding sample collection or shipping, please contact us by phone (0131-4455111) or e-mail (Philip.skuce@moredun.ac.uk or Ruth.zadoks@moredun.ac.uk).

4.3 Composite evaluation sampling instructions

Use of faecal samples for detection of liver fluke infection and treatment success in sheep

SAMPLING INSTRUCTIONS

1. Select animals that have not been treated against liver fluke since spring. If this is not possible, please contact us to discuss alternative options.
2. Pen 2 groups with 12 animals per group, ideally from different fields because fluke burden may differ between fields.

3. Collect 10 faecal samples from the floor of each pen and put each sample in a separate pot. If possible, fill the pot beyond the black ring marked on the outside. u
4. Write the pen identification on each pot.

5. Weigh animals.
6. Treat animals with flukicide of choice and dose to individual weight or to the weight of the heaviest animal in the group.
7. Mark animals so that the same 2 groups of 12 animals can be gathered 21 days later.
8. Record the method of weighing, the treatment product and the date of treatment on the return slip.
9. Return pots on the day of sampling to the address listed on the return slip.
10. Collect the second set of samples from the same 2 groups of animals 21 days after the first set. We will send you a new set of pots for this purpose.

If you have any questions regarding sample collection or shipping, please contact us by phone (0131-4455111) or e-mail (Philip.skuce@moredun.ac.uk or Ruth.zadoks@moredun.ac.uk).

4.4 Composite evaluation post-treatment letter to farmers

21 October 2014

<ADDRESS>

Moredun Research Institute

Pentlands Science Park
Bush Loan
Penicuik, near Edinburgh
EH26 0PZ
Scotland, UK

Tel: +44 (0)131 445 5111
Fax: +44 (0)131 445 6111
Philip.Skuce@moredun.ac.uk
www.moredun.ac.uk

Re: Use of faecal samples for detection of liver fluke infection and treatment success in sheep

Dear <NAME>,

Thank you for your continued participation in our study. Please find enclosed your kit for sampling at 21 days after treatment. We have calculated that your next sampling should take place on <DATE>. Please use the same animals as at the previous sampling.

In your sampling kit you will find

a) 21 sample collection pots (two sets of 10 pots and one spare)
b) A return slip with a few questions
c) An envelope with freepost label for shipping of samples to Moredun
d) Gloves
e) A report of your pre-treatment results

After receipt of your samples, we will process them at the Moredun Research Institute and we will contact you with the results.

We have incorporated initial feedback into the new sampling kit. Once again we would be very interested in any feedback you have on the procedure.

We are trying to make this as simple and robust as possible and value your time and effort. If you have any questions regarding the study, please contact us by phone (0131-4455111) or e-mail (u or Ruth.zadoks@moredun.ac.uk).

Sincerely,

Philip Skuce, PhD Danielle Gordon, MSc Ruth Zadoks, DVM, PhD

4.5 Composite evaluation post-treatment information slip

Use of faecal samples for detection of liver fluke infection and treatment success in sheep – information to be included with samples on the day of treatment

1. Your name and address:

2. Date of treatment and sample collection:

3. Treatment product:

4. Method used to determine weight of sheep:

5. Comments on ease of use of the sampling materials and the sampling method (optional):

After collection, please return the faecal samples and this information slip to:

Danielle Gordon
The Moredun Foundation
FREEPOST EH1686
Penicuik
EH26 0FG

If you have any questions regarding sample collection or shipping, please contact us by phone (0131-4455111) or e-mail (Philip.skuce@moredun.ac.uk or Ruth.zadoks@moredun.ac.uk).

4.6 Group average and composite FEC and FECR

		Average					Composite				
		Pre-treatment		Post-treatment			Pre-treatment		Post-treatment		
Group	Farm	epg	Outcome	epg	Outcome	FECR (%)	epg	Ouctome	epg	Outcome	FECR (%)
1	3	2.3	Positive	0.0	Negative	100.00	2.0	Positive	0.0	Negative	100.00
2	3	2.4	Positive	0.0	Negative	100.00	4.7	Positive	0.0	Negative	100.00
3	6	41.4	Positive	0.9	Positive	97.87	35.5	Positive	2.5	Positive	92.96
4	6	46.3	Positive	126.7	Positive	-173.36	39.5	Positive	229.8	Positive	-481.86
5	7	0.4	Positive	0.0	Negative	100.00	0.3	Positive	0.0	Negative	100.00
6	7	1.0	Positive	0.0	Negative	100.00	1.3	Positive	0.0	Negative	100.00
7	10*	56.8	Positive	17.0	Positive	70.13	54.0	Positive	9.2	Positive	83.03
8	11	1.3	Positive	0.0	Negative	100.00	0.8	Positive	0.0	Negative	100.00
9	11	1.7	Positive	0.0	Negative	100.00	1.0	Positive	0.0	Negative	100.00
10	12	15.0	Positive	131.9	Positive	-776.58	10.8	Positive	136.7	Positive	-1161.57
11	12	139.8	Positive	0.2	Positive	99.87	102.5	Positive	0.0	Negative	100.00
12	16	0.0	Negative	0.0	Negative	No Fluke	0.0	Negative	0.0	Negative	No Fluke
13	16	0.0	Negative	0.1	Positive	No Fluke	0.0	Negative	0.0	Negative	No Fluke
14	17	3.8	Positive	0.7	Positive	81.50	2.7	Positive	0.0	Negative	100.00
15	17	3.5	Positive	0.6	Positive	81.83	2.0	Positive	0.7	Positive	66.70
16	18	0.1	Positive	-	-	-	0.0	Negative	-	-	-
17	18	16.3	Positive	-	-	-	22.2	Positive	-	-	-
18	19	0.0	Negative	0.0	Negative	No Fluke	0.0	Negative	0.0	Negative	No Fluke
19	19	0.7	Positive	0.1	Positive	88.41	1.0	Positive	0.0	Negative	100.00
20	20	0.0	Negative	0.0	Negative	No Fluke	0.0	Negative	0.0	Negative	No Fluke
21	20	1.1	Positive	0.4	Positive	61.78	0.2	Positive	0.7	Positive	-301.20
22	24*	7.1	Positive	0.0	Negative	100.00	4.8	Positive	0.0	Negative	100.00
23	26*	0.0	Negative	0.0	Negative	No Fluke	0.0	Negative	0.0	Negative	No Fluke
24	26*	1.3	Positive	0.0	Negative	100.00	1.2	Positive	0.0	Negative	100.00
25	27	2.6	Positive	-	-	-	5.2	Positive	-	-	-
26	27	38.8	Positive	5.1	Positive	86.99	32.3	Positive	4.8	Positive	85.05
27	28	13.1	Positive	59.9	Positive	-357.54	23.0	Positive	70.0	Positive	-204.35
28	28	48.2	Positive	0.1	Positive	99.86	36.5	Positive	0.0	Negative	100.00
29	29	4.6	Positive	4.8	Positive	-4.30	4.3	Positive	3.0	Positive	30.76
30	29	0.0	Negative	0.1	Positive	No Fluke	0.0	Negative	0.0	Negative	No Fluke
31	30	2.6	Positive	0.0	Negative	100.00	3.8	Positive	0.0	Negative	100.00
32	30	1.6	Positive	0.0	Negative	98.00	1.3	Positive	0.0	Negative	100.00
33	31*	0.2	Positive	-	-	-	0.0	Negative	-	-	-
34	31*	0.0	Negative	-	-	-	0.0	Negative	-	-	-
35	32	0.1	Positive	0.1	Positive	-28.45	0.3	Positive	0.0	Negative	100.00
36	33	7.0	Positive	0.0	Negative	100.00	7.3	Positive	0.0	Negative	100.00
37	33	21.1	Positive	0.0	Negative	100.00	16.3	Positive	0.0	Negative	100.00
38	34	0.0	Negative	0.0	Negative	No Fluke	0.0	Negative	0.0	Negative	No Fluke
39	34	0.0	Negative	0.0	Negative	No Fluke	0.0	Negative	0.0	Negative	No Fluke
40	36	0.0	Negative	-	-	-	0.0	Negative	-	-	-
41	37	2.1	Positive	0.1	Positive	95.28	2.0	Positive	0.3	Positive	83.35
42	37	0.8	Positive	0.0	Negative	95.60	0.3	Positive	0.0	Negative	100.00
43	38*	1.3	Positive	-	-	-	0.2	Positive	-	-	-
44	41	0.2	Positive	-	-	-	0.0	Negative	-	-	-

*indicates farms which did not report estimating weight prior to dosing

4.7 Group average and composite cELISA and CR

		Average					Composite				
		Pre-treatment		Post-treatment			Pre-treatment		Post-treatment		
Group	Farm	PD	Outcome	PD	Outcome	CR (%)	PD	Ouctome	PD	Outcome	CR (%)
1	3	28.679	Positive	0.298	Negative	98.96	33.589	Positive	1.364	Negative	95.94
2	3	46.938	Positive	0.675	Negative	98.56	76.332	Positive	0.754	Negative	99.01
3	6	72.007	Positive	0.683	Negative	99.05	85.949	Positive	0.711	Negative	99.17
4	6	87.086	Positive	75.839	Positive	12.91	103.171	Positive	93.767	Positive	9.11
5	7	0.883	Negative	2.080	Negative	No Fluke	0.423	Negative	1.825	Negative	No Fluke
6	7	1.168	Negative	1.375	Negative	No Fluke	1.321	Negative	2.433	Negative	No Fluke
7	10*	95.377	Positive	65.628	Positive	31.19	106.913	Positive	75.161	Positive	29.70
8	11	10.828	Positive	1.130	Negative	89.57	14.015	Positive	1.170	Negative	91.65
9	11	7.706	Positive	0.234	Negative	96.96	10.524	Positive	-0.153	Negative	101.45
10	12	73.327	Positive	65.013	Positive	11.34	70.864	Positive	93.925	Positive	-32.54
11	12	97.417	Positive	0.197	Negative	99.80	106.193	Positive	0.376	Negative	99.65
12	16	3.721	Negative	0.462	Negative	No Fluke	3.685	Negative	0.664	Negative	No Fluke
13	16	1.192	Negative	3.105	Negative	No Fluke	1.445	Negative	1.680	Negative	No Fluke
14	17	10.820	Positive	3.342	Negative	69.11	18.567	Positive	1.644	Negative	91.15
15	17	14.123	Positive	4.135	Negative	70.72	14.601	Positive	6.985	Positive	52.16
16	18	-1.212	Negative	-	-	-	2.389	Negative	-	-	-
17	18	13.578	Positive	-	-	-	11.364	Positive	-	-	-
18	19	0.790	Negative	0.468	Negative	No Fluke	1.003	Negative	0.847	Negative	No Fluke
19	19	5.870	Negative	1.048	Negative	No Fluke	3.512	Negative	0.052	Negative	No Fluke
20	20	0.463	Negative	0.620	Negative	No Fluke	0.349	Negative	1.181	Negative	No Fluke
21	20	2.569	Negative	0.738	Negative	No Fluke	3.940	Negative	1.181	Negative	No Fluke
22	24*	34.384	Positive	0.153	Negative	99.56	35.762	Positive	0.153	Negative	99.57
23	26*	0.122	Negative	0.136	Negative	No Fluke	0.316	Negative	0.369	Negative	No Fluke
24	26*	14.390	Positive	-0.105	Negative	100.73	24.040	Positive	3.616	Negative	84.96
25	27	51.820	Positive	-	-	-	63.633	Positive	-	-	-
26	27	73.160	Positive	27.086	Positive	62.98	52.422	Positive	35.408	Positive	32.45
27	28	31.051	Positive	49.926	Positive	-60.79	33.780	Positive	31.993	Positive	5.29
28	28	71.345	Positive	1.049	Negative	98.53	88.401	Positive	0.393	Negative	99.56
29	29	25.326	Positive	4.941	Negative	80.49	34.197	Positive	5.038	Negative	85.27
30	29	0.013	Negative	0.438	Negative	No Fluke	0.084	Negative	6.819	Positive	No Fluke
31	30	9.814	Positive	0.705	Negative	92.82	11.576	Positive	0.888	Negative	92.33
32	30	15.634	Positive	1.857	Negative	88.12	15.266	Positive	4.840	Negative	68.30
33	31*	1.450	Negative	-	-	-	1.922	Negative	-	-	-
34	31*	0.220	Negative	-	-	-	-0.110	Negative	-	-	-
35	32	1.006	Negative	0.015	Negative	No Fluke	1.143	Negative	0.149	Negative	No Fluke
36	33	25.915	Positive	0.222	Negative	99.15	29.436	Positive	-0.180	Negative	100.61
37	33	38.517	Positive	0.032	Negative	99.92	43.207	Positive	0.451	Negative	98.96
38	34	0.053	Negative	0.603	Negative	No Fluke	0.680	Negative	-0.260	Negative	No Fluke
39	34	0.151	Negative	0.140	Negative	No Fluke	-0.529	Negative	0.052	Negative	No Fluke
40	36	0.158	Negative	-	-	-	4.367	Negative	-	-	-
41	37	4.791	Negative	3.306	Negative	No Fluke	7.464	Positive	3.733	Negative	49.99
42	37	2.912	Negative	1.202	Negative	No Fluke	2.807	Negative	1.436	Negative	No Fluke
43	38*	8.069	Positive	-	-	-	11.281	Positive	-	-	-
44	41	1.108	Negative	-	-	-	-0.052	Negative	-	-	-

*indicates farms which did not report estimating weight prior to dosing

Appendix 5

5.1 Summary of DNA amplification by PCR and LAMP

Sample	Farm	FEC (epg)	cELISA (PD)	DNA extraction method	PCR	LAMP
F2897	10	**4**	**17.26**	DNEasy® Blood and Tissue	✗	✗
F2904	10	**37.17**	**58.32**	DNEasy® Blood and Tissue	✗	✓
F2994*^	44	**95**	**121.71**	QIAmp® DNA Stool Mini	✗	✗
F2995*	44	**112.67**	**68.45**	QIAmp® DNA Stool Mini	✓	✓
F2996*	44	**143.83**	**57.6**	QIAmp® DNA Stool Mini	✗	✓
F2997*^	44	**103.5**	**115.52**	QIAmp® DNA Stool Mini	✗	✓
F2998*	44	**150.5**	**29.87**	QIAmp® DNA Stool Mini	✓	✗
F2999*	44	**249.83**	**60.67**	QIAmp® DNA Stool Mini	✗	✓
F3000*	44	**318.5**	**109.69**	QIAmp® DNA Stool Mini	✗	✓
F3205	44	**208**	**104.57**	Freeze/thaw	ND	✓
F3207	44	**121**	**10.11**	Freeze/thaw	ND	✗
F3208	44	**82**	**80.75**	Freeze/thaw	ND	✓
F3543	45	**17.83**	0.59	Freeze/thaw	✗	✓
F3578	23	0	1.12	QIAmp® FAST DNA Stool Mini	ND	✗
F3582	45	**0.67**	-2.79	QIAmp® FAST DNA Stool Mini	ND	✓
F3586	10	**3.83**	2.79	QIAmp® FAST DNA Stool Mini	ND	✗
F3590	10	**34.5**	-6.7	QIAmp® FAST DNA Stool Mini	ND	✓

* indicates a composite sample, ^ indicates rumen fluke eggs present in faecal sample, **bold** indicates a positive test result, ✓ indicates DNA amplification, ✗ indicates no DNA amplification. ND = not done.

5.2 Individual animal FEC, cELISA, LAMP and PCR results 0 - 18 wpc

						LAMP	PCR	
Animal ID	Liver burden	Faecal ID	wpc	FEC (epg)	cELISA (PD)	Fh ITS-2	ITS-2+	Cox_1
A1330	44	F3064	0	0.00	0.535	×	×	×
		F3081	1	0.00	-0.049	×	×	×
		F3099	2	0.00	-0.181	×	×	×
		F3117	3	0.00	-0.072	×	×	×
		F3135	4	0.00	0.072	×	×	×
		F3153	5	0.00	-0.298	✓	×	×
		F3172	6	0.00	0.179	✓	×	×
		F3189	7	0.00	1.432	×	×	×
		F3218	8	0.00	3.401	×	×	×
		F3236	9	0.00	**13.795**	✓	×	×
		F3254	10	**0.83**	**39.077**	×	×	×
		F3281	11	**11.17**	**13.419**	✓	×	×
		F3305	12	**8.67**	**49.656**	✓	×	×
		F3328	13	**11.50**	**60.233**	✓	✓	×
		F3352	14	**22.83**	**51.177**	✓	×	✓
		F3378	15	**25.83**	**39.026**	✓	×	✓
		F3412	16	**42.00**	**42.638**	✓	×	×
		F3430	17	**37.00**	**31.879**	✓	×	×
		F3454	18	**87.67**	**60.301**	ND	ND	ND

						LAMP	PCR	
Animal ID	Liver burden	Faecal ID	wpc	FEC (epg)	cELISA (PD)	Fh ITS-2	ITS-2+	Cox_1
A1331	22	F3065	0	0.00	0.243	×	×	×
		F3082	1	0.00	1.362	×	×	×
		F3100	2	0.00	0.121	×	×	×
		F3118	3	0.00	1.514	✓	×	×
		F3136	4	0.00	0.577	✓	×	×
		F3154	5	0.00	0.358	✓	×	×
		F3173	6	0.00	0.298	✓	×	×
		F3190	7	0.00	**9.248**	×	×	×
		F3219	8	0.00	**21.882**	×	×	×
		F3237	9	0.00	**19.324**	×	×	×
		F3255	10	**1.83**	**39.936**	×	×	×
		F3282	11	**5.00**	**22.437**	×	×	×
		F3306	12	**10.67**	**76.626**	✓	×	×
		F3329	13	**9.33**	**72.448**	✓	✓	×
		F3353	14	**9.33**	**64.696**	✓	×	✓
		F3379	15	**14.67**	**37.876**	×	×	×
		F3413	16	**22.67**	**65.955**	✓	×	×
		F3431	17	**13.83**	**71.621**	✓	×	×
		F3455	18	**39.50**	**40.559**	ND	ND	ND

Bold indicates a positive test result, ND indicates no data, ✓ indicates DNA amplification, × indicates no DNA amplification

						LAMP	PCR	
Animal ID	Liver burden	Faecal ID	wpc	FEC (epg)	cELISA (PD)	Fh ITS-2	ITS-2+	Cox_1
A1333	41	F3067	0	0.00	0.097	×	×	×
		F3084	1	0.00	0.486	×	×	×
		F3102	2	0.00	0.121	×	×	×
		F3120	3	0.00	0.360	✓	×	×
		F3138	4	0.00	0.288	×	×	×
		F3156	5	0.00	0.298	✓	×	×
		F3174	6	0.00	0.895	✓	×	×
		F3192	7	0.00	1.909	×	×	×
		F3221	8	0.00	**29.478**	×	×	×
		F3239	9	0.00	**32.313**	×	×	×
		F3257	10	**10.00**	**56.575**	×	×	×
		F3284	11	**13.33**	**31.669**	✓	×	×
		F3308	12	**36.50**	**81.438**	×	×	×
		F3331	13	**38.33**	**52.565**	✓	×	×
		F3355	14	**34.17**	**52.983**	✓	×	✓
		F3381	15	**42.83**	**39.901**	✓	×	✓
		F3415	16	**53.33**	**75.753**	✓	×	✓
		F3433	17	**47.00**	**68.282**	×	✓	✓
		F3457	18	**29.50**	**59.010**	ND	ND	ND

						LAMP	PCR	
Animal ID	Liver burden	Faecal ID	wpc	FEC (epg)	cELISA (PD)	Fh ITS-2	ITS-2+	Cox_1
A1337	36	F3071	0	0.00	3.533	×	×	×
		F3088	1	0.00	-0.380	×	×	×
		F3106	2	0.00	0.423	×	×	×
		F3124	3	0.00	0.865	✓	×	×
		F3142	4	0.00	0.433	×	×	×
		F3160	5	0.00	0.537	×	×	×
		F3175	6	0.00	0.656	×	×	×
		F3196	7	0.00	1.644	×	×	×
		F3225	8	0.00	3.118	×	×	×
		F3243	9	0.00	3.060	×	×	×
		F3261	10	**1.00**	**18.680**	×	×	×
		F3288	11	**1.83**	**23.956**	✓	×	×
		F3312	12	**4.67**	**82.708**	×	×	×
		F3335	13	**18.17**	**62.718**	✓	×	×
		F3359	14	**36.83**	**74.767**	✓	×	✓
		F3385	15	**48.33**	**5.309**	✓	×	×
		F3419	16	**28.67**	**49.865**	✓	×	×
		F3437	17	**31.00**	**70.921**	✓	×	✓
		F3461	18	**36.8**	**51.641**	ND	ND	ND

Bold indicates a positive test result, ND indicates no data, ✓ indicates DNA amplification, × indicates no DNA amplification

						LAMP	PCR	
Animal ID	Liver burden	Faecal ID	wpc	FEC (epg)	cELISA (PD)	Fh ITS-2	ITS-2+	Cox_1
A1339	42	F3073	0	0.00	0.097	×	×	×
		F3090	1	0.00	0.489	×	×	×
		F3108	2	0.00	0.060	×	×	×
		F3126	3	0.00	0.216	✓	×	×
		F3144	4	0.00	0.937	✓	×	×
		F3162	5	0.00	0.298	✓	×	×
		F3176	6	0.00	0.239	×	×	×
		F3198	7	0.00	0.794	×	×	×
		F3227	8	0.00	4.025	×	×	×
		F3245	9	0.00	**12.131**	✓	×	×
		F3263	10	**1.00**	**52.281**	✓	×	×
		F3290	11	**9.00**	**40.243**	✓	×	×
		F3314	12	**12.83**	**86.092**	✓	×	×
		F3337	13	**33.50**	**53.041**	✓	×	×
		F3361	14	**34.17**	**68.747**	✓	×	✓
		F3387	15	**51.17**	**52.326**	✓	×	✓
		F3421	16	**51.50**	**48.358**	✓	×	×
		F3439	17	**48.00**	**70.652**	✓	×	×
		F3463	18	**110.33**	**89.672**	ND	ND	ND

						LAMP	PCR	
Animal ID	Liver burden	Faecal ID	wpc	FEC (epg)	cELISA (PD)	Fh ITS-2	ITS-2+	Cox_1
A1342	29	F3076	0	0.00	0.243	×	×	×
		F3093	1	0.00	0.217	×	×	×
		F3111	2	0.00	-0.060	×	×	×
		F3129	3	0.00	0.577	✓	×	×
		F3147	4	0.00	1.081	✓	×	×
		F3165	5	0.00	0.179	✓	×	×
		F3183	6	0.00	0.537	×	×	×
		F3201	7	0.00	0.624	×	×	×
		F3230	8	0.00	3.685	×	×	×
		F3248	9	0.00	**7.676**	×	×	×
		F3266	10	**1.50**	**36.715**	×	×	×
		F3293	11	**6.17**	**29.561**	✓	×	×
		F3317	12	**7.50**	**66.208**	✓	×	×
		F3340	13	**15.17**	**51.243**	✓	×	×
		F3364	14	**23.00**	**66.174**	×	×	✓
		F3390	15	**26.50**	**64.039**	×	×	×
		F3424	16	**31.67**	**75.552**	✓	×	×
		F3442	17	**35.00**	**64.890**	✓	×	×
		F3466	18	**38.5**	**49.919**	ND	ND	ND

Bold indicates a positive test result, ND indicates no data, ✓ indicates DNA amplification, × indicates no DNA amplification

Appendix 6

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On farm evaluation of the coproantigen ELISA and coproantigen reduction test in Scottish sheep naturally infected with *Fasciola hepatica*

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ABSTRACT

The liver fluke, *Fasciola hepatica*, is a cause of significant economic losses in sheep farming. Lack of convenient and sensitive diagnostic tests in the live animal hampers the ability to monitor infection status and treatment efficacy. Use of a coproantigen ELISA and coproantigen reduction test, based on this ELISA, may address these issues but has, to date, only been evaluated in experimental challenge studies. We evaluated the coproantigen ELISA under working farm conditions in Scotland to assess its usefulness as a diagnostic test for liver fluke infection and as a diagnostic test to help determine the efficacy of flukicide treatment in sheep. First, liver fluke infection status was monitored longitudinally in a group of lambs, using monthly blood samples for biochemical assays and serum antibody ELISA and using monthly faecal samples for faecal egg count (FEC) and coproantigen ELISA. The average serum antibody ELISA titre became positive in September, two months ahead of faecal indicators of fluke infection. In contrast to results from experimental challenge studies, FEC and coproantigen ELISA became positive at the same time point. Secondly, treatment efficacy was measured in 100 ewes, from two farms, after treatment with triclabendazole (TCBZ) or closantel. Group level estimates of treatment efficacy were similar between faecal egg count reduction testing and coproantigen reduction testing at 7, 14 and 21 days post treatment. For individual animals, some inconsistencies between tests were observed. TCBZ treatment failure was noted on both farms, despite accurate weighing of animals and dosing of treatment products. We conclude that (1) coproantigen ELISA is a more convenient test than faecal egg counts and holds promise as a diagnostic tool for natural fluke infections in sheep but further evaluation of interpretation criteria may be needed; (2) the coproantigen ELISA has performed differently in the field compared with experimental challenge studies in sheep and (3) TCBZ-resistant fluke were present on both farms.

1. Introduction

The liver fluke, *Fasciola hepatica*, is a common trematode parasite of ruminants, which causes significant economic losses to the sheep industry. In Scotland, fluke have traditionally been seen in the wetter western areas, however, over recent years, cases have been reported in the eastern and north-eastern regions in increasing numbers (Mitchell, 2002; Kenyon et al., 2009). Factors that are thought to contribute to the increasing prevalence of liver fluke include climate change, animal movements, treatment failure and changes in farming practices associated with climate change (e.g. extended grazing periods) or

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environmental regulations (e.g. preservation of wetlands) (Daniel and Mitchell, 2002; Pritchard et al., 2005; Kenyon et al., 2009). With the increase in prevalence, accurate diagnostics are needed to manage and control the spread of this parasite.

Diagnosis in the live animal is commonly performed via one of the following methods; abnormal liver (glutamate dehydrogenase (GLDH)) or bile duct (gamma-glutamyl transferase (GGT)) enzyme levels (Mitchell, 2002; Matanović et al., 2007), detection of anti *F. hepatica* antibodies in serum (serum antibody ELISA) (Levieux and Levieux, 1994) and faecal egg count (FEC) (Dennis et al., 1954). A coproantigen ELISA, (BIO K201, Bio-X Diagnostics), based upon the MM3 monoclonal antibody (Mezo et al., 2004) is now commercially available. This has shown promise in experimental infections of sheep due to the reported ability to detect antigen from a single parasite (Mezo et al., 2004) and the ability to detect infection from 5 weeks post infection (wpi) (Flanagan et al., 2011b), considerably earlier than FEC, which becomes positive at 9–15 wpi (Valero et al., 2006).

However, these tests are not without their limitations. For example, due to the persistence of anti-*F. hepatica* antibodies in serum after successful treatment (Ibarra et al., 1998; Sánchez et al., 2001), the serum antibody ELISA is limited in its ability to determine current infections and is, rather, an indicator of previous exposure. An increase in GLDH and GGT levels is not pathognomonic for fasciolosis and should not be used for fluke diagnosis in isolation. In addition, tests which require blood samples are invasive and require the presence of a trained professional for sample collection. Faecal samples are more convenient and less invasive in terms of collection. However, eggs are typically not released until months after infection, at which point they may be excreted intermittently from the host (Mezo et al., 2004), leading to potential false negatives by FEC. Furthermore, due to sequestration in the gall bladder, eggs may be seen up to 3 weeks post successful treatment (Chowaniec and Darski, 1970), leading to potential false positives, as here the eggs are indicators of past infection. Whilst the coproantigen ELISA shows promise as a sensitive and specific indicator of live infection with *F. hepatica* (Mezo et al., 2004, 2007; Ubeira et al., 2009; Valero et al., 2009; Flanagan et al., 2011a,b), it has yet to be evaluated in natural infections of sheep.

The main strategies for liver fluke control have been grazing management to avoid intermediate host snail habitats and the use of strategic flukicide treatments (Sargison and Scott, 2011a). The most commonly used flukicide is triclabendazole (TCBZ) due to its unique ability to target fluke from 1 wpi in sheep (Boray et al., 1983). It is immature fluke which are responsible for acute cases of fasciolosis and hence, a large portion of the economic losses. However, there have been widespread reports of treatment failure and suspected resistance to TCBZ (Overend and Bowen, 1995; Anon, 1998; Lane, 1998; Moll et al., 2000; Thomas et al., 2000; Mitchell, 2002; Álvarez-Sánchez et al., 2006; Olaechea et al., 2011). Although there is debate in the veterinary press as to the usefulness of such declarations (Fairweather, 2011b; Sargison and Scott, 2011b), it is clear that reliable diagnostic tests to monitor treatment outcomes are urgently needed.

The only tests currently available to evaluate drug efficacy in fluke infections are dose and slaughter trials (Coles et al., 2006). Because this test requires the euthanasia of animals, it is highly impractical for farmers. The most widely used indicator of treatment failure is the faecal egg count reduction test (FECRT) (Fairweather, 2011b). Whilst detailed guidelines exist for conducting and interpreting FECRT of nematodes, no equivalent guidelines exist for trematodes (Coles et al., 1992, 2006; Torgerson et al., 2005). As an alternative to the FECRT, use of a coproantigen reduction test (CRT), based on the coproantigen ELISA, has been proposed for evaluation of flukicide efficacy (Flanagan et al., 2011a). This was shown to be effective in experimental infections of sheep, using isolates of known resistance status (Flanagan et al., 2011a). However, it has yet to be trialled under field conditions in naturally infected sheep.

In this study, we report the kinetics of coproantigen detection alongside other standard diagnostic tests including biochemistry, serology and FEC, in lambs with natural exposure to *F. hepatica*. In addition, we compared the performance of the FECRT and CRT, under genuine farm conditions with sheep naturally infected with suspected TCBZ resistant fluke.

2. Materials and methods

2.1. Longitudinal monitoring of infection status

A convenience sample of 27 Scottish Blackface × Blueface Leicester lambs was selected from a flock in Dumfries and Galloway (Farm 1). Lambs were born in April and sampled at the end of each month from June through November 2010. Individual blood and faecal samples were collected and investigated by biochemical assay, serum antibody ELISA, FEC and coproantigen ELISA, as detailed below. Serum samples were processed on the day of collection and faecal samples were stored at 4 °C until investigation. One lamb died after the August sampling (not due to fasciolosis).

A subset of 10 animals could be followed to slaughter in November or January and livers were investigated for the presence of parasites as, described below, to obtain an indication of fluke burden.

2.2. Treatment trial

Two farms with an anecdotal history of TCBZ treatment failure were selected for the treatment trial. Farm 1 is described in Section 2.1 and housed the ewes for this study. Farm 2 was located in the southeast of Scotland and kept Scottish Blackface × Blueface Leicester ewes on pasture. Animals from both farms ($n = 70$ and $n = 68$, respectively) were pre-screened by FEC and coproantigen ELISA for evidence of fluke infection in December 2010, using individual faecal samples.

In January 2011, 56 ewes from Farm 1 and 44 ewes from Farm 2, which had tested positive by both coproantigen ELISA and FEC at pre-screening, were alternately allocated to a TCBZ (Fasinex®, Novartis Animal Health) or a

closantel (Flukiver®, Janssen Animal Health) treatment group, based upon the order in which they were sampled. Farm 1 ewes were weighed individually and dosed according to the manufacturer's guidelines, with the modification that the dosage was rounded to the nearest ml and 1 ml added to allow for loss in the syringe during dosing. Farm 2 ewes were dosed to 80 kg as no animals were estimated to exceed this weight. All dosing was performed using a 20 ml syringe to ensure accuracy (Coles et al., 1992). Because the study took place on working farms with in-lamb ewes, animals that did not respond to the initial treatment were treated with the alternative product at 21 days after the initial treatment. This ensured that the welfare of the animals was not compromised. For the same reason, an untreated control group was not included (Mooney et al., 2009). Rectal faecal samples were taken at 0, 7, 14, 21 and 56 days post treatment (dpt) to observe the kinetics of egg and antigen production post-treatment. Samples were collected using clean gloves for each animal and individually stored in 50 ml Falcon tubes at 4 °C until testing.

2.3. Diagnostic tests

2.3.1. Diagnosis of infection using invasive tests

Serum was tested using commercial biochemical assay kits for the determination of GLDH and GGT levels (Randox Ltd. and Instrumentation Laboratory UK Ltd., respectively) on an IL600 random access analyser using standard methods. Current Scottish Agricultural College Consulting: Veterinary Services (SAC C VS) reference ranges are <25 IU/L and <50 IU/L, for GLDH and GGT, respectively. The Pourquier *Fasciola hepatica* serum ELISA (Institut Pourquier, France) was performed and interpreted according to the manufacturer's guidelines.

Fluke were extracted from livers at slaughter following the method outlined in Clery et al. (1996), with the addition that livers were frozen on receipt for investigation at a later date, as examination was not always possible on the day of collection. Retrieved fluke were placed in water and examined under a light microscope. Indicators of adult status, such as development of the reproductive system and width of shoulders, were noted. For partial flukes recovered, the numbers of anterior and posterior ends were counted and the larger figure added to the number of whole fluke seen in order to determine liver burden.

2.3.2. Diagnosis of infection using faecal samples: FEC and coproantigen ELISA

FECs were performed on all samples within 1 week of collection using 3 g of faeces per animal. For the longitudinal study, FEC was performed by SAC C VS, Dumfries, using standard methods. Briefly, samples were homogenised with water, sieved through 710, 150 and 38 μm sieves in sequence and sedimented in water for 4 min. The supernatant was then removed and the sediment re-suspended in water. This was repeated as necessary until the supernatant was clear. The final sediment was viewed in a petri dish, with a few drops of 0.1% aqueous malachite green.

For the treatment trial, FEC was performed at the Moredun Research Institute using a modified version of the method outlined by McCaughey and Hatch (1964). Briefly, 3 g of each sample was homogenised with 42 ml of water and poured through a strainer into a beaker. The filtrate was then poured through a 150 μm sieve into a conical measure. The beaker was half-filled with water and poured through the sieve again to wash any remaining eggs through to the conical measure. The conical measure was allowed to sit at a slight angle for 3 min at which point the supernatant was siphoned off. A drop of 1% (w/v) methylene blue was added to the sediment, which was examined on a marked petri dish at 16× magnification using a light microscope. Each sample dish was counted twice and an average taken, which was divided by 3 to calculate the epg. An animal was considered positive when at least 1 egg was detected.

The coproantigen ELISA was performed according to manufacturer's guidelines with the following modifications. All samples were homogenised prior to weighing out the 0.5 g (±0.03 g) used in the ELISA and all samples were vortexed for 10 s prior to centrifugation. Samples were prepared to supernatant stage and stored in 1.5 ml Eppendorf tubes at −20 °C until testing, no longer than 6 weeks following collection. A titre of ≥0.15 is considered positive by the BIO K201 kit criteria.

2.3.3. Diagnosis of treatment efficacy using faecal samples: FECRT and CRT

The reduction in the FEC from 0 dpt to each subsequent sampling point was calculated using the formula below (Coles et al., 1992), which was developed for nematodes, and where FEC_0 is the group arithmetic mean FEC at 0 dpt and FEC_t is the group arithmetic mean FEC at a subsequent time point (7, 14, 21 or 56 dpt) (Levecke et al., 2011).

$$\%\,\text{reduction} = \left(\frac{FEC_0 - FEC_t}{FEC_0}\right) \times 100$$

The CRT was interpreted as proposed by Flanagan et al. (2011b), who stated that if all animals are negative at 14 dpt, the treatment is deemed successful. In addition, the proportion of animals testing negative by FEC and coproantigen ELISA was noted at all sampling points

2.4. Statistics

All statistical tests were performed using GraphPad Prism. Because the data were not normally distributed, a non-parametric *t*-test (Mann–Whitney) was used to compare differences between treatment groups and farms.

3. Results

3.1. Longitudinal study

3.1.1. Diagnosis of infection using invasive assays

Blood samples were collected from all animals at each sample point ($n = 27$ from June through August, $n = 26$ from September through November). Mean GLDH and GGT levels are shown in Fig. 1. Mean values for GLDH were normal in June and July and increased beyond reference levels (<25 IU/L) from August onwards, indicating onset of infection in July (Sandeman and Howell, 1981; Ferre et al., 1994, 1996, 1997; Phiri et al., 2007; Raadsma et al., 2007). At an

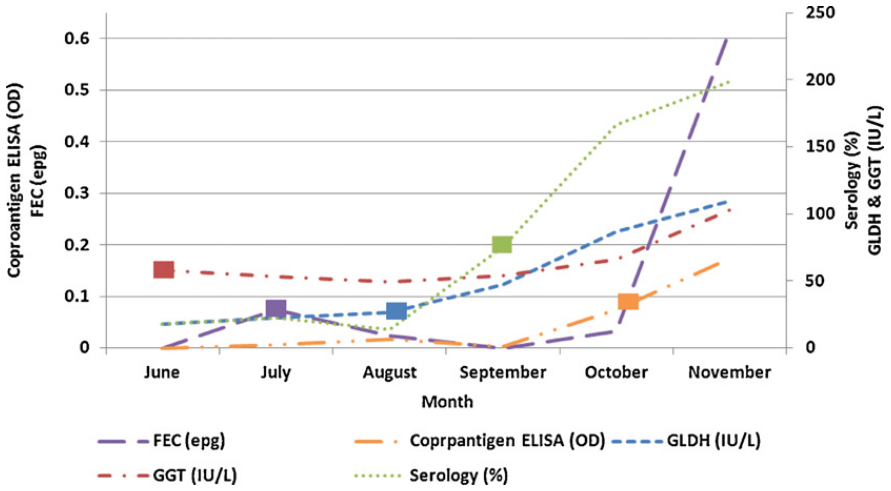

Fig. 1. Changes in group mean test results are shown on the left axis for FEC (epg), coproantigen ELISA (Optical Density) and on the right axis for biochemical changes (GLDH, GGT) and serum antibody ELISA (serology). ■ indicates the mean value at which the group first exceeded reference value. Stated detection limits indicate infection occurring in April (GGT), May (FEC), July (GLDH), or September (antibody ELISA and coproantigen ELISA).

individual level, 4 animals were positive in June, 6 in July, 11 in August, 16 in September, 22 in October and 23 at the last sampling in November (Supplementary Table S1).

The mean level of GGT was above reference levels (<50 IU/L) at the start of the study with marginally normal mean values in August (49.33 IU/L), followed by an increase from then on. Positive results in June indicate that onset of infection would have been in April (Sandeman and Howell, 1981; Ferre et al., 1994, 1996, 1997; Phiri et al., 2007; Raadsma et al., 2007). At the individual level, results fluctuated by this test, with 23 animals positive in June, 17 in July, 8 in August, 11 in September, 16 in October and 20 in November (Table S1).

The results of the serological antibody ELISA are shown in Fig. 1. Mean values were above the positive cut-off value by late September and remained so until the end of the study. The estimated point of infection was early September based on this test (Reichel, 2002). At the individual level, the number of positive animals per month was 4, 4, 3, 16, 18 and 24 for the months June through November. The infection status of three animals fluctuated between months, changing from positive to negative and back. In November, all animals except one were positive; this animal had not achieved a positive result throughout the study. The results of individual animals are presented in Supplementary Table S1.

Ten livers from lambs in this study were examined at slaughter. Fluke burdens ranged from 2 to 10 adult parasites (mean = 5.71, median = 5). No juvenile fluke were seen (Table S1).

3.1.2. Faecal tests

Rectal faecal samples could be collected from 27 animals in June and July, 25 in August, 26 in September and 23 in October and November, although the quantity of material was occasionally insufficient to determine FEC (4 and 3 samples, respectively, in October and November; Table S1). The results of the FEC are shown in Fig. 1. All animals were negative in June. A low mean egg count was seen in July (0.07 epg) and August (0.03 epg). No eggs were seen in the group in September and low group egg counts were seen in October and November (0.03 and 0.61 epg, respectively). Individual animals first had a positive egg count in July, resulting from 3 animals (mean = 0.66 epg). In August, only 1 animal was positive, with a FEC of 0.66 epg. No eggs were seen in the samples collected in September, but in October two animals were positive (mean = 0.33 epg). In November, 14 animals were positive (mean = 0.88 epg) and the group mean was 0.33 epg. The estimated point of infection, based on a group level positive faecal egg count in July, was May (Valero et al., 2006).

The changes in mean coproantigen titres are shown in Fig. 1. The mean value was above the positive threshold in November. One animal tested positive in July (Table S1), but negative in August, September and October. The number of positive animals started to increase in October ($n = 4$) and was highest in November ($n = 13$). The estimated point of infection by coproantigen ELISA, based on group level positive results in November, was September (Flanagan et al., 2011b).

3.2. Treatment trial

3.2.1. FEC and FECRT

All animals in this study had a positive FEC at 0 dpt (0.17 to 229.6 epg). All TCBZ treated ewes retained a positive egg count, until they were treated with closantel 21 days after the initial TCBZ treatment. A proportion of closantel treated ewes, ranging from 13 to 38%, also retained a positive, albeit low, egg count. The proportion of animals in each treatment group testing positive at each sampling point on each farm is shown in Table 1.

On Farm 1, individual epg ranged from 0.17 to 165 at 0 dpt with a mean of 33.43 and a median of 23.08. The individual epg on Farm 2 ranged from 1.5 to 229.67 at 0 dpt with a mean of 36.06 and a median of 21.5. Group epg prior to treatment was not different between the two treatment groups on either farm ($P = 0.08$ and $P = 0.24$ for Farms 1 and 2, respectively), as can be seen in Fig. 2.

Table 1
Faecal egg count reduction and proportion of animals positive by faecal egg count (FEC), on Farms 1 and 2, treated with TCBZ or closantel, at 0, 7, 14, 21 and 56 days post treatment.

dpt	Faecal egg count reduction[a]				Proportion of animals FEC positive			
	Farm 1		Farm 2		Farm 1		Farm 2	
	TCBZ	Closantel	TCBZ	Closantel	TCBZ	Closantel	TCBZ	Closantel
0	–	–	–	–	100%	100%	100%	100%
7	47.4%	97.5%	14.6%	96.8%	100%	30%	100%	38%
14	12.2%	99.3%	58.9%	97.8%	100%	19%	100%	13%
21	36.7%	99.7%	19.2%	99.3%	100%	11%	100%	13%
56*	99.6%	99.4%	100.0%	99.4%	22%	22%	6%	21%

* indicates 35 days post closantel treatment of the TCBZ group.

[a] Based on 29 observations for the TCBZ group on Farm 1, with the exception of 56 dpt ($n = 27$); 27 observations for the closantel group on Farm 1; 20 observations for the TCBZ group on Farm 2, with the exception of 14 dpt ($n = 19$) and 56 dpt ($n = 16$); 24 observations for the closantel group on Farm 2, with the exception of 21 dpt ($n = 23$) and 56 dpt ($n = 14$).

At 7 dpt, a significant difference in epg between treatment groups was seen on both farms ($P = 0.0001$), with the mean epg in the TCBZ group being 23.03 and 37.41 compared to the closantel group at 0.56 and 0.94 epg on Farms 1 and 2, respectively. At 14 dpt, mean epg in the TCBZ group was 38.49 and 18.03 compared to 0.17 and 0.64 epg in the closantel groups on Farms 1 and 2, respectively ($P = 0.0001$). At 21 dpt, mean FEC decreased in the TCBZ group on Farm 1 (27.74 epg), but increased on Farm 2 (35.41 epg). Both values were significantly higher than those of the closantel groups, 0.07 and 0.19 epg, respectively ($P = 0.0001$).

As FEC indicated that TCBZ treatment had not been successful, TCBZ treated ewes on both farms were treated with closantel at 21 dpt, with the exception of two ewes on Farm 1. Thus, the last sampling was 56 dpt relative to the original TCBZ and closantel treatments and 35 dpt relative to the closantel treatment of the TCBZ groups. At this point the mean epg in the TCBZ + closantel treated groups decreased to 0.15 and 0.02 epg on Farms 1 and 2, respectively. The mean FEC of the closantel-only treated groups remained low at 0.14 and 0.17 epg. The two ewes that had not been treated with closantel received additional TCBZ treatment at 35 dpt and 126 dpt and continued to shed eggs at high levels. Data for those animals were not included in group level calculations for 56 dpt.

The results of the group FECRT for all groups on both farms at all sampling points are shown in Table 1. On Farms 1 and 2, a >95% reduction in FEC was seen in the closantel

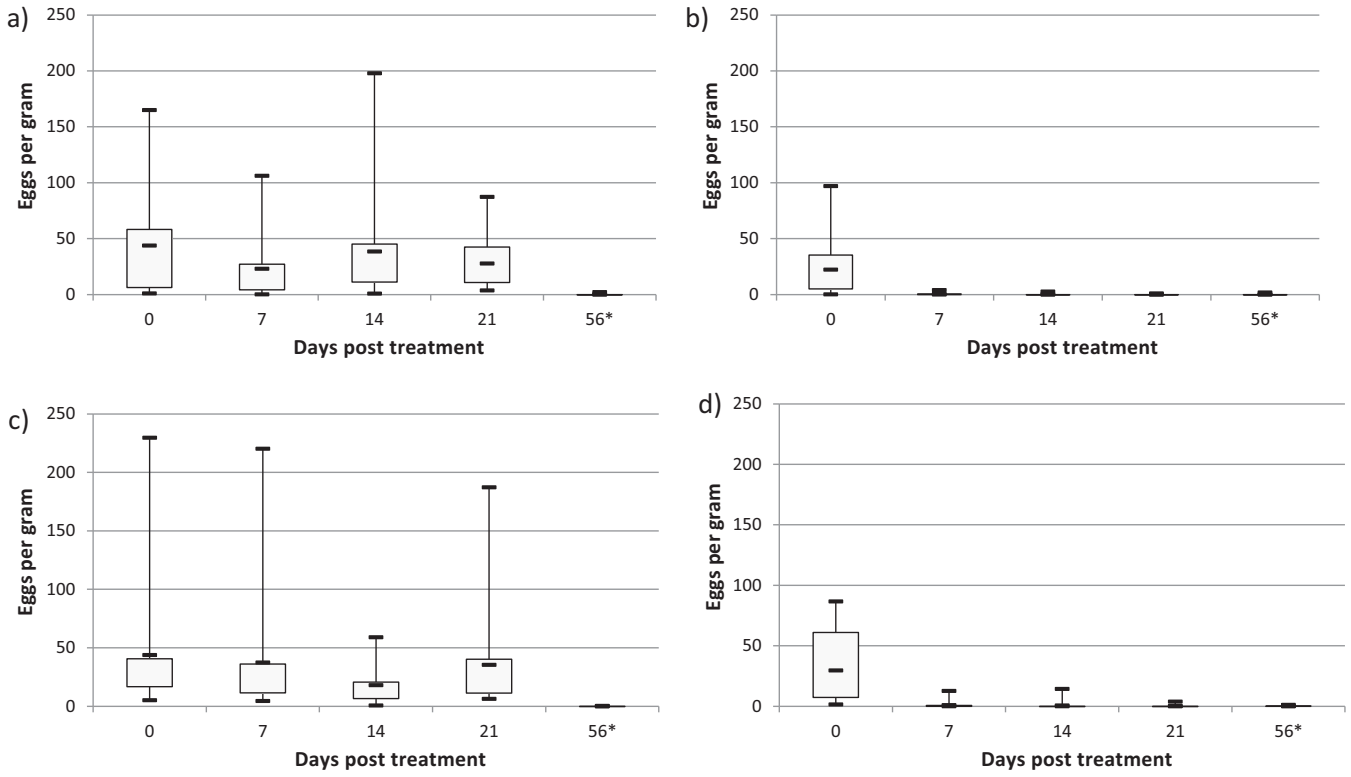

Fig. 2. Eggs per gram (epg) of the Farm1 TCBZ (a) and closantel (b) and the Farm 2 TCBZ (c) and closantel (d) treatment groups at 0, 7, 14, 21 and 56 days post treatment. * indicates 35 days post closantel treatment of the TCBZ group. Whiskers indicate minimum and maximum values, boxes indicate 25th and 75th percentile values and the dash indicates the mean values.

groups at each sampling point. The TCBZ treated groups ranged in percent reduction, from 12.2% to 58.9%, between 7 and 21 dpt, indicating treatment failure. At 56 dpt, a >95% reduction was seen in both the TCBZ + closantel treated groups and the closantel-only treated groups.

3.2.2. Coproantigen ELISA and CRT

All animals tested positive by coproantigen ELISA at 0 dpt. Prior to treatment, ELISA optical densities (OD) did not differ between TCBZ and closantel treated groups on Farm 1 (range 0.21–2.46, mean 1.36, median 1.6 vs. range 0.15–2.46, mean 1.1, median 1.08, respectively, $P = 0.15$) or Farm 2 (range 0.21–3.91, mean 2.30, median 2.35 vs. range 0.18–4.02, mean 2.18, median 2.98, respectively, $P = 0.86$). The coproantigen ELISA results of TCBZ and closantel treated ewes on both farms over the duration of the study are shown in Fig. 3.

At 7 dpt, all TCBZ treated animals were positive by ELISA with a mean OD of 1.55 on Farm 1 and 1.01 on Farm 2. There was a significant difference between the TCBZ and closantel treated groups on both farms ($P = 0.0001$), with the mean OD of the closantel treated groups being 0.02 on both farms, with all closantel treated animals testing negative.

At 14 dpt, the differences between TCBZ treated groups and closantel treated groups were also significant ($P = 0.0001$). Once again all TCBZ treated animals tested positive, with a mean OD of 1.18 on Farm 1 and 1.41 on Farm 2, and all closantel treated animals tested negative with mean OD of 0.02 on both farms.

On 21 dpt, the TCBZ treated animals were once again all positive, with a mean OD of 1.89 and 1.47 on Farms 1 and 2, respectively. All closantel treated animals on Farm 1 remained negative with a mean OD of 0.01. However, one animal was coproantigen ELISA positive in the closantel treated group on Farm 2 (mean group OD of 0.02).

At 56 dpt, all TCBZ + closantel treated animals (now at 35 dpt with regards to closantel) tested negative with the mean group ODs being 0.04 and 0.03 on Farms 1 and 2, respectively. On Farm 1, one closantel-only treated animal tested positive and the mean group OD was 0.06. The closantel-only treated animal on Farm 2 which had been positive at 21 dpt, returned to negative status and the closantel-only treated group had a mean OD of 0.02.

4. Discussion

This study aimed to investigate the usefulness of the coproantigen ELISA as a method for detecting fluke infection in naturally infected lambs and to assess the potential of the coproantigen ELISA reduction test to measure treatment efficacy in naturally infected ewes.

In the lambs followed longitudinally, levels of GGT were above SAC C VS guidelines from the start and rose over the course of this study, whilst levels of GLDH rose from August onwards. As expected based on the migration route of fluke through the liver, the GLDH level, which is an indicator of liver damage (Mitchell, 2002), started to increase before the GGT level, which is an indicator of damage to the bile ducts (Matanović et al., 2007). This implies that whilst GLDH and GGT follow the same kinetics in the fluke infection of lambs as they do in older sheep, different reference ranges may need to be set for this test to be of use in young animals. It also supports the notion that these tests, with their current reference ranges, should not be used in isolation for the diagnosis of fasciolosis.

The detection of anti-*F. hepatica* antibodies was inconsistent in some lambs, i.e. lambs that were negative subsequently became positive before testing negative again. It is expected that antibody titres would persist for many months after clearance of parasites rather than fluctuate between apparent positive and negative status. It is possible that early positive results are due to antibodies from colostrum (Petzoldt and Von Benten, 1978). There is also the possibility for false positive results as the ELISA has a reported specificity of 99.4% (Molloy et al., 2005).

Fluctuations were seen in lamb FEC, where animals changed from positive to negative to positive again over the course of the study. The natural fluctuation in epg seen in fluke infections is well documented (Chowaniec and Darski, 1970; Mezo et al., 2004) and a known limitation of the test. However, the positive egg counts in July would imply exposure of the animals soon after birth. It is possible that this is the result of a true infection, with lambs ingesting grass, and hence cysts, from a young age. Alternatively, it is possible that this is due to the ingestion and passage of eggs from contaminated teats. This has been documented as a potential cause of false positive FEC in humans after ingestion of liver (Hillyer, 1988). Oral inoculation and faecal shedding in the absence of infection have also been described for mycobacteria (Sweeny et al., 1992). Finally, there is also the possibility of cross contamination during sample processing, leading to false positive results.

Despite considerable variation in age and diet of animals, results from experimental infections indicate that the coproantigen ELISA consistently becomes positive weeks prior to the FEC (1–5 weeks, Mezo et al., 2004; 4–7 weeks, Valero et al., 2009). By contrast, the mean coproantigen ELISA became positive at the same time as the FEC in our study of naturally infected animals. Some experimental infections have involved challenge doses of ca. 200 metacercariae, which may have contributed to the large interval between the first positive results for coproantigen ELISA and FEC in that study (Valero et al., 2009). Other experimental infections have involved low doses of metacercariae, i.e. 5–40 per animal, and resulted in liver fluke burdens of 1–36 flukes (Mezo et al., 2004). Within this range, fluke burdens of 5 or fewer are associated with delayed onset of positive coproantigen ELISA results compared to fluke burdens over 5 (Mezo et al., 2004). Of 10 livers evaluated in our study, 7 had fluke counts of 5 or lower (Table S1), suggesting that low fluke burdens may provide a partial explanation for the fact that coproantigen ELISA did not become positive before FEC in our study. The fact that we sampled at monthly rather than weekly intervals may also have affected our ability to detect positive coproantigen ELISA results ahead of positive FEC.

As with lambs, the FECs of ewes in this study fluctuated greatly, both between animals at a single sampling point and between sampling points for individual animals. This impacts on the test's ability to detect treatment failure. Although a FECRT is often used, there has been no standardisation of the method for fluke infections, unlike

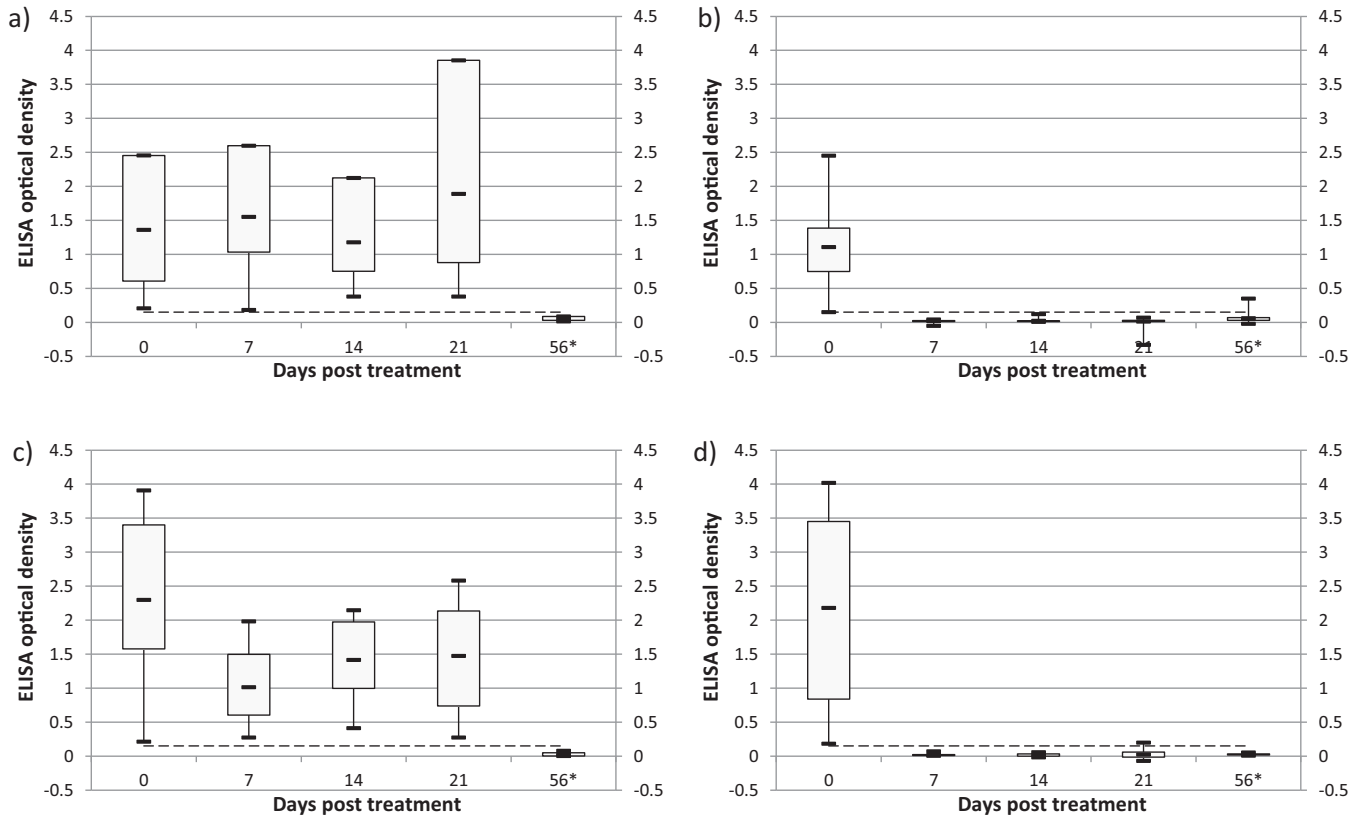

Fig. 3. ELISA optical density (OD) of the Farm1 TCBZ (a) and closantel (b) and the Farm 2 TCBZ (c) and closantel (d) treatment groups at 0, 7, 14, 21 and 56 days post treatment. * indicates 35 days post closantel treatment of the TCBZ group. ELISA ODs are considered positive if ≥0.15, indicated by the dashed line. Whiskers indicate minimum and maximum values, boxes indicate 25th and 75th percentile values and the dash indicates the mean values.

for nematode infections, and it is an imperfect test. This is highlighted in the results presented here. Although the closantel groups on both farms maintained a >95% reduction in FEC throughout the study, >10% of animals remained FEC positive, excreting eggs at a low level for the duration of the study. Excretion of eggs at low level may be due to sequestration of eggs in the gall bladder, although it could also be an early indicator of treatment failure. The coproantigen ELISA yielded unambiguous results at 7 and 14 dpt, with all animals testing negative for presence of fluke.

During the course of this investigation it became apparent that the coproantigen ELISA was superior to the FEC in terms of convenience. Per animal, the ELISA took much less time to process than the FEC, required less faecal matter and it was also possible to store the supernatants for later use if time did not allow for immediate testing. For gastrointestinal nematodes, use of FEC data can lead to failure to detect low anthelmintic efficacy with commonly used mathematical techniques such as the FECRT (Torgerson et al., 2005). Whether the coproantigen ELISA has advantages over FEC in robustness as an indicator of treatment outcome for fluke infections remains to be established.

It is clear from the results presented here that TCBZ was not effective on either farm. The reduction in epg of the TCBZ treated animals fluctuated considerably, and was never greater than 59% at the group level. In contrast, the closantel treated groups achieved >95% reduction in epg by 7 dpt at group level and this was maintained throughout the study with a steady increase in reduction. At an individual animal level, a 95% reduction in FEC was not always achieved, particularly not for animals with low initial epg. This is in agreement with results reported by Flanagan et al. (2011a) and underscores the need for a minimum start value for epg when performing an FECRT. It also emphasises the need for a minimum group size to ensure that high and low epg values are distributed evenly across treatment groups (Torgerson et al., 2005).

The CRT gave the same indication of treatment success as the FECRT at 7 and 14 dpt. At 21 dpt, one animal in the closantel group tested positive on Farm 2, where ewes were housed outside. This ewe was coproantigen ELISA negative again at 56 dpt. No treatment had been administered in the interim. A different animal in the closantel group on Farm 2 tested coproantigen ELISA positive at 56 dpt. It is possible that the latter animal became coproantigen ELISA positive due to maturation of early immature fluke that had not been affected by the closantel treatment. Re-infection was unlikely during this study because of the weather conditions, i.e. deep snow for prolonged periods. Because 56 dpt was the last sampling point of the study, it could not be determined whether this animal remained positive.

It has been suggested that the definition of treatment success as measured by CRT should be based on 100% negative results after treatment (Fairweather, 2011c). Both our study and reports from others show that intermittent positive results do occur after treatment that is deemed successful based on FECRT, with positive results

occurring at 7, 8, 14 and 21 dpt (Flanagan et al., 2011a,b; our study). Fluctuations in coproantigen ELISA results have been attributed to disintegrating fluke releasing antigen. Initially, it was suggested that such fluke disintegration and antigen release is unlikely to occur to the extent that it would be an issue for diagnosis. Based on our study of natural fluke infection and the reports on experimental fluke infection cited above, the strict criteria for treatment success, i.e. 100% negative results by coproantigen ELISA, are not compatible with results from treatment trials. We believe that this is an area of concern that must be addressed if the CRT is to be of genuine utility in the field. It is believed that this test will be very useful in monitoring drug efficacy but more work is needed to standardise methodology and to provide quantitative support for interpretation of results before it can see widespread uptake in the veterinary community. False positive and false negative results will continue to occur by FEC and coproantigen ELISA, as with all tests, but this can be taken into account by using a minimum reduction threshold alongside a minimum sample size, rather than an all positive or all negative criterion, allowing more confidence to be placed in both FECRT and CRT.

Good diagnostics are a necessity if a distinction is to be made between failure of a treatment and resistance of a parasite population to a drug. Apparent treatment failure could result from numerous factors, including under-dosing, reduced bioavailability of drug, inadequate diagnostics or poor drug formulation (Fairweather, 2011a). In practice, if treatment failure is observed, animals will be treated with an alternative flukicide and, if the second treatment is successful, 'resistance' will be declared, regardless of the possibility of inaccurate timing or dosing. In our study, two ewes from Farm 1 were given TCBZ rather than closantel at 21 dpt. Both ewes were FEC and coproantigen ELISA positive before and 35 days after the second TCBZ treatment. A third TCBZ treatment was administered in June and the animals were euthanized 7 days later. At each time point, animals had been dosed by weight and using a syringe to rule out inaccurate timing or dosing. At post-mortem, large numbers of live, undamaged fluke were recovered from the livers of both animals, confirming the presence of TCBZ resistant fluke.

5. Conclusions

Our evaluation of the coproantigen ELISA and comparison with established diagnostic methods for detection of liver fluke in naturally infected lambs and sheep showed that the coproantigen ELISA is superior to the FEC with regards to convenience characteristics and provides similar results with regards to infection status. The coproantigen ELISA did not give an earlier indication of fluke infection when used at monthly intervals, but it did provide an unambiguous indication of treatment success at 7 and 14 dpt. FECRT and CRT were useful in assessment of treatment efficacy but guidelines for the conduct and interpretation of results from both tests need further development. Lastly, we report the observation of TCBZ treatment failure and the presence of TCBZ resistant liver fluke in sheep in Scotland as well as the effectiveness of closantel against TCBZ-resistant fluke.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetpar.2012.02.009.

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Confirmation of triclabendazole resistance in liver fluke in the UK

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Letters

LIVER FLUKE

Confirmation of triclabendazole resistance in liver fluke in the UK

WE would like to report confirmation of triclabendazole resistance (TCBZ-R) in liver fluke, *Fasciola hepatica*, from the UK, based on a dose and slaughter trial. Recently, there has been considerable debate regarding the usefulness of reports of triclabendazole treatment failure in liver fluke infected sheep and cattle as an indicator of TCBZ-R (Fairweather 2011, Sargison and Scott 2011). It has been suggested that the diagnosis of TCBZ-R in the field should be based on a combination of diagnostic tests (Fairweather 2011). We used a combination of faecal egg count reduction testing (FECRT) and coproantigen reduction testing (CRT) to demonstrate TCBZ-R in liver fluke in sheep from Scotland (Gordon and others 2012). We have now confirmed this diagnosis based on a dose and slaughter trial, the only method that was put forward by a panel considering new World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines on the detection of anthelmintic resistance (Coles and others 2006).

F hepatica eggs were obtained at postmortem examination from the gall bladder of two naturally infected ewes from Dumfries and Galloway, Scotland. Large numbers of live adult fluke were present in the liver of both animals (46 and 75, respectively) despite three triclabendazole treatments. *Fasciola* eggs were propagated through *Galba truncatula* snails and metacercariae were harvested for reinfection of sheep. Ethical approval for the study was obtained through the Moredun Research Institute's Experiments and Ethical Review Committee.

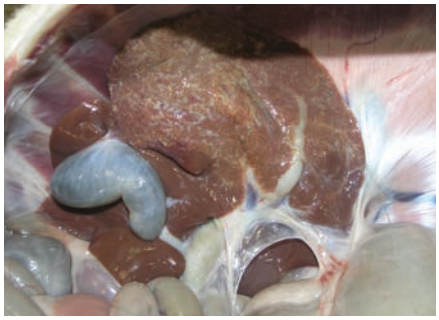

FIG 1: Sheep liver in situ showing damage to the liver tissue and thickening of the bile ducts 17 weeks after challenge with *Fasciola hepatica*

Six animals (one-year-old males) were challenged with an estimated 150 cysts each. Infection status was monitored for 17 weeks by means of a serum antibody ELISA, a coproantigen ELISA and faecal egg count (first positive at three, three and nine weeks after challenge, respectively). At 12 weeks after challenge, infection was patent in all animals. The animals were then treated with triclabendazole. Calibrated scales were used to measure bodyweight and calculate adequate dosage. Treatment failure was confirmed based on FECRT and CRT, as described by Gordon and others (2012). Three weeks later, animals entered a dose and slaughter trial, that is, triclabendazole treatment was repeated and the numbers of fluke present were counted at postmortem examination 14 days later (Fig 1). Between 19 and 70 live adult fluke were retrieved from the liver and bile ducts of each animal, confirming the TCBZ-R status of the fluke isolate.

The dose and slaughter trial-based confirmation of TCBZ-R in liver fluke in the UK, which was suspected on the basis of FECRT and CRT, should be seen as a further incentive to apply the best possible management strategies for control of liver fluke on sheep and cattle farms. This should include use of preventive measures where possible, treatment where necessary and the evaluation of treatment efficacy. Before declarations of TCBZ-R are made in the field, problems due to inadequate dosing, poor product storage or inferior quality products need to be ruled out, as triclabendazole is still the drug of choice for migrating juvenile fluke, particularly in sheep.

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Identification of the rumen fluke, *Calicophoron daubneyi*, in GB livestock: possible implications for liver fluke diagnosis

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ABSTRACT

The liver fluke, *Fasciola hepatica*, is common in many parts of Great Britain. To detect liver fluke infection and to assess whether fasciolicide treatment has been successful, the faecal egg count (FEC) and faecal egg count reduction test (FECRT) are widely used. Rumen fluke is also increasingly reported from Great Britain, but its species identity is yet to be determined. Liver fluke and rumen fluke eggs are morphologically similar, which may lead to erroneous diagnoses of liver fluke infection or treatment failure. As an alternative to FEC, a coproantigen ELISA (cELISA) can be used. The potential for this test to cross-react with rumen fluke species from Great Britain has not been evaluated. Rumen fluke specimens from cattle and sheep in Scotland were identified to species level using DNA sequencing of the ITS-2 region. Subsequently, rumen and liver fluke obtained from naturally co-infected sheep were subjected to immunohistochemistry using antibodies from a commercially available cELISA kit for *F. hepatica*. Finally, faecal samples from naturally co-infected sheep flocks were examined by FEC and cELISA. Rumen fluke from imported and home-bred cattle and sheep in Scotland belonged to the species *Calicophoron daubneyi*, rather than *Paramphistomum cervi*, the species presumed to be most common in Great Britain. Intense staining of the gastrodermis was observed in *F. hepatica* but cross-reactivity with *C. daubneyi* was not seen. Faecal samples that contained rumen fluke eggs but not liver fluke eggs were all negative by cELISA. We conclude that *C. daubneyi* is the most common rumen fluke of domestic ruminants in Scotland and that cELISA reduction testing may be a valuable alternative to FECRT in herds or flocks that are co-infected with liver and rumen fluke.

1. Introduction

The liver fluke, *Fasciola hepatica*, is a zoonotic trematode parasite with a worldwide distribution (Rojo-Vázquez et al., 2012). Liver fluke disease or fasciolosis limits production in sheep, goats, cattle and buffalo, with economic losses due to reduced growth and fertility, death and condemnation of livers at slaughter (Mezo et al., 2011; Sargison and Scott, 2011b; Borji et al., 2012). In recent years, there has been an increase in the incidence of this disease in Great Britain and the parasite has spread from western areas, where it has been known to occur for a long time, to the east (Mitchell, 2002; Anonymous, 2012). This has possibly been facilitated by warmer winters and wetter summers, favouring the liver fluke's life-cycle, but animal movement may also be implicated (Kenyon et al., 2009; Taylor, 2012). Environmental schemes that encourage wet grassland conditions for breeding and migrating birds and invertebrates have also been linked with a higher risk of liver fluke in livestock (Pritchard et al., 2005). In addition, resistance of liver fluke to the drug of choice for killing juvenile stages, triclabendazole, has been confirmed in sheep in Great Britain

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and elsewhere (Moll et al., 2000; Gordon et al., 2012a), in cattle (Olaechea et al., 2011) and, most recently, in a sheep farmer from the Netherlands (Winkelhagen et al., 2012).

The detection of *F. hepatica* and determination of the outcome of fasciolicide treatment are commonly based on standard sedimentation faecal egg counts (McCaughey and Hatch, 1964). Due to the biology of the parasite, faecal egg counting (FEC) can lack sensitivity, e.g. as a result of sporadic egg shedding by adult fluke and the long (9–15 weeks) pre-patent period. In addition, FEC may lack specificity, e.g. due to release of eggs from the gall bladder several weeks after elimination of the adult parasite (Chowaniec and Darski, 1970; Mezo et al., 2004; Valero et al., 2009). A commercially available coproantigen ELISA (cELISA), BIO K 201 (BIO-X Diagnostics Belgium), aims to alleviate some of the difficulty in determining infection status and treatment outcome in *F. hepatica* infections. The cELISA is based on a monoclonal antibody (MM3 MAb) assay that recognises cathepsin L protease, secreted from the luminal surface of the fluke's gut (Mezo et al., 2004; Muiño et al., 2011). The test is claimed to be able to be sensitive to a single fluke in sheep and to only detect presence of living fluke. Evidence of liver fluke can be detected as early as 5 weeks post-infection after experimental challenge, but this interval can be longer following natural infection (Mezo et al., 2004; Flanagan et al., 2011; Gordon et al., 2012b).

To compound the problems with detection of *F. hepatica* and assessment of treatment outcome, there has been a rise in reports of paramphistome (or rumen fluke) infections in sheep and cattle in Great Britain and Ireland, often as co-infections with liver fluke (Foster et al., 2008; Murphy et al., 2008; Mason et al., 2012). The eggs of rumen fluke are morphologically similar to those of *F. hepatica* and may cause false positive results in the FEC for liver fluke (Silvestre et al., 2000; Rojo-Vázquez et al., 2012). Most treatments that are commonly used against liver fluke, with the exception of oxyclozanide, are not effective against paramphistomes (Rolfe and Boray, 1987, 1988). Thus, rumen fluke eggs may remain present in faeces after treatment with a fasciolicide. If rumen fluke eggs are mistaken for liver fluke eggs, this could result in a false diagnosis of liver fluke treatment failure. The MM3 antibody based cELISA has been tested for cross-reactivity to the small lancet fluke *Dicrocoelium dendriticum* and the rumen fluke *P. cervi* (Mezo et al., 2004). However, it is not known whether the most common rumen fluke in Great Britain is *P. cervi*, as often assumed, or whether it is *C. daubneyi*, which is the main rumen fluke of cattle, sheep and goats in mainland Europe (Abrous et al., 2000; Rinaldi et al., 2005; Díaz et al., 2006). Therefore, this study aimed to determine the species identity of rumen fluke from cattle and sheep in Great Britain using molecular methods, and to assess the potential impact of rumen fluke presence on the specificity of liver fluke diagnostics.

2. Materials and methods

2.1. Species identification of rumen fluke

Genomic DNA was extracted from four batches of adult rumen fluke. Batch 1 was recovered in 2012 from a homebred Scottish heifer. Batch 2 was recovered in 2012 from a cow, which had been imported from Ireland and grazed in Scotland. Batch 3 was from a 47 month old homebred Aberdeen Angus cross cow submitted for postmortem examination after it died from ruminal bloat in 2009. Batch 4 was recovered in 2011 from sheep that had been grazed in Scotland. The latter sample was also used for immunohistochemistry as described in Section 2.2. We also processed a pool of immature rumen fluke recovered from the duodenum of calves that had died of clinical paramphistomosis (Millar et al., 2012).

For each batch, parasites were washed in physiological saline and stored in 70% ethanol prior to molecular analysis. Genomic DNA was extracted from at least 3 individual fluke per batch, or from a pool of the immatures, using the DNEasy extraction kit (Qiagen, Germany), according to the manufacturer's recommendations. DNA was eluted in 100 μl nuclease-free water and stored at −20 °C prior to use. Genomic DNA was also extracted from adult *F. hepatica* to act as a control.

The ITS-2 region of rDNA plus the flanking 5.8S and 28S sequences were amplified from the rumen fluke specimens by PCR using the generic primers, ITS-2For 5′-TGTGTCGATGAAGAGCGCAG-3′ and ITS-2Rev 5′-TGGTTAGTTTCTTTTCCTCCGC-3′, as described by Rinaldi et al. (2005). PCR was conducted in 25 μl reaction volumes comprising ~10 ng genomic DNA template, 25 pmol of each primer (Eurofins, Germany), 0.2 mM of each dNTP (Bioline, UK), 2 mM $MgCl_2$ and 0.1 U platinum *Taq* polymerase in 1× platinum *Taq* buffer (Invitrogen). The PCR was carried out on an applied biosystems 7270 PCR machine under the following conditions: 94 °C for 5 min; 40 cycles of 94 °C for 1 min; 53 °C for 1 min; 72 °C for 1 min; followed by 72 °C for 10 min. PCR products were separated on a 1.2% agarose gel prepared in Tris–acetate–EDTA (TAE) buffer incorporating GelRed (Cambridge Bioscience, UK) and visualised on an ultraviolet transilluminator.

PCR products of the appropriate size (~500 bp) amplified from individual rumen fluke were excised from agarose gels and DNA extracted using a QiaQuick gel extraction kit (Qiagen, Germany) according to the manufacturer's recommendations. PCR products were eluted using 30 μl nuclease-free water and 1 μl used for ligation into the pGEM-T Easy plasmid vector (Promega, USA), as instructed by the manufacturer. 1 μl of each ligation reaction was transformed into chemically competent JM109 *Escherichia coli* (Stratagene) and the resultant transformants plated on Luria Bertani (LB) agar plates impregnated with 0.5Mm 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal) and 80 μg/ml isopropyl β-D-1-thiogalacto-pyranoside (IPTG). Plates were incubated overnight at 37 °C and colonies containing insert identified based on blue/white selection. Individual white colonies (at least 3 per individual fluke) were picked into 10 ml LB medium containing 100 μg/ml ampicillin and grown overnight at 37 °C with shaking. Mini-prep DNA was prepared from overnight cultures using a Wizard Plus SV Miniprep DNA kit (Promega, USA), according to manufacturer's instructions. The DNA concentration and quality of purified plasmids were assessed using a Nanodrop spectrophotometer ND-1000 at 260 and 280 nm. Plasmids were

then diluted to 15 ng/μl and submitted to MWG Eurofins for sequencing using the SP6 vector primer. Sequences obtained were compared to reference sequences in GenBank using BLASTn at the European Bioinformatics Institute website (http://www.ebi.ac.uk/).

2.2. Immunohistochemistry

Rumen fluke from batch 4 as described in Section 2.1 as well as *F. hepatica* from the same ewes were recovered at post-mortem. The parasites were washed in phosphate-buffered saline (PBS) then fixed in 10% formol saline prior to being blocked in paraffin wax. Sections 5 μm thick, were cut on a rotary microtome and mounted onto SuperFrost® Plus (Menzel-Glaser, Germany) microscope slides and dried at 37 °C overnight. Sections were taken to water through graded alcohols prior to application of immunohistochemistry (IHC). Endogenous peroxidase was removed by immersing slides in 3% H_2O_2 (Sigma, UK) in methanol for 20 min at room temperature (RT), after which the slides were washed in water, placed in a Sequenza slide rack and cover plate system (Thermo Electron Corporation, USA) and washed twice with PBS. Non-specific binding sites were blocked by applying 100 μl 25% Normal goat serum (Vector Laboratories, UK) for 30 min at RT. The sections were then incubated with 100 μl primary antibody, in this case the anti-*F. hepatica* coproantigen MM3 monoclonal antibody (MAb) from the BIO K201 *Fasciola* coproantigen ELISA kit (Bio-X Diagnostics, Jemelle, Belgium), diluted 1:50 in PBS and incubated at 4 °C overnight. Sections were washed twice with PBS before 100 μl goat anti-mouse horseradish peroxidase (Dako Envision system polymer, Dako Ltd., UK) was applied for 30 min. Again, sections were washed twice with PBS and 100 μl Dako Envision substrate-chromagen (20 μl DAB chromagen to 1 ml substrate buffer) was added and incubated for 8 min. Slides were rinsed in water, removed from the Sequenza system and washed in running tap water for 5 min. Nuclei were counterstained with haematoxylin (Thermo Electron Corporation) for 1 min and slides were washed before being dehydrated through graded alcohols, immersed in xylene and mounted using Consul mount (Thermo Shandon, UK). Negative control sections were prepared as above, except that PBS was applied instead of the primary MAb.

2.3. Field evaluation of cELISA

As part of a study on the diagnosis of liver fluke infection and flukicide treatment outcome, sampling packs were distributed to 38 sheep farmers throughout Great Britain. Eighteen farmers returned complete sets of faecal samples, i.e. sets of 10 pre- and 10 post-treatment samples for each of 2 groups of 12 sheep. Faecal samples could be individually collected or picked up from the floor following protocols for composite FECRT (Daniel et al., 2010). Pre-treatment samples were taken on the day of flukicide treatment and post-treatment samples were collected approximately 3 weeks later. Flukicide treatments were administered by the farmers using on-farm protocols and their fasciolicide of choice. On receipt by the laboratory, faecal samples were tested for presence of liver fluke and rumen fluke eggs using standard sedimentation faecal egg count (FEC) (Gordon et al., 2012b). Liver fluke eggs were differentiated from rumen fluke egg based on colour and shape following methylene blue staining (Silvestre et al., 2000). The faecal egg count reduction test (FECRT) for liver fluke was performed for each group on each farm (Gordon et al., 2012b). In addition, all individual pre- and post-treatment samples were tested by the Bio-X K201 cELISA (Gordon et al., 2012b).

3. Results

3.1. Species identification of rumen fluke

A ~500 bp fragment of ITS-2 and its flanking regions was amplified using generic primers (Rinaldi et al., 2005) from 4 separate batches of rumen fluke (see Section 2.1). The amplicons proved to be identical to each other (both within and between samples) and had 100% (428/428 bp) homology with *C. daubneyi* (GenBank accession number AY790883; Rinaldi et al., 2005). Homology with the next best match, *P. cervi*, was 97.2% (277/286 bp; GenBank accession number HM026462; Bazsalovicsová et al., 2010). The alignment is shown in Supplementary Fig. 1.

3.2. Immunohistochemistry

Using IHC, the presence of immunoreactive antigen was clearly demonstrated in *F. hepatica*. The MM3 MAb bound specifically to the lamellae lining the gastrodermis whilst no specific staining was observed in other fluke tissues or in negative control sections (Fig. 1). Specific staining was not observed in any of the rumen fluke sections examined (at least 3 sections from each of 3 specimens), which were processed in exactly the same way (Fig. 2).

3.3. Field evaluation of cELISA

Among the ovine faecal samples submitted by 18 farmers, samples from 3 farms showed evidence of co-infection with liver fluke and rumen fluke based on FEC (Fig. 3).

On farm A, which was located in Scotland, most samples from both groups were positive for liver fluke and rumen fluke prior to treatment (Table 1). After treatment of group A.1 with a closantel-based product, the majority of samples still contained liver fluke eggs but counts were low. Based on FECRT, which indicated a 97.9% reduction in FEC, the treatment was deemed successful against liver fluke. The number of rumen fluke positive samples was higher after treatment than it had been before treatment (Table 1). Group A.2 was treated with a TCBZ-based product and showed an increase in the number of samples that were positive for liver fluke and rumen fluke as well as an increase in liver fluke FEC after treatment, resulting in a negative value for the FECRT (Table 1).

On farm B, located in a different region of Scotland, most samples from group B.1 were positive for liver fluke and rumen fluke prior to treatment, whereas group B.2 was positive for rumen fluke only (Table 1). After treatment of both groups with a TCBZ-based product, group B.1 was still positive for both types of fluke, whereas group B.2 was still

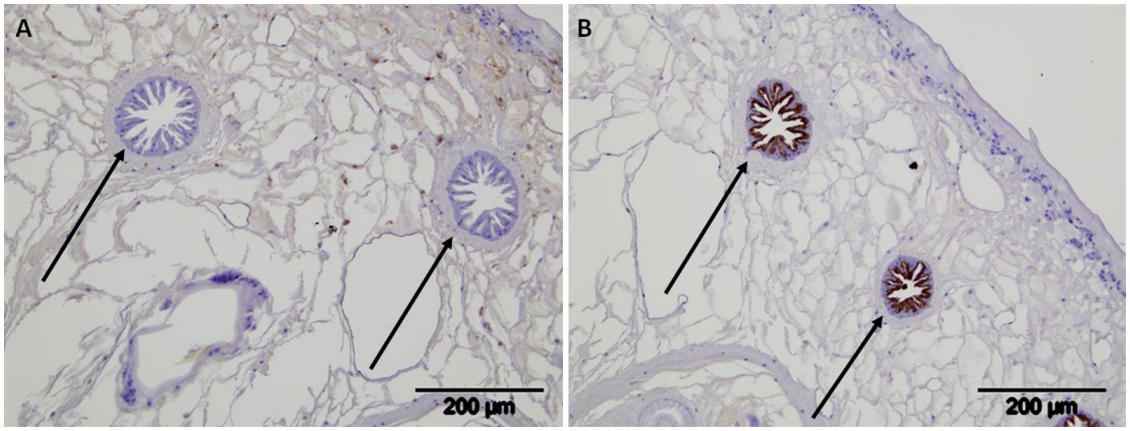

Fig. 1. Immunohistochemical staining of *Fasciola hepatica* with MM3 monoclonal as primary antibody. The gastrodermis in the negative control shows no labelling of the brush border (panel A), whereas intense staining of the lamellae lining the gastrodermis is observed in the positive control (panel B). Arrows indicate gut profiles.

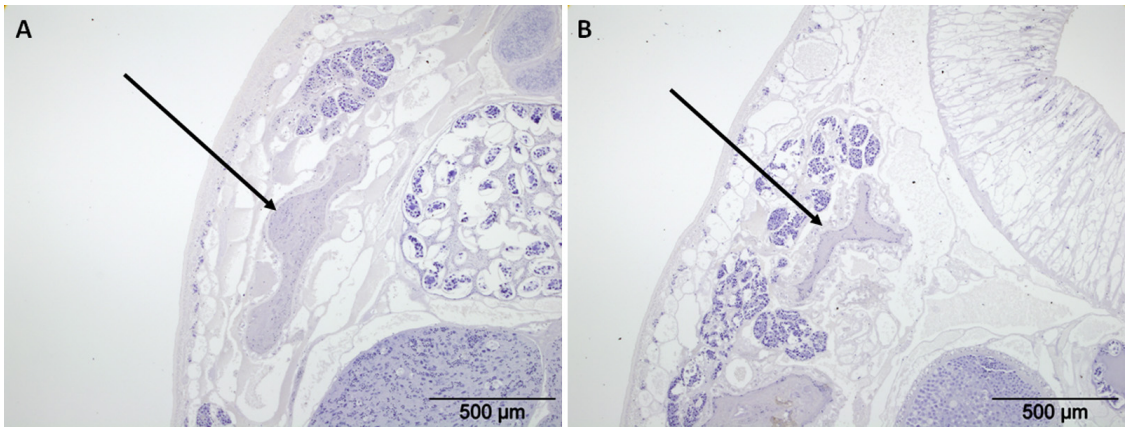

Fig. 2. Immunohistochemical staining of rumen fluke with MM3 monoclonal as primary antibody. Neither negative controls (panel A), nor rumen fluke with MM3 MAb applied (panel B) show specific staining. Arrows indicate gut profiles.

largely negative for liver fluke and remained positive for rumen fluke.

On farm C, located in Wales, a single post-treatment sample was positive for rumen fluke. This sample was negative for liver fluke based on FEC and cELISA.

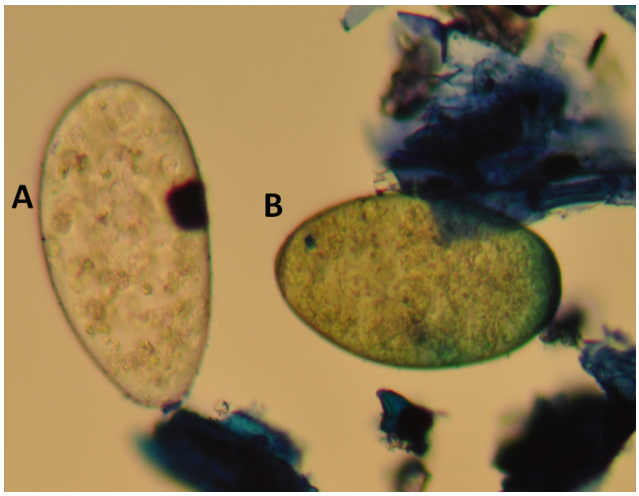

Fig. 3. Rumen fluke egg (A, light colour) and liver fluke egg (B, dark colour) obtained by the faecal sedimentation technique.

In total, 20 samples were positive for rumen fluke eggs but negative for liver fluke eggs (2 from farm A, 17 from farm B, 1 from farm C; Supplementary Table S1). None of those samples were positive by cELISA, indicating that there was no cross-reactivity with rumen fluke.

4. Discussion

Liver fluke is increasingly common in the UK, and the number of reported and confirmed cases of flukicide resistance is growing (Fairweather, 2011; Sargison and Scott, 2011a). In practice, flukicide resistance is diagnosed based on the FECRT. Presence of rumen fluke eggs, if mistaken for liver fluke eggs, could lead to erroneous interpretation of FECRT results as indicative of treatment failure. Using standard FEC methods, co-infections with liver fluke and rumen fluke were detected in 3 of 18 sheep farms in our study. Liver fluke and some species of rumen fluke (including *C. daubneyi*) may occur together in ruminants as they can use the same intermediate host snail species and have similar life-cycles (Abrous et al., 2000; Díaz et al., 2006). This would suggest that the increase in incidence and geographic distribution that has been described for liver fluke (Pritchard et al., 2005; Kenyon et al., 2009) may also occur for rumen fluke, making accurate differentiation

Table 1
Results of pre- and post-treatment testing of faecal samples from 2 Scottish sheep flocks for liver fluke and rumen fluke showing results for faecal egg counts (FEC), coproantigen ELISA (cELISA) and faecal egg count reduction testing (FECRT). The number of positive samples is shown relative to the number of samples tested. A negative result for the FECRT means that FEC increased after treatment.

Farm	Group	Outcome	Pre-treatment		Post-treatment	
			Liver fluke	Rumen fluke	Liver fluke	Rumen fluke
A	1	Individual FEC	10/10	5/10	8/10	7/10
		Individual cELISA	9/10	n/a	0/10	n/a
		Average FEC	41.4	n/a	0.88	n/a
		FECRT	n/a	n/a	97.9%	n/a
	2	Individual FEC	9/10	7/10	10/10	10/10
		Individual cELISA	9/10	n/a	9/10	n/a
		Average FEC	46.4	n/a	126.7	n/a
		FECRT	n/a	n/a	−173.1%	n/a
B	1	Individual FEC	7/10	7/10	10/10	10/10
		Individual cELISA	7/10	n/a	3/10	n/a
		Average FEC	4.7	n/a	6.0	n/a
		FECRT	n/a	n/a	−27.7%	n/a
	2	Individual FEC	0/10	7/10	1/10	6/10
		Individual cELISA	0/10	n/a	0/10	n/a
		Average FEC	0	n/a	0.1	n/a
		FECRT	n/a	n/a	n/a	n/a

of the two types of fluke increasingly important. Until recently, reports of rumen fluke from Great Britain were rare (Foster et al., 2008; Mason et al., 2012) but the high within-flock prevalence of rumen fluke in home-bred ewes in the current study implies that that rumen fluke is established rather than imported on a number of Scottish sheep farms. Liver fluke treatments used on those farms were not effective against rumen fluke, as previously observed for a range of treatments in sheep and cattle (Rolfe and Boray, 1987, 1988; Foster et al., 2008). Thus, there is a genuine risk that the increasing prevalence of rumen fluke may compromise the usefulness of standard FECRT methods as an indicator of treatment success for liver fluke.

Until now, it was assumed that the predominant rumen fluke species in the British Isles was *P. cervi* (Willmott, 1950; De Waal, 2010). Historically, rumen fluke was identified based on phenotypic methods. This led to designation of apparently new species of rumen fluke from Scotland and Ireland as *P. scotiae* and *P. hiberniae* (Willmott, 1950). Both species names, as well as *P. leydeni*, were later recognised as being synonyms of *P. cervi*, showing the potential for inaccurate species identification based on morphology (Odening, 1983). In the present study, rumen fluke from cattle and sheep were identified using modern molecular methods. All specimens were identified as *C. daubneyi*, previously known as *P. daubneyi*, whilst *P. cervi* was not detected. Included among these specimens were samples from a recently reported fatal bovine clinical case, which were also confirmed to be *C. daubneyi* by ITS-2 sequencing (Millar et al., 2012; unpublished data). *C. daubneyi* has also been reported from cattle, sheep and goats in mainland Europe, e.g. from France (Abrous et al., 2000; Silvestre et al., 2000), Italy (Rinaldi et al., 2005; Biggeri et al., 2007) and Spain (Díaz et al., 2006). Despite published reports of morbidity and mortality due to rumen fluke (Foster et al., 2008; Mason et al., 2012) and anecdotal reports of clinically favourable responses after rumen fluke treatment, the economic impact of *C. daubneyi* and other paramphistomes is yet to be determined. Accurate determination of the disease burden caused by rumen fluke species will require accurate differentiation of *C. daubneyi* and *P. cervi* and ITS-2 sequencing would be an appropriate method to achieve this (Rinaldi et al., 2005; this study).

There have been various attempts to overcome the difficulties associated with FECRT as a measure of treatment efficacy for liver fluke, including the use of composite sampling (Daniel et al., 2012) and egg hatch tests (Fairweather et al., 2012). Another approach is the use of the coproantigen reduction test (CRT), which was successful in experimental challenge models and field studies (Flanagan et al., 2011; Gordon et al., 2012b). The specificity of the MM3 antibodies used in a commercially available coproantigen ELISA was previously evaluated and confirmed in relation to the trematodes *D. dendriticum*, *P. cervi* and the cestode, *Taenia hydatigena* (Mezo et al., 2004; Kajugu et al., 2012). Our *in vitro* experiments using IHC on rumen fluke derived from cattle and sheep show that *C. daubneyi* can be added to this list. Testing of faecal samples from naturally infected sheep did not yield any false positive results for samples that were positive for rumen fluke eggs but negative for liver fluke eggs. Thus, the CRT would be a viable alternative to FECRT for the assessment of liver fluke treatment outcome in flocks or herds with a high rumen fluke burden.

5. Conclusion

The rumen fluke *C. daubneyi* is present on sheep and cattle farms in Scotland. The within-farm prevalence of *C. daubneyi* in home-bred animals can be high. Eggs of *C. daubneyi* are similar in appearance to those of liver fluke and may continue to be present after successful treatment of liver fluke. This could result in an incorrect diagnosis of liver fluke treatment failure based on FEC. *C. daubneyi* does

not cross-react with the Bio X K 201 coproantigen ELISA, making it a useful test for evaluation of treatment efficacy on farms with co-infections due to rumen fluke and liver fluke. Further study using accurate species identification methods would be required to determine the prevalence and impact of *C. daubneyi* and *P. cervi* in livestock in Great Britain.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2013.01.014.

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