The Detection and Occurrence of Triclabendazole Resistance in *Fasciola hepatica* in Sheep in England and Wales

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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August 2015

AUTHOR'S DECLARATION

Apart from the help and advice acknowledged, this thesis represents the unaided work of the author

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August 2015

This research was carried out in the Department of Infection Biology and School of Veterinary Science, University of Liverpool

This thesis is dedicated to my husband.
For your endless love, support and encouragement

Truly thankful to have you in my life

ACKNOWLEDGEMENTS

It has been a great opportunity for me to study for my PhD degree in Veterinary Parasitology at the University of Liverpool, UK. The project was funded by the Ministry of Education Malaysia (Malaysian Government) for three and half years with association with Gloworm.

Living and studying in a country with a different language (Scouse), weather and food than I was used to have provided many opportunities and also some challenges while I have been here. I am greatly indebted to large number of individual people and organisations in Liverpool and Malaysia for their support and trust which made my PhD journey possible up to the end. Therefore, I would like to say:

I am profoundly grateful to the Vice-Chancellor of Universiti Putra Malaysia and the Dean of the Faculty of Agriculture and Food Sciences for allowing me to pursue my doctoral studies in the UK and also for their support during the entire tenure of my study.

I would like to express my deep sense of gratitude to my supervisors Dr Jane Hodgkinson and Professor Diana Williams who have provided me with parasitology nous and for their valuable guidance, without which I am not sure I could have started! They have each contributed in their unique way to encourage me in undertaking a PhD and using their expertise to assist me through the various stages. They have always been there for me, lending both support and time. Thank you!

My heartfelt thanks also go to Dr Gina Pinchbeck (third supervisor) for her fruitful discussion and assistance during my time at Liverpool.

My special thanks go to Catherine Hartley and Katherine Allen for being helpful from day one of my PhD. I am very grateful for their technical assistance as well as for their friendship. It was a great pleasure working in the ‘dirty’ laboratory and in the field with both of you and you were fun to be with.

I extend my appreciation to the members of the PCR team - Krystyna and Stephanie who gave freely of their time to get me started on PCR assay protocol.

I would like to express my sincere thanks to my group members for being cheerful colleagues; Alison, Cara, Christina, Corrado, John, Laura, Nicola, Patrick, Tess, Tonk (note the alphabetical order) for their friendship and encouragement during my study. Thank you guys for your help and guidance: proofreading the thesis, data analysis and using statistical software and also for Nicola's home baked cupcakes. I enjoyed the time I had with other groups and staff members in the department (especially Jill Hudson) who I would like to thank for their kindness and help. I hope that we are able to keep in touch in the future.

A special thank you to the sheep farmers who contributed many hours of their time to provide the sheep faecal samples for our project. Thanks are extended to the staff of APHA, especially Tilly Stephens, for their willingness to work on this project and for their assistance during faecal sample collection which facilitated my data collection. Their kindness and patience have been very much appreciated.

A heartfelt thank you to my overseas/ shopping friends, Alia, Mazlinda, Ayuni, Kitty and Ila who made my stay in Liverpool joyful and full of happy memories.

Last but not least, I greatly thank my beloved parents; Kamaludeen and Aminah, my parents- in-law, Ahmad and Nancy and my family for their love and moral support when I was miles away from home. Without their encouragement and advice it would have been hard to make my student journey this far.

And lastly thank you to my husband, the one and only until Jannah, Hakim, for being by my side through all the highs, lows and in-betweens. Thank you Hakim for your sacrifice on my best and worst days on which you are always at my side. I love you so much.

ABSTRACT

The Detection and Occurrence of Triclabendazole Resistance in *Fasciola hepatica* in Sheep in England and Wales

Juriah Kamaludeen

Sheep are at risk from the disease fasciolosis, caused by *Fasciola hepatica*. For sheep, triclabendazole (TCBZ) is the drug of choice as it kills the early, immature stages of the parasite. However, TCBZ is becoming increasingly ineffective due to emergence of resistance. The aim of this thesis was to identify the most appropriate diagnostic test to detect infection with *F. hepatica* in sheep, the optimum faecal egg count reduction method to detect TCBZ resistance, and thirdly to assess the prevalence of TCBZ resistance in Britain. In Chapter 2, three different techniques that have been described in the literature to diagnose *F. hepatica* infection: faecal egg counts (FEC), coproantigen ELISA and a PCR-based assay, were compared. The objectives were to compare the sensitivity of these techniques to detect early infection in experimentally infected sheep and measure the efficacy of TCBZ against a susceptible isolate. For this, a total of 12 sheep were infected with 200-215 TCBZ susceptible metacercariae and infection was tracked on a weekly basis from 0 to 14 weeks post-infection (wpi) at which point they were divided into two groups of six. Group 1 was left untreated and Group 2 animals were treated with TCBZ (Fasinex®; 10 mg/kg) per os. Faecal samples were collected on the day of treatment and daily until 10 days post treatment (dpt). At 10 dpt, all sheep were killed for liver fluke recovery and enumeration. Results showed that the coproantigen ELISA could detect infection from 5 wpi (2/12 sheep), and all sheep were positive by 8 wpi. FEC was less sensitive at detecting early infection, 1/12 animal became positive at 7 wpi and all sheep were positive at 11 wpi. The PCR failed to detect infection at any time point. The efficacy of TCBZ against *F. hepatica* was 97% with $p<0.001$. There was no correlation between the coproantigen levels and FEC for all sheep, $R^2 = 0.031$ (Spearman's test $p = 0.21$) at 14 wpi. The study confirmed that the coproantigen ELISA is more sensitive in detecting early fluke infection compared to FEC. All six sheep in Group 2 were positive by FEC and 4 of these were also positive by coproantigen ELISA at 10 days post treatment with TCBZ; at post-mortem, between one and 13 flukes were recovered in all but one animal.

Chapter 3 describes a pilot study conducted to improve the design of a previously described composite faecal egg count reduction test (cFECRT). Firstly we determined if it was necessary to sample the same 20 sheep before and post treatment, or if two random groups of 20 sheep could be sampled for the two counts. Samples from 44 sheep from Farm 1 and 105 sheep from Farm 2 were collected and individual egg counts determined. Bootstrap analysis showed that the same 20 sheep had to be sampled pre-treatment and at 21 dpt. The coproantigen ELISA and FEC were compared on individual samples and composite samples from 20 sheep on five farms. Faecal samples were collected prior to treatment, 7 and 21 dpt to determine the optimum time to collect the

post-treatment sample. A comparison revealed that the results of the individual coproantigen ELISA were more informative compared to composite ELISA values for detection of infected sheep. The results also showed that sample testing at 21 dpt can help avoid false positive results.

Chapter 4 describes two TCBZ resistance prevalence studies. Firstly a total of 20 farms in the county of Cumbria were approached. Sixteen farms submitted samples and 13 farms had pre-treatment counts sufficiently high to conduct the FECRT, and evidence of TCBZ failure was detected on all 13 farms. Secondly a survey of TCBZ resistance was conducted in three regions of Britain, North East England, South West England and South Wales. Two hundred and fifty farms were contacted, 42 farms took part in the study. Of the 42 farms who submitted pre-treatment samples, seven had pre-treatment counts of 100eggs per gram or higher. Evidence of TCBZ failure was observed in 4 of those 7 farms, all from North East England. Using questionnaire data, descriptive statistics showed that there was no significant difference (T-test $p = 0.82$) between resistance status and total number of ewes on a farm. Evidence from the present study suggests that TCBZ resistance is common on sheep farms in some parts the UK but further work is needed to establish a national prevalence.

Overall, this study demonstrated that the coproantigen ELISA test is able to detect pre-patent fluke infections when compared to FEC. However, this work highlights that the coproantigen ELISA performs differently in experimentally infected sheep compared with naturally infected sheep. Further evaluation of the coproantigen ELISA is needed if it is to be used with confidence for detecting TCBZ resistance in the field. Whilst this study identified TCBZ to be a problem on some sheep farms it was not possible to determine the prevalence of TCBZ resistance in England and Wales, This highlights the need to monitor TCBZ efficacy on a farm by farm basis in England and Wales.

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celcius
µm	Micrometre
µl	Microlitre
µg	Microgram
APHA	Animal and Plant Health Agency
cELISA	Coproantigen ELISA
cFECRT	Composite Faecal Egg Count Reduction Test
CRT	Composite Reduction Test
CFN	Cumbria Farmers Network
DNA	Deoxyribonucleic acid
dpt	Day post treatment
ELISA	Enzyme-linked immunosorbent assay
EHA	Egg Hatch Assay
epg	Egg per gram
FEC	Faecal egg counts
FECRT	Faecal Egg Count Reduction Test
ml	Millilitre
NBD	Negative Binomial Distribution
PCR	Polymerase Chain Reaction
PP	Percentage Positivity
pi	Post infection
pt	Post treatment
RADAR	Rapid Analysis and Detection of Animal-related Risks
RT	Room temperature
SCOPS	Sustainable Control of Parasites in Sheep
TCBZ	Triclabendazole
TCBZ_SO	TCBZ sulphoxide
TCBZ_SO_2	TCBZ sulphone
VIDA	Veterinary Investigation Diagnosis Analysis
WAAVP	World Association for the Advancement of Veterinary Parasitology
wpi	week post infection

CHAPTER 1
INTRODUCTION

1.1 The importance of *Fasciola hepatica*

Liver fluke disease or known as fasciolosis, is an economically important disease of sheep and cattle worldwide. Fasciolosis, caused by the trematode parasite *Fasciola hepatica*, results in tremendous economic loss to farmers in the UK livestock industry due to rejection of infected cattle and sheep livers (McKenna et al., 2002; Sanchez-Vazquez and Lewis, 2013). Liver fluke infection also causes economic losses due to costs of drug treatment, the need for farm management strategies and adverse effects on productivity, including reduction on milk and meat production, wool production, animal health, growth rates, development and fertility (Mezo et al., 2011; Oakley et al., 1979; Sargison and Scott, 2011b). Fasciolosis is also recognised as an important disease in humans (Winkelhagen et al., 2012). Infection with *F. hepatica* and its sister species *Fasciola gigantica* constitutes a major public health problem and fasciolosis is now considered as a neglected tropical disease (Gonzalez et al., 2011; Mas-Coma, 2005). These two trematodes have different geographical distributions. In the low-altitude tropics, fasciolosis is usually caused by *F. gigantica*, whereas *F. hepatica* is more common in the high-altitude tropics and in temperate regions throughout the world. Outbreaks of *F. hepatica* have a wider range in Europe, the Americas and Oceania (Bennema et al., 2009) whereas *F. gigantica* is typically limited to tropical regions and is mainly found in Southern Asia, Eastern Asia, Middle East, Eastern Europe and Africa (Torgerson and Claxton, 1999). In the UK, only *F. hepatica* species is present and infects a large variety of livestock commonly cattle and sheep, in which infection rates may reach 90% (Boray, 1999). Whilst sheep and cattle are the main host species for liver fluke it can infect pigs and donkeys (Valero et al., 2001) and other hosts such as deer, hares and rabbits (Walker et al., 2011).

In sheep, fasciolosis can cause morbidity and mortality, which can be divided into acute, sub-acute and chronic disease. Acute fasciolosis occurs two to six weeks after sheep

ingest large numbers of metacercariae in a short period of time. The acute form of the disease is a result of immature larvae migrating from the duodenum to the liver causing severe damage and haemorrhage to the liver parenchyma due their migration. Acute fasciolosis is far more common in sheep than in cattle. The clinical signs are characterized by anaemia and sudden death. When considering options for diagnosis and treatment, the primary target is considered these early stages of the parasite. Similarly the subacute form of disease is caused by migration of immature fluke through the liver parenchyma after sheep ingest very large numbers of metacercariae over a period of several weeks or months. The lesions are less severe, but sudden death of previously healthy animals could occur. Other evidence of problems includes reduced grazing activity and also lethargy, pallor, abdominal pain and dyspnoea (Sargison and Scott, 2011b). Outbreak of the disease in the UK is mostly seen in October through to spring after animals become infected in the summer or from late spring until early summer if initially infected during winter (Table 1.1). However, acute fasciolosis can occur throughout the year (Kenyon et al., 2009; Sargison and Scott, 2011b). Subacute fluke infection in sheep may manifest as poor reproductive performance such as fertility problem, reduced twinning rates and protracted lambing periods (Sargison, 2008). Other clinical signs reported in sheep is liver enlargement but this can only be detected by using ultrasonograpahic examination (Scott et al., 2005). In addition, fasciolosis may predispose to secondary bacterial infection with *Clostridium novyi* type B or D (known as black disease), which causes sudden death in unvaccinated sheep (Sargison and Scott, 2011b). The chronic phase begins after approximately four to five months after ingestion of metacercariae, due to the presence of mature adults in the bile ducts. Weight loss and reduced wool quality are commonly seen in chronic fasciolosis due to blood feeding activity of adult flukes in the bile ducts (Sargison and Scott, 2011b). There are concerns about the effects of a chronic fluke infection with abortion in heavily pregnant sheep (Anon, 2010). Outbreak of the disease is mostly seen during late winter and spring (following summer infection of snails). Thus, *F. hepatica* has a big impact on the sheep industry; not only in of terms of animal welfare but also major economic losses through mortality, ill-thrift, treatment and veterinary costs. Table 1.1 summarises three clinical scenarios of fasciolosis, the season and stages of liver fluke involved.

Table 1.1 Disease type, seasonality and liver fluke stage within UK sheep flocks

Clinical sign	Peak incidence (month)	Fluke stages
Acute	July to December	Immature
Sub-acute	October to January	Adult and immature
Chronic	January to April	Adult

Source: Adapted from SCOPS website. (http://www.scops.org.uk/endoparasites-liver-fluke.html).

Fasciolosis is now recognised as a major parasitic infection responsible for production losses in the UK which amount to approximately £300 million per year to the cattle industry; £3 million per year of which is due to liver condemnation at slaughter itself (http://www.eblex.org.uk/). Estimation of financial losses of subacute fasciolosis in sheep industry is being £8.73 per ewe, due to death and costs of drug treatments (Sargison and Scott, 2011b).

1.2 The life cycle of *Fasciola hepatica*

The life cycle of *F. hepatica* is complex due to the need for an intermediate snail host. In western Europe, Great Britain specifically, the lymnaeid snail responsible for *F. hepatica* transmission is *Galba* (*Lymnaea*) *truncatula* (Taylor, 1949). In the UK, *G. truncatula* are primarily responsible for spreading fasciolosis but in Ireland *F. hepatica* have been detected in other molluscs; *Succinea* sp. and *Radix peregra* (Relf et al., 2009). The definitive host range for *F. hepatica* is very broad including sheep, cattle, buffalo, goat, horse, donkey and rabbit or humans. The life cycle of *F. hepatica* is shown in Figure 1.1. The mature adult flukes are localized in the bile ducts of the liver of ruminant hosts (Andrews, 1999). Sheep acquire infection by ingestion of metacercariae, the infective stage. Metacercarial cysts can be found attached to the grass blades or on vegetation such as watercress (Boray, 1969). Following ingestion, the metacercariae

excyst in the small intestine after approximately one hour, then burrow through the intestinal wall. They then migrate through the peritoneal cavity to the liver parenchyma and into the bile ducts where the flukes complete their maturation. *Fasciola hepatica* are hermaphrodites capable of both self- and cross-fertilization and large proportion of each adult fluke consists of reproductive organs (Hanna et al., 2006). Adult parasites have the potential to produce up to 25, 000 eggs per day (Happich and Boray, 1969b). Adult flukes in the bile ducts of sheep shed eggs into the host faeces where they are then passed out onto pasture. The eggs embryonate in 9 to 10 days given warm (above 10 °C) temperatures and with the presence of moisture (Schmidt and Roberts, 2005). The miracidia (free-living larval stages) hatch out from the eggs and swim actively to find and penetrate an intermediate host, *G. truncatula*. Once inside the infected snails, miracidia undergo three developmental stages from sporocyst, to rediae and finally, cercariae. These stages represent an extensive multiplication within the intermediate host. The free-swimming cercariae are shed from the snail and become encysted on vegetation near the surface of water (Kendall and McCullough, 1951) and then develop to the infective metacercariae stage. The liver fluke life cycle is completed when the definitive host, typically sheep and cattle, ingest metacercariae on pasture (Andrews, 1999). The metacercariae then undergo encystation into newly excysted juvenile stage in the host small intestine and migrate to the liver and to the bile duct system where they fully develop into egg laying adults and the life cycle continues. The pre-patent period is approximately 10 to 12 weeks. Estimation of life-span of adult *F. hepatica* in humans is 9-13 years, in cattle is 1-2 years and in sheep is up to 20 years (Andrews, 1999; Keiser and Utzinger, 2009; Robinson and Dalton, 2009).

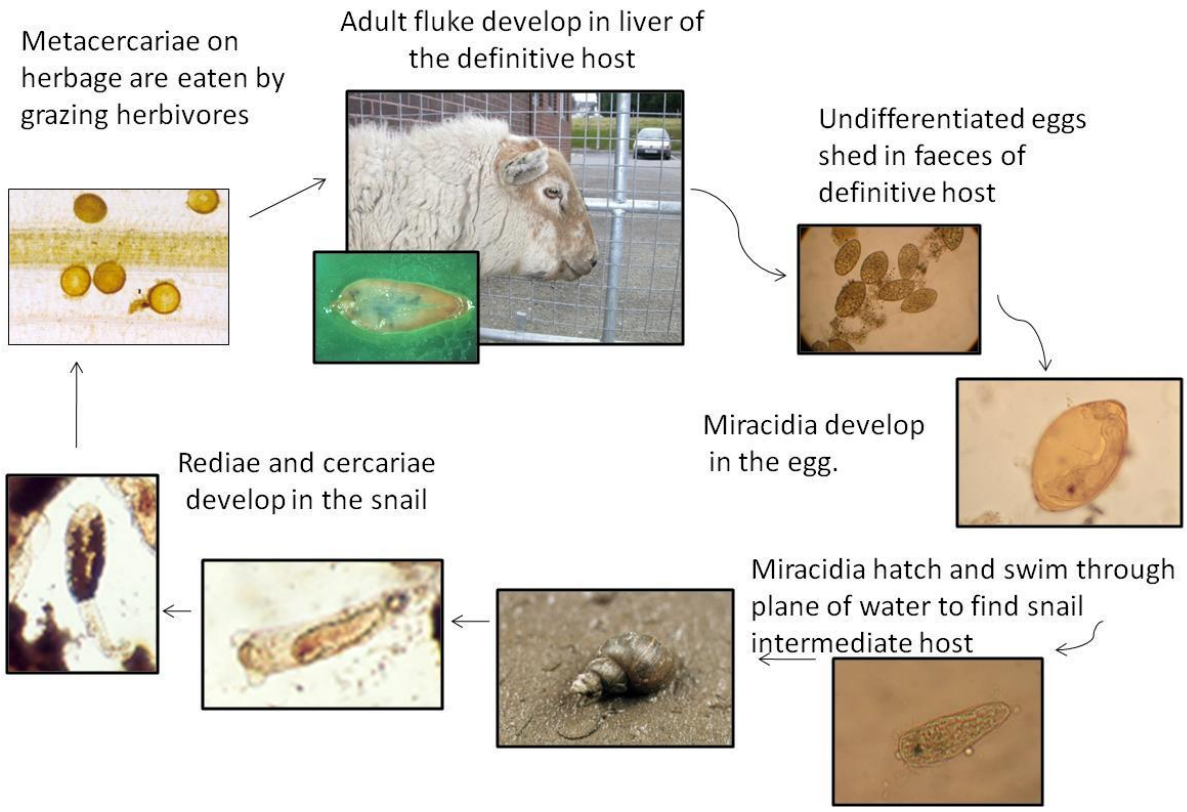


Figure 1.1 The life cycle of *Fasciola hepatica* (provided by DJL Williams).

1.3 Epidemiology and transmission of *Fasciola hepatica*

The geographical distribution of *F. hepatica* is mainly determined by the distribution patterns of the snail intermediate hosts (Mas-Coma, 2005), which is tied to suitable snail habitats. Moreover, transmission of *F. hepatica* is influenced by the biology of the parasite, the movement of farmed animals, climatic and environmental conditions, and herd management (Bennema et al., 2011; McCann et al., 2010a). Few, if any comprehensive epidemiological studies on the prevalence of liver fluke infection have been conducted in sheep however several studies for cattle include information that is directly relevant to transmission of the parasite to sheep and these studies will be referred to in the section below.

1.3.1 The role of snail development in the transmission of *Fasciola hepatica*

In order to understand the epidemiology and transmission patterns of *F. hepatica* and initiate efficient disease control, a comprehensive knowledge of the ecology of the intermediate snail host, *G. truncatula* is very important. Optimum conditions required for snail development are suitable temperature, light, soil pH, vegetation, depth of water, current of the water, soil moisture, and chemical composition of the soil, which can affect snail populations in the ecology system (Ollerenshaw and Rowlands, 1959). Attention is given to the suitability and availability of snail habitats on pasture. *F. hepatica* egg development and larval stages in the snails can take place over a few months within a 7 to 9 month period of the year (Boray and Enigk, 1964). Temperate climate affects the prevalence of *F. hepatica* infection in *G. truncatula* (Charlier et al., 2011). In the UK, snail populations have shown seasonal variations in summer and winter infection. In a wet summer, many juvenile snails develop rapidly and they are invaded by hatching miracidia from May to July. During July to October, snails shed a large numbers of cercariae onto pasture. During a dry cold summer, small densities of snail populations will appear in May, June and July. Exposure of sheep to heavily contaminated-pasture in July to October may increase the incidence of acute fasciolosis due to ingestion of massive doses of metacercariae. Therefore, levels of pasture contamination in autumn can be much lower due to fewer numbers of fluke eggs hatching and fewer snails available for fluke infection and subsequent completion of the life cycle. Winter infection of snails is less common compared to summer infection. In addition, snails deactivate and hibernate in the winter months and development commences again in spring and leads to metacercariae on pasture to challenge sheep in May and June.

The absence of *G. truncatula* in Northern Bolivian Altiplano have shown that the populations of this species inhabited mainly permanent water bodies (Lake Titicaca) for the survival of *G. truncatula* throughout the year (Mas-Coma et al., 1999b). However more typically in the UK, the habitat of the intermediate snail host of *F. hepatica* requires comparatively little water as it can breed well in muddy conditions and also depending on the type of soil. Poorly drained pasture also influences the abundance of

G. truncatula and may provide suitable habitats for the snails (Charlier et al., 2011; Sargison, 2012) thus can make a significant contribution to liver fluke infection in sheep.

1.3.2 The role of *Fasciola hepatica* biology on parasite transmission

Under favourable circumstances, fully developed miracidia will hatch from the eggs in two to three weeks and actively seek and penetrate *G. truncatula* or die within 24 hours (Andrews, 1999). They generally successfully infect a snail within three hours of hatching (Kalbe et al., 1997). The ideal temperature of the development of miracidia in the eggs has been reported as a minimum temperature of 9.5 to 10°C (Rowcliffe and Ollerenshaw, 1960). Several studies have been carried out to determine the time of egg hatching (Over, 1982; Rowcliffe and Ollerenshaw, 1960). The eggs were incubated at a constant temperature of 15°C and it was found that miracidia were fully developed and able to hatch around 40 days and at a higher constant temperature of 25°C, egg hatching occurred in 30 days. The second important factor for the development of the eggs is moisture and humidity. The eggs need to be washed out of the faeces by either rain, surrounding water, insects or trampling, and to be constantly surrounded by a surface film or moisture (Rowcliffe and Ollerenshaw, 1960). Eggs can be killed by desiccation, but they may survive for months in moist faeces and over the winter season. For example, in the UK, *F. hepatica* eggs can survive up to 10 weeks in the summer and up to six months in winter (Ollerenshaw, 1971). Throughout the winter (<10°C), the climatic conditions do not allow the development of the eggs but studies have shown that overwintering of infection inside snails could occur (Boray, 1969; Gaasenbeek et al., 1992).

Miracidia need light, specific temperatures and cold, fresh water to stimulate the hatching process (Torgerson and Claxton, 1999). A previous study on temperature has shown that 5 to 6°C is the lowest temperature threshold for miracidia to survive once hatched; mean survival time at 8°C was 24 to 30 hours, whereas at 24°C this increased

to 13 to 20 hours (Christensen and Nansen, 1976). A similar experiment has been conducted in the UK, to determine the survival temperature for miracidia and the study showed that high temperature may reduce the miracidium life-span; 6 hours at 25°C (Smith and Grenfell, 1994). The miracidia respond to light (phototropic) and chemotactic reactions to locate the snail with a distance of 15 cm increasing their chances of encountering *G. truncatula* (Andrews, 1999; Neuhaus, 1953).

Not many studies have been carried out for the mollusc stages of development. It is known that the development of mollusc stages of *F. hepatica* is crucially dependent on temperature; the lowest threshold temperature for development to occur being 10°C (Kendall and McCullough, 1951; Ollerenshaw, 1959). Other studies have shown that the time of infection of the snail until cercarial shedding may decrease from 80 days at 15°C to around 20 days at 30°C (Gettinby and Byrom, 1991; Over, 1982). Laboratory studies suggested that *G. truncatula* infected by a single-miracidium led to production of more metacercariae than those infected by two, five, 10 or 20 miracidia (Dreyfuss et al., 1999). Nevertheless, one snail has the potential to produce multiple metacercariae compared to the number of miracidia it was infected with. This study also showed that the numbers of metacercariae produced depends on several factors such as height of the snail, amount of food the snail received and survival rate of the infected snails (Dreyfuss et al., 1999).

Studies carried out on the survival of metacercariae, indicated that, about two-thirds of the metacercariae were attached to various objects near the water surface (Ueno and Yoshihara, 1974) whereas the rest become floating cysts (Dreyfuss and Rondelaud, 1994). The floating cysts may flow with the water to find a suitable object to become attached to or if not, they may die. Metacercariae of *F. hepatica* are relatively resistant to unfavourable climatic conditions and remain viable on the pasture for several months, particularly in cool and damp environments (Enigk and Hildebrandt, 1964). Without water, survival of metacercariae is dependent on relative humidity of approximately 70% (Hodasi, 1972). On the other hand, at a constant temperature of 20°C with a high relative humidity of 90% encysted metacercarial survival rates were limited to only 14 days (Boray and Enigk, 1964). In tropical countries, if exposed to direct sunlight for

more than eight hours, survival of metacercariae is 0% (Suhardono et al., 2006). Metacercariae have the ability to survive at temperatures between 0°C to -20°C and are able to survive the freezing and thawing processes (Boray and Enigk, 1964). Previous studies in The Netherlands have shown that metacercariae can survive in the winter (both mild and wet and dry winters) under natural conditions on commercial farms (Gaasenbeek et al., 1992) although this study did not record the exact temperature in winter. The overwintering of metacercariae of *F. hepatica* has been reported elsewhere in the UK and Australia (Anon, 2009; Boray, 1963; Ollerenshaw, 1959). Warm and moist soil surface conditions also favour the propagation of the snail and infective stages on pasture.

Historically hay, which is used as animal fodder during winter, has been reported as an important source of *F. hepatica* infection. Metacercariae successfully encysted on hay stalks can persist in moist conditions with a minimum of 70% relative humidity (Hodasi, 1972) or 90% relative humidity (Boray and Enigk, 1964). Metacercariae can be killed within two days or in eight hours times by exposing them to direct sunlight at 37°C and above (Suhardono et al., 2006).

1.3.3 The effect of environment on *Fasciola hepatica* transmission

Epidemiological studies have found a high prevalence of exposure to *F. hepatica* infections in dairy herds in England and Wales is significantly related to environmental factors such as the elevation and slope, soil type and also soil pH (McCann et al., 2010b). Other studies pointed out soil condition as a risk factor for transmission of bovine fasciolosis in Switzerland (Rapsch et al., 2008), specifically the water retention capacity and ground water of soils. Clay soils are more water retentive compared to sandy soils, and are therefore associated with the presence of *G. truncatula*. Slope was found to be a negative risk factor for fluke in dairy herds (Howell et al., 2015; McCann et al., 2010b). Generally, the greater the slope gradient, the better the drainage and hence providing poorer snail habitats. Similarly soil pH was identified as a potential protective

risk factor (McCann et al., 2010a). Although several risk factors have been identified, it is essential to understand why these factors are so important to *F. hepatica* life cycle and habitat for *G. truncatula.*

Soil minerals are another factor that influences *F. hepatica* transmission in farmed animals. Snails grow in soil conditions rich with calcium and magnesium minerals for their shell formation, however it has been reported that iron and phosphorus may influence the snail biology as well as the parasite life cycle (McCann et al., 2010a). Nevertheless, further understanding and investigations are needed since very little research has been carried out on the effect of soil mineral contents on the parasite life cycle. Soil pH may also have its effect on *F. hepatica* transmission because a neutral pH is essential for propagation of the snail. Various studies have been carried out on this factor in woodlands, for example, in England, Sweden and Finland and suggested that snail richness was increased by 2 to 5 species per pH unit (Millar and Waite, 1999; Valovirta, 1968; Waldén, 1981). According to Frömming, E. (1956), the essential conditions for these snail with the range of pH 5.6 to 8.6 which could have impact on the snail richness. Interestingly, Kirk et al. (2010) reported that soil pH across England and Wales has increased, for instance, soils became less acid under all land uses (arable, managed grass, semi-natural grass and coniferous wood) from 1978 to 2003. These results suggest that the increase in soil pH was due to the decreased sulphur deposition from the atmosphere and changes of liming practices on arable land during the survey period. On the other hand, the details of soil pH for grassland in England of 6.0 to 6.5 were obtained from Department of Agriculture and Rural Development (http://www.dardni.gov.uk). Therefore, it is most likely that changing soil pH conditions could favour *F. hepatica* transmission in England and Wales.

1.3.4 The effect of climate on *Fasciola hepatica* transmission

British climates are locally influenced by the Atlantic Ocean and latitude. Regions which are closest to the Atlantic i.e. western parts of England, Wales, Scotland and

Northern Ireland, are wettest regions of the UK whereas Eastern parts generally have drier and cooler weather. As for Northern parts, the general pattern of the climate is cooler and has wetter weather compared to Southern parts. For example, Wales has a high rainfall, and North East England has a drier climate (McCann et al., 2010a). Studies have shown that climatic-change scenario may be inextricably linked to fasciolosis and is particularly relevant in years when summer rainfall is high (Skuce et al., 2014). This prediction has now been confirmed in the UK, where fasciolosis patterns have been changing for the last 20 years due to the climate change and also the amount and frequency of rainfall (Fox et al., 2011). Studies reported in England and Wales (McCann et al., 2010a) and in Belgium (Bennema et al., 2011) have provided evidence that climatic factors have a large effect on *F. hepatica* transmission. As prevalence of fasciolosis is highest in areas where winters are wetter and parts of the country receiving high summer rainfalls in the UK, these conditions favour the *F. hepatica* life cycle and snail development in many parts of the UK (Fox et al., 2011; Kenyon et al., 2009; McCann et al., 2010b; Van Dijk et al., 2010). Risk of infection of fasciolosis in sheep is associated with the changeable weather in the UK (Van Dijk et al., 2010) especially if it has been a wetter summers that favours the propagation of parasite life cycle and support larger snail populations. According to the authors, climate change in south eastern Scotland has confirmed that the prevalence of fluke infections in the drier east of the country has been reported on most sheep farms. This part of the country has received more rainfall than usual. Hence, it is possible that flooding might have affected the development of the intramolluscan and free-living stages of the parasite. However, other studies have shown that rainfall has negative association with infection risk of *F. hepatica* due to heavy rainfall or flooding which washed away the free-living stages and snails (Bennema et al., 2011; Rapsch et al., 2008). These authors reported the development success rate of *F. hepatica* is not influenced by rainfall but it depends on the presence of sufficient humidity.

There is now a consensus that climate change (warmer winters and wetter summers), which are predicted to occur in the UK over the next 50 years may play a significant role in the changing epidemiology of fasciolosis (increase the prevalence of *F.*

hepatica), along with growing concerns about drug resistance (Charlier et al., 2012). Summer outbreaks of fasciolosis are rare but occur when snails are infected in late spring and early summer in any year. In the UK most infection results from appearance of the metacercariae on the pasture in the autumn, as a result of summer infection of snails (Ollerenshaw and Rowlands, 1959). If pastures have poor drainage, it is likely that the pasture will be wet for many weeks to come, especially in years when summer rainfall is high. Boggy or poached fields will provide suitable snail habitats, increasing infection of snails and leading to high infection risk in the autumn. Sheep are also at risk when they are forced to graze flukey areas in March-April as metacercariae can survive over winter. It has been predicted that liver fluke infection could extend from being a seasonal disease to a year round threat due to predicted temperature increases in the winter months, with the greatest rise in exposure in Wales. Furthermore, winter season are predicted to become even milder and through the year, the mean temperature will be above 10°C development threshold, thus fasciolosis outbreaks can be expected in late spring/early autumn. The studies also showed that Scotland and Wales are predicted to have serious epidemics of fasciolosis up to 2070 (Fox et al., 2011). The future maps have showed that serious epidemics are expected in Scotland by 2020 and by 2050 in part of Wales (Fox et al., 2011). To conclude, high annual rainfall, high soil moisture, suitable temperature and rainfall, affecting farming practices and parasites life cycle. These factors together with increased of anthelmintic resistance (Charlier et al., 2012) creating a really worrying scenario.

1.3.5 The effect of animal management on *Fasciola hepatica* transmission

It has been proposed that snail densities and richness on the grassland can be also affected due to mowing mechanisms (Martin, 1987; Schmid, 1983). Mowing mechanism or grazing is essential to maintain the soil structure, soil fertility as well as the balance and diversity in grassland. Bennema et al. (2011) suggested that greater numbers of metacercariae are found when there are no mowing activities on the pasture; a possible reason being that mowing can disturb the snail habitat. Furthermore, other

damage of the soil surface that is caused by mowing practise is through fertilizing and compacting by farm vehicles (Martin and Sommer, 2004). A few studies in Belgium have reported that farm management may also play an important role in spreading the disease (Bennema et al., 2009; Bennema et al., 2011; Charlier et al., 2011). The type of pasture, length of grazing season and proportion of grazed grass in the diet have all been shown to influence the transmission of *F. hepatica* in farmed animals (Bennema et al., 2011). Notable under farm management is the length of grazing season (as mentioned above) which can cause high levels of infection in livestock (Bennema et al., 2011). This suggests that the longer the grazing season the higher the risk of *F. hepatica* infection at the farm level. In order to avoid this, livestock are not allowed to graze on pastures heavily contaminated with metacecariae as the proportion of grazed grass in the diet of the herd has posed an elevated risk for high *F. hepatica* infection levels (Bennema et al., 2011). Thus, restricted grazing area for the herd is likely to minimize the disease risk for *F. hepatica*. By preventing the animals from grazing on the contaminated pastures or areas close the river bank this may reduce their risk of ingesting the metacercariae. Similarly cleaning out ditches on the farm or fencing off wet areas in order to reduce the chances of sheep coming into contact with infective metacercariae at these favourable for *G. truncatula* breeding habitats (Howell et al., 2015).

1.4 The increasing prevalence of *Fasciola hepatica* infection in the UK

Fasciolosis is one of the most common parasitic diseases in the UK as this country has wetter summers and warmer winters, suggesting that climate change is partly responsible for the increase in the prevalence of *F. hepatica* in this region. According to the Veterinary Investigation Diagnostic Analysis (VIDA) database submissions made to the Animal Health and Veterinary Laboratories Agency (AHVLA) or now known as Animal and Plant Health Agency (APHA), diagnoses of fasciolosis have increased rapidly from 1999 to 2009 (Anon, 2009a). The APHA has documented an increase in the diagnosis of acute and chronic liver fluke infections in sheep and cattle over the past

10 years. Liver fluke infection in sheep has been reported across Great Britain and the number of outbreaks was increased from 1995 to 2001 as shown in Figure 1.2 (Mitchell, 2002). This increase in the number of cases has led to concerns over the increased risk of fascioliasis in sheep. Sheep and cattle in western and eastern regions of the UK are now being exposed to the liver fluke infection based on the findings of several studies conducted. McCann et al. (2010b) indicated that the seroprevalence of *F. hepatica* infection in dairy herds in England was estimated at 72% whereas in Wales it was 84%. Changing climate patterns over recent years may explain the different findings between these studies to the studies done by Salimi-Bejestani et al. (2005a) in 2003, indicating that the prevalence of fasciolosis in dairy herds was found at 48% and 86% in England and Wales respectively. Within the UK, *F. hepatica* appears to have expanded in its geographical distribution and transmission range. This parasite was traditionally associated with wetter western parts of the UK. However, evidence of increasing levels of subacute fasciolosis was reported in the eastern parts of the UK; East Anglia and eastern Scotland (Pritchard et al., 2005). This is supported by studies that have documented the emergence of fasciolosis in cattle in East Anglia; this area is among the driest in the UK (Pritchard et al., 2005). However, due to recent high summer rainfall, increased sheep movement for seasonal grazing and wetter conditions; these provided an ideal climate for the development of free living stages of the life cycle of *F. hepatica* as well as the stages involving the intermediate snail hosts, *G. truncatula* (Pritchard et al., 2005). Similarly *F. hepatica* appears to be successfully completing its life cycle on most farms in south east Scotland (Kenyon et al., 2009). The climate change has favoured the intermediate host, *G. truncatula* and has the potential to alter survival rate of fluke stages in the environment (Van Dijk et al., 2010). These hypotheses agreed with other studies, indicating that changing climate, changing farming practices and increased livestock movement or introduction of infected sheep or cattle into unaffected areas in these regions is another possible way of spreading the disease (Bennema et al., 2011; Howell et al., 2015). Over the next 50 years, climate change forecasts predict warmer winters and wetter summers in the UK.

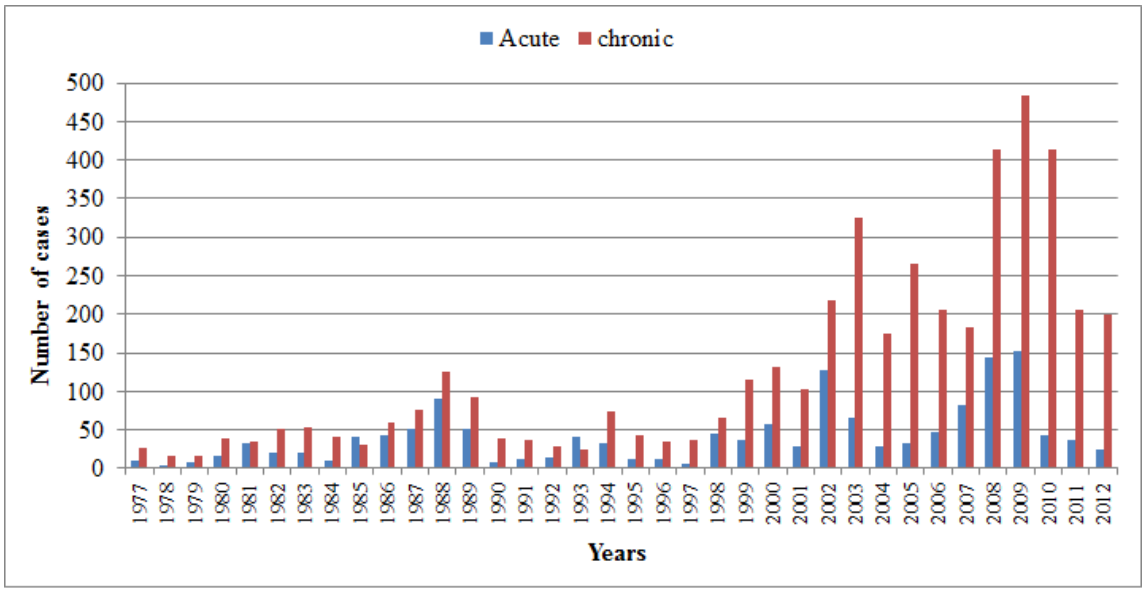


Figure 1.2 Acute and chronic cases of fasciolosis in sheep in Great Britain from 1977 to 2012. Source: Modified from Animal Health and Veterinary Laboratories Agency (AHVLA)/APHA.

The risk of infection has increased directly as there has been greater animal movement between farms (Fairweather, 2005). Introduction of newly bought-in sheep and hence their parasites, was suggested to have caused the spread of *F. hepatica* across the UK over the past few years. It is likely that animal movement has spread resistance of *F. hepatica* and may be responsible for the introduction of fluke into new areas or into new environments. Therefore, farmers need to know the health status of new stock before they enter their premises. Farmers are recommended to do a quarantine treatment especially if sheep are bought from known fluke endemic areas, e.g: South Wales, North West England and West Scotland.

1.5 Detection of *Fasciola hepatica* infection

Liver fluke infections can be detected using various diagnostic tools. Under field conditions, faecal egg count (FEC) has been considered the most reliable method of

diagnosing *F. hepatica* infection in animals and is often the method of choice to detect the efficacy of anthelmintic treatments (Flanagan et al., 2011a). Liver fluke eggs in faeces have been reported to be detected from as early as 7 week post-infection (wpi) (Paz-Silva et al., 2002), whilst other studies report eggs found at 9 wpi (Martinez-Perez et al., 2012), 8 to 10 wpi (Martinez-Valladares et al., 2010a; O'Neill et al., 2000), 10 and 12 wpi (Dumenigo and Mezo, 1999) or even later at between 11 to 16 wpi (Zimmerman et al., 1982). The limitation of the test sensitivity of FEC is that it can only detect patent infection once adult fluke are producing eggs. This means that the test is not suitable for detecting fluke infection prior to 7-8 wpi and represents a major drawback given that fasciolosis in sheep is due to the early stages of the parasite migrating through the liver 1-2 wpi. Even when detecting patent infection various studies have highlighted the unreliable and intermittent shedding of fluke eggs in the host faeces which affects the FEC and results in under-diagnosis of infection (Conceicao et al., 2002; Rokni et al., 2002).

More recently the coproantigen ELISA, cELISA, (Espino et al., 1998; Espino et al., 1997; Mezo et al., 2004; Valero et al., 2009) has been developed for the early detection of *F. hepatica* infection and a PCR assay has also been used as a diagnostic tool (Martinez-Perez et al., 2012; Robles-Pérez et al., 2013). Coproantigen ELISA, which uses *F. hepatica* antigens in host faeces, allows early detection of infection from 4 wpi onwards. Under experimental studies in sheep, it has been proven that this test has 100% sensitivity and specificity, even when sheep were only infected with a single fluke (Mezo et al., 2004). As is mentioned above, due to the lower sensitivity of FEC, (Charlier et al., 2008; Rapsch et al., 2006) compared to cELISA, the latter is potentially a convenient tool for the early diagnosis of *F. hepatica* infection in sheep. Similarly, PCR of faecal samples, is also a promising tool for the detection of *F. hepatica* as early as 2 wpi (Robles-Pérez et al., 2013). A comprehensive evaluation of these tests under experimental conditions forms the basis of chapter 2 and a more detailed introduction of the relative merits of each test is provided there.

1.6 Control of *Fasciola hepatica*

The control of *F. hepatica* can be achieved by using various options such as pasture management strategies and the use of molluscicides. Historically molluscicides were used by farmers to control snail populations in the short-term (Crossland, 1976), but this was both expensive and environmentally unsound (Urquhart et al., 1970). Due to their toxicity and negative effects on biodiversity, the use of molluscicides was made illegal in the UK. Fasciolosis control might be attained with environment approaches such as draining or fencing-off wet areas on pasture to reduce access of grazing sheep and cattle to snail habits (Charlier et al., 2011; Howell et al., 2015). In reality the use of anthelmintic drugs to treat *F. hepatica* has formed the basis of effective strategies in controlling liver fluke infections in sheep as summarised below.

1.7 Anthelmintic control of *Fasciola hepatica*

For over 30 years, triclabendazole (TCBZ; 6-chloro-5-(2, 3-dichlorophenoxy)-2-methylthiobenzimidazole), a benzimidazole derivative, has been the the drug of choice for controlling *F. hepatica* infection in both sheep and cattle due to its efficacy against both immature and mature flukes (Boray et al., 1983; Fairweather and Boray, 1999a). Currently there are a few fasciolicides on the market that are available for the control of liver fluke infections (see Table 1.2 and 1.3). Closantel and nitroxynil are also available to control fasciolosis in livestock but these two anthelmintics have no effect on immature fluke less than 6 week old as highlighted below.

Table 1.2 Drugs currently available in sheep.

Active Compound	Product	Type	Efficacy
Triclabendazole	Fasinex, Tribex	Fluke only	All stages from two-day-old immature flukes to adult
Closantel	Flukiver, Supaverm,	Fluke *Haemonchus contortus*	Adult and immature flukes from three to four weeks, *Haemonchus contortus*
Nitroxynil	Trodax	Fluke *Haemonchus* species	Adult and immature flukes, *Haemonchus* species and gutworms
Albendazole	Valbazen, Allverm	Fluke and worm	Adult flukes, gutworms, lungworms and tapeworms
Triclabendazole + Levimisole	Combinex	Fluke and worm (Combination)	Adult and immature flukes, gutworms and lungworms
Levimisole+ oxyclozanide	Nilzan Gold	Fluke and worm (Combination)	Adult flukes, gutworms and lungworms

Source: Modified from Fairweather and Boray, Fasciolicides: Efficacy, actions, resistance and its management (Fairweather and Boray, 1999a).

Table 1.3 Percentage efficacy spectrum of drugs at recommended dose rates against *Fasciola hepatica* in sheep.

	Age of fluke (weeks)													
Drug	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
Albendazole, Oxyclozanide, Niclofolan, Bithionol, Clorsulon+Ivermectin(inj)										50 - 70%		80 - 99%		
Clorsulon (oral)										90-99%				
Nitroxynil, closantel							50 - 90%			91 - 99%				
Rafoxanide				50-90%			91-99%							
Triclabendazole		90 - 99%		99- 100%										

Source: Modified from Fairweather and Boray, Chapter 7, Fasciolosis, JP Dalton editor, CABI Publishing,1998 (Dalton, 1998).

1.7.1 Triclabendazole (TCBZ)

Triclabendazole demonstrated activity against both mature and immature stages of *F. hepatica* at 10 mg/kg in sheep (Boray et al., 1983). Oral administration of TCBZ by dosing in sheep delivers the drug directly into the rumen of the host where TCBZ is metabolised into the TCBZ sulphoxide (TCBZ-SO) and the sulphone (TCBZ-SO_2) and hydroxy derivatives (Virkel et al., 2006). Evidence of fluke involvement was noted in drug metabolism of TCBZ to TCBZ-SO and TCBC-SO_2 with the fluke microsomal fraction capable of generating the active metabolites from the TCBZ parent drug (Mottier et al., 2004; Robinson et al., 2004). Based from these findings, it was hypothesised that, in addition to the host species liver, the flukes themselves play an important role in metabolising the parent drug.

It has been proposed that TCBZ displays the same mode of action as other BZs involving the binding of the drug to tubulin, which is a constituent protein in microtubules and mitochondrial membranes. The BZs have been shown to inhibit the polymerization of microtubules and therefore most likely interfere with microtubule-based processes in helminths (Lacey, 1988). For *F. hepatica*, this has led to suggestions that TCBZ and its metabolites cause uncoupling of oxidative phosphorylation (Carr et al., 1993). Alternatively, it may result in a failure to form microtubules and disrupt the transportation process in the tegument of flukes (Stitt and Fairweather, 1993), leading to the loss of the tegument of flukes (Halferty et al., 2009) and death of the fluke (Brennan et al., 2007). It has been shown that 48 hours after treating the host with TCBZ, morphological changes were observed in fluke (Hanna et al., 2010). Triclabendazole significantly inhibited microtubule polymerization, preventing the production of a tegumental secretion layer which covers the surface of the fluke and maintains apical membrane function (Halferty et al., 2008; Shareef et al., 2014; Tansatit et al., 2012). Therefore, the interruption of this layer may damage the tegument severely by 48 hours post treatment (Hanna et al., 2010). The destructive lesions observed on the tegument of flukes by SEM following treatments with TCBZ comprised tegument swelling, followed by blebbing, loss of spines and tegumental sloughing leading to complete disruption of the latter organ. These studies were carried out using *in vitro* and *in vivo* experiments on

both stages of juveniles and adults *F. hepatica* and F. *gigantica* (Halferty et al., 2008; Tansatit et al., 2012; Toner et al., 2010). However, other studies on TCBZ-resistance have shown that TCBZ does not target the tubulin molecule. It is believed that TCBZ-resistant isolates processed TCBZ more rapidly and their may be a role for drug detoxification pathways, specifically P-glycoprotein (Fairweather, 2011b).

1.7.2 Other flukicides

Nitroxynil is a narrow spectrum anthelmintic and it belongs to the halogenated phenol group of fasciolicides. Nitroxynil has been reported to have a high efficiency against adult flukes, lower efficacy (50-90%) against fluke aged 6 to 8 weeks and are not effective against earlier stages (Boray, 1986; Rapic et al., 1988). Experimental studies have shown that nitroxynil is active against adult TCBZ-resistant *F. hepatica*, with 100% efficacy ($P < 0.001$) by day 14 post treatment (Coles and Stafford, 2001; Mooney et al., 2009) and most recently similar results were reported in sheep farms in Northern Ireland (Hanna et al., 2015). The mechanism of action of the drug is unclear but the mechanism of action of nitroxynil is suggested to be due to uncoupling of oxidative phosphorylation, consequently leading to depletion of parasite ATP (Martin, 1997). This is based on the similarity of the chemical structure between halogenated phenols and 2, 4-dinitrophenol, a known uncoupler of oxidative phosphorylation in mammals. Extensive pharmacokinetics of nitroxynil studies have been undertaken on the plasma proteins, especially albumin both *in vivo* and in *vitro* (Alvinerie et al., 1991; Beretta and Locatelli, 1969). After drug administration in the sheep, the plasma levels increase and remain high for three days, then decreases precipitously although levels of two to three parts per million (ppm) can be still be detected after 60 days post treatment (Parnell, 1970). It is suggested that the flukes ingest the drug from the plasma and this is probably the primary route of nitroxynil ingestion by the parasites. In addition, this slow rate of elimination and the persistence of nitroxynil in the plasma may affect the efficacy of this drug against fluke in sheep.

Closantel and oxyclozanide have similar pharmacologic effects that act by uncoupling oxidative phosphorylation. They belong to the salicylanilide group of fasciolicides. The processes involved increasing the glucose uptake (Kane et al., 1980) and a decrease in fluke intra-tegumental pH (6.8 to 6.5) (Pax and Bennett, 1989). Because of these processes, parasites become paralyzed and starve to death (Skuce and Fairweather, 1990). Closantel has marked activity against liver flukes (Boray, 1986). It is highly lipophilic compound that extensively bound to plasma protein and is known to shuttle protons across membranes, in particular the inner mitochondrial membrane (Alvarez et al., 2007). Studies have been conducted looking at activity of closantel against adult TCBZ-resistant *F. hepatica* (Coles et al., 2000; Hanna et al., 2015). The results suggested that closantel works effectively against adult TCBZ-resistant *F. hepatica* with 100% efficacy (Coles et al., 2000) and was fully effective against TCBZ-resistant *F. hepatica* on sheep farms in Northern Ireland *(*Hanna et al., 2015). In a separate experiment using oxyclozanide, similar drug efficacy (99.6%) was reported against adult TCBZ-resistant *F. hepatica* (Coles and Stafford, 2001).

1.8 Definition of Anthelmintic resistance

Resistance is inherited; resistance genes are present in populations of parasites, which allow parasites to survive anthelmintic treatment, and when they the reproduce pass these anthelmintic resistance genes on to the next generations (Sangster, 1999). Resistance is defined as, 'a greater frequency of individuals within a parasite population that have been affected by a dose or a concentration of compound, are no longer affected; therefore, a greater concentration of drug will be needed (Prichard et al., 1980). Higher doses are needed in order to reach a certain level of drug efficacy. The World Association for the Advancement of Veterinary Parasitology (WAAVP) has drawn up specific guidelines for defining resistance, however, presently there is no 'gold standard' available for defining different isolates of *F. hepatica* in terms of drug sensitivity either *in vitro* or *in vivo*.

The cause of resistance in parasites is often difficult as many factors are involved in the evolution of this process. Since drug resistance is inherited and selective, the survivors following drug treatment pass resistance genes to their offspring and whilst it is believed that these genes are initially rare, their proportion in the parasite population increases under drug selection (Kaplan, 2004). A parasite that is able to respond to selective pressure, i.e drug pressure, is likely to carry the resistance alleles in the parasite population. This process of selection identifies that the rate of selection of resistant parasites is dependent on the frequency of drug use. The reliance on TCBZ as the drug of choice due to its high efficacy against immature and mature *F. hepatica* in sheep has inevitably led to TCBZ resistance. Since its introduction in the early 1980s, heavy reliance on TCBZ has resulted in resistance in sheep that has become a worldwide problem.

1.9 The incidence of triclabendazole resistance in the UK and worldwide in sheep

Triclabendazole resistance in liver flukes now poses major problems to small ruminant farmers throughout the world. The first case of TCBZ resistance has been documented in Australia in the mid 1990s (Overend and Bowen, 1995). Since then there have been several reports of TCBZ resistance on sheep farms in the UK; reviewed by Fairweather et al. (2005) and there is a broader concern that over-reliance on TCBZ resulted in increased levels of resistance in sheep in the UK (Daniel et al., 2010; Gordon et al., 2012a; Mitchell et al., 1998; O'Brien, 1998) and overseas such as The Netherlands (Gaasenbeek et al., 2001; Moll et al., 2000), South America (Ortiz et al., 2013) and Spain (Alvarez-Sanchez et al., 2006) and elsewhere in the world (see Table 1.4 and 1.5). Evidence from preliminary studies in England and Wales showed that TCBZ resistance was found on seven out of 25 sheep farms by using a composite fluke egg count reduction test (Daniel et al., 2012). However we do not know how extensive the problem of TCBZ resistance is at the national level.

Given the importance of TCBZ resistance a number of TCBZ-resistant (TCBZ-R) and susceptible (TCBZ-S) laboratory isolates have been derived and form the basis of experimental studies to identify mechanisms of drug resistance and all these *F. hepatica* isolates have been reviewed by Hodgkinson et al. (2013). In Ireland, the Sligo isolate was originally derived from the County of Sligo in 1998 and described as being TCBZ-R (Coles et al., 2000; Fairweather, 2011a). Other isolates that are listed; Cullompton which originated from Cullompton, Devon has been shown to be TCBZ-S *in vivo* (Halferty et al., 2008; McConville et al., 2009) and Fairhurst is also TCBZ-S (Walker et al., 2004). The Oberon isolate was confirmed to be TCBZ-resistant and Walker et al. (2004) have reported that the disruption was more severe in the Fairhurst than the Oberon isolate, based on *in vitro* study. For other TCBZ isolates, Leon, originated from North West Spain and studies have confirmed that this is a resistant isolate (Alvarez-Sanchez et al., 2006; Martinez-Valladares et al., 2010b). However, studies by Flanagan et al. (2011a) have shown that results from FECRT and the coproantigen reduction test, suggesting that the Leon fluke isolate was in fact susceptible to TCBZ treatment and this was confirmed at necropsy.

Other studies question whether failure of the efficacy of TCBZ to remove a parasite population from the liver is due to anthelmintic resistance or the inability of a fluke-damaged liver to metabolize the drug into its active forms and thus reduces the concentration of the active sulphoxide metabolites of TCBZ (Fairweather, 2011a). However, Sargison et al. (2011b) consider that liver function in metabolising TCBZ to ensure its efficacy may not be as important as previously assumed given that both TCBZ and its sulphoxide (TCBZ-SO_2) metabolite are capable of disrupting adult *F. hepatica in vitro (*Halferty et al., 2009). These studies highlight some of the problems of interpreting TCBZ resistance studies in *F. hepatica* populations in sheep.

Table 1.4 Reports of triclabendazole resistance in *Fasciola hepatica* in the United Kingdom (UK**)** and Ireland.

Country (region)	Sheep population tested	Anthelmintic tested	Test Used	Drug efficacy/result	References
Ireland	Not stated	**TCBZ**	Not stated	Failure of **TCBZ** treatment	(Lane, 1998)
Ireland	Not stated	**TCBZ**	Not stated	Failure of **TCBZ** treatment	(O'Brien, 1998)
West of Ireland	Naturally infected sheep on farms	**-TCBZ (Fasinex)** - Nitroxynil (Trodax) -Closantel (Flukier) -Oxyclozanide (Zanil)	FECRT	**- TCBZ: 49%-66%** efficacy (7-56 days pt) -Nitroxynil: 100% efficacy -Closantel: 100% efficacy -Oxyclozanide: 100% efficacy	(Mooney et al., 2009)
UK	Naturally infected sheep on farm and experimentally infected sheep	**TCBZ**	-FECRT -CRT	Failure of TCBZ treatment	(Gordon et al., 2012a)
UK (England, Wales and Scotland)	Natural infection sheep farms	**TCBZ (Fasinex)**	CFECRT	-Full validation of cFECRT - Loss of efficacy of TCBZ in 6/13 farms in SW Wales and one farm in Scotland	(Daniel et al., 2012)
Northern Ireland	Natural infection sheep farms	**-TCBZ (Fasinex)** -Nitroxynil (Trodax) -Closantel (Flukier)	-FECRT -Coproantigen ELISA	**-TCBZ was ineffective** -Nitroxynil and closantel were full effective	(Hanna et al., 2015)

CRT = Coproantigen reduction test, pt = post treatment, cFECRT = Composite faecal egg count reduction test, TCBZ = Triclabendazole, FECRT = Faecal egg count reduction test

Table 1.5 Reports of triclabendazole resistance in *Fasciola hepatica* outside the United Kingdom.

Country (region)	Sheep population tested	Anthelmintic tested	Test Used	Drug efficacy/result	References
Australia (Victoria)	Natural infection sheep farms/ Experimental infection	**TCBZ**	-FECRT - Fluke recovery	First case of TCBZ resistance reported worldwide	(Overend and Bowen, 1995)
Netherlands	Natural infection sheep farms	**-TCBZ (Fasinex)** -Closantel (Flukier)	FECRT	-TCBZ: 15.3% efficacy -Closantel: 99.7% efficacy	(Moll et al., 2000)
Netherlands	Experimental infection	**TCBZ (Fasinex)**	FECRT	TCBZ: 10.8% efficacy	(Gaasenbeek et al., 2001)
Spain	Sheep farm	**TCBZ (Fasinex)**	FECRT	TCBZ: 75.7% efficacy (30 days pt)	(Alvarez-Sanchez et al., 2006)
Peru	Experimental infection	**TCBZ (Fasinex)**	Fluke recovery	TCBZ: 25.2% efficacy	(Ortiz et al., 2013)

TCBZ = Triclabendazole, FECRT = Faecal egg count reduction test

1.10 Mechanisms of resistance

The mechanism of drug resistance is thought to be influenced by genetic changes in drug uptake, drug metabolism that inactivates / removes the drug or prevents its activation and a change in the distribution of the drug in the target organism that prevents the drug from accessing its site of action. In liver fluke, it is suggested that drug metabolism is up-regulated in TCBZ-resistant *F. hepatica* (Alvarez et al., 2005; Robinson et al., 2004). The active form of TCBZ-SO, blocks the movement of tegumental secretion, leading to widespread sloughing of the tegument. With regard to the uptake of drugs (TCBZ to TCBZ-SO); the uptake by TCBZ-R fluke isolates was shown to be significantly lower than that of the TCBZ-S isolates (Alvarez et al., 2005; Mottier et al., 2006), suggesting that uptake of TCBZ and its metabolites is altered in resistant fluke. Furthermore, the well-known drug transporters, p-glycoprotein-linked drug efflux pumps, were suggested to be involved in the resistance mechanism. It is likely that p-glycoprotein expression can have important effects on drug absorption, distribution and elimination; this is specific to TCBZ as it is not seen in albendazole (Mottier et al., 2006). In addition, p-glycoprotein inhibitors have potential to lead to a 'reversion' from resistance flukes to susceptible flukes. Similarly, inhibition of p-glycoprotein by verapamil, appears to enhance TCBZ action in TCBZ-R isolates, but such findings were not observed for TCBZ-S isolates (Fairweather, 2009).

In terms of what is known about genetic changes or mutations responsible for the development of resistance this is complicated because the genes involved are not known although, extrapolating from the situation of BZ resistance, it was suggested that tubulin was involved (Robinson et al., 2002). However, studies on TCBZ have indicated that the same mutation does not seem to cause resistance in *F. hepatica* (Fuchs et al., 2013). Mutations in p-glycoprotein genes have been implicated (Wilkinson et al., 2012) but again other studies do not support their involvement (Elliott and Spithill, 2014). Essentially the mode of action and mechanisms of TCBZ resistance remain unknown and remain the focus of research; reviewed by Hodgkinson et al. (2013).

1.11 Diagnosing triclabendazole resistance

As highlighted above diagnosing TCBZ resistance can be challenging, particularly in the field. In the absence of WAAVP guidelines there are a number of approaches that can be used, for example faecal egg count reduction test (FECRT), controlled efficacy tests and the coproantigen ELISA test. These tests rely on accurate methods of detecting *F. hepatica* infection, as highlighted in section 1.5 above. The relative merits of each approach are highlighted below along with the ongoing need to evaluate and validate these tests. The tests differ in their ability to detect TCBZ resistance *in vivo* or *in vitro*.

1.11.1 *In Vivo* Diagnostic Tests

1.11.1.1 Faecal egg count reduction test

The FECRT has become the principle means of diagnosing the efficacy of anthelmintic treatments under field conditions. To conduct the test, faecal samples can be collected from individual sheep per rectum and then coproscopy examinations performed but this procedure is not suitable on commercial farms as the sedimentation technique is time consuming when processing a large number of faecal samples and needs trained personnel.

Using a subset of the flock, 10 animals per treatment group, has become a well-established method to diagnose anthelmintic efficacy of different classes of drugs against nematodes in sheep based on the guidelines of the WAAVP (Wood et al., 1995). This method has been adapted for detecting TCBZ resistance in experimentally infected sheep (Flanagan et al., 2011a). Other studies also suggested that the FECRT is a useful diagnostic test to investigate the true anthelmintic failure for use in the field (Gordon et al., 2012a; Hanna et al., 2015; Jones et al., 2014). A separate study has validated the use of a composite FECRT (cFECRT) in groups of 10 sheep to detect TCBZ resistance status on sheep farms (Daniel et al., 2012). The cFERCT, requires sampling of faecal samples from a group of 20 sheep, followed by flukicide treatment, and 21 days post-

drenching sampling of the same 20 sheep for assessing TCBZ efficacy (Daniel et al., 2012). The cFECRT approach was shown to be just as sensitive as using the traditional method, which is the individual counts (Daniel et al., 2012). Both tests have limitations in that they may give essentially different results due to egg losses during processing and counting stages. Unlike roundworms, liver flukes do not release eggs consistently into faeces as eggs may be retained in the gall bladder, so even after the removal of mature flukes from the liver of sheep (i.e. following drug administration) a positive FEC can be recorded, however the fate of adult fluke that are killed by the drug is unclear (Sargison, 2012). Therefore, for an anthelmintic to be fully effective and to avoid misinterpretation of anthelmintic efficacy it is recommended that FECRT be taken 14 and 21 days post treatment to exclude the problem associated with retention of *F. hepatica* eggs in the bile ducts of sheep (Flanagan et al., 2011a).

1.11.1.2 Controlled slaughter test

This *in vivo* test is most reliable for all types of drugs and is the gold standard for evaluating anthelmintic efficacy. However, it is also the most costly test as animals have to be sacrificed so that total worm counts (nematode parasites) and liver fluke burden (*F. hepatica*) can be performed, it is therefore not suitable for diagnosing resistance in the field but ideal for research work such as dose confirmation studies or for confirmation of resistance. The WAAVP provided the guidelines for conducting this test for gastrointestinal nematode parasites (Wood et al., 1995). The only reliable and useful test for TCBZ resistance in *F. hepatica* is a dose and slaughter study (Coles et al., 2006). The controlled slaughter test is conducted when both control and infected groups in the trial have been treated with an anthelmintic in which the parasites have reached patency in the host. The percentage efficacy is calculated by comparing the mean of parasites recovered in treated and control animals (Wood et al., 1995). FEC and coproantigen reduction tests (CRT) have been validated as tests for diagnosis of TCBZ resistance in sheep and cattle by using the WAAVP approved dose and slaughter trials for nematodes (as official guidelines for interpretation of the CRT do not exist)

(Brockwell et al., 2013; Gordon et al., 2012a). In nematode parasites, resistance is confirmed when the reduction in worm burdens is less than 90% or if 1000 or more worms survived anthelmintic treatment (Wood et al., 1995).

1.11.1.3 Coproantigen ELISA test (BIO K 201)

As discussed in section 1.5 above the coproantigen ELISA (cELISA) has been developed for the early detection of *F. hepatica* infection and the levels of coproantigens were no longer detected in the faeces within 2 weeks of anthelmintic treatment identifying its potential for diagnosing TCBZ resistance (Dumenigo and Mezo, 1999; Espino et al., 1997; Gordon et al., 2012b; Mezo et al., 2004). This test has been applied in a number of experimental and field studies reporting cases of TCBZ resistance (see Table 1.6). However, studies done by Novobilsky et al. (2012) found that following TCBZ and albendazole treatment, fluke eggs appeared in two individual ewes on day 0, 7 and 24, however the presence of coproantigen were not detected. It may thus be concluded that the release of coproantigen by this test may cause false negative results. This is also supported by other studies that reported in naturally infected sheep, 2 to 6 flukes were found in liver of 5 out of 27 slaughtered sheep, however they were found to be negative for the cELISA (Gordon et al., 2012b). Most recently, fluke eggs were found in one animal, on day 7 post treatment with TCBZ but negative result was reported by using cELISA (Robles-Pérez et al., 2013). The cELISA has not been fully validated under field conditions and therefore requires further evaluation.

1.11.1.4 Polymerase Chain Reaction (PCR)

Molecular and genetic tests are limited and available for detecting benzimidazole resistance in *Haemonchus contortus* in sheep (Taylor et al., 2002). Recently, PCR assay is a newly developed molecular technique for diagnosis of *F. hepatica* infection in sheep which may prove a useful method of detecting anthelmintic resistance in naturally and

experimentally infected sheep (Martinez-Perez et al., 2012; Robles-Pérez et al., 2013). The sensitive and specific detection of *F. hepatica* DNA by PCR has been investigated as a diagnostic tool to monitoring the efficacy of TCBZ treatment and detect cases of TCBZ resistance. The assay, claims to detect liver fluke infection as early as 2 wpi, which is two weeks earlier than detection using cELISA (Robles-Perez et al., 2013). However, this diagnostic tool has limitations and every step in preparing the samples must be carefully done to achieve the specificity. DNA extraction and amplification reaction from faecal samples is crucial and one limiting factor is the presence of PCR inhibitors in faecal samples which can inhibit PCR amplification of target DNA. The inhibitors that have been identified are from gut materials, complex polysaccharides derived from plants in the diet (Fernando et al., 2003) or food debris (Greenfield and White, 1993), the break down products of heme (eg: bilirubin, bile salts) (Widjojoatmodjo et al., 1992) and DNA present in the faeces could also inhibit the *Taq* polymerase (Weyant et al., 1990). Some also indicate that inhibitors are from the materials and reagents such as KCL, NaCl and other salts, ionic detergents such as sodium deoxycholate, sarkosyl and SDS (Weyant et al., 1990), ethanol and isopropanol (Loffert, 1997) and phenol (Katcher and Schwartz, 1994) that come into contact with DNA samples during processing. There have been reports of other reagents used for cultivating microorganisms that could potentially be PCR inhibitors (Rossen et al., 1992); other inhibitors are still unknown.

To eliminate the PCR inhibitors, there are several options that can be applied during DNA extraction such as dilution of template DNA (1:10) as recommended by Monteiro et al. (1997). QIAamp tissue method extraction is the kit that was proposed in this study (Monteiro et al., 1997). According to Wehausen et al. (2004), the use of magnetic beads in DNA extraction was superior compared to other materials.

1.11.2 *In Vitro* Diagnostic Tests

Another test that is used for gastrointestinal nematodes is the egg hatch assay (EHA) that has been developed to detect benzimidazole resistance (Coles et al., 2006; von Samson-Himmelstjerna et al., 2009). Although the EHA was originally developed to detect anthelmintic resistance in nematode parasites, this test can be also be used in fluke. Several studies have been conducted in *F. hepatica* for validation of EHA (Alvarez et al., 2009; Canevari et al., 2014; Fairweather et al., 2012).

1.11.2.1 Egg Hatch Assay

The egg hatch assay (EHA) is used to detect benzimidazole resistance in sheep gastrointestinal nematodes and relies on ovicidal activity of the drugs. Eggs of resistant isolates embryonate and hatch in higher concentrations of the drug than those of a susceptible isolates (Coles et al., 2006; Whitlock et al., 1980). Proof of concept for EHA to detect TCBZ resistance in *F. hepatica* was carried out (Alvarez et al., 2009; Fairweather et al., 2012). In their work, fresh eggs are recovered from the gall bladder and the hatch rate compared for eggs at various concentrations of TCBZ, albendazole (ABZ) and their sulfoxide metabolites. Most recently, the EHA was developed to detect resistance of *F. hepatica* to ABZ by using eggs from faecal samples of ABZ-susceptible and -resistant *F. hepatica* isolates (Robles-Perez et al., 2014). It has been shown that susceptible and resistant isolates can be differentiated at serial dilutions of drug (Fairweather et al., 2012; Robles-Perez et al., 2014). It is a simple diagnostic tool as it does not require any expensive equipment, however, the limitation of this technique is that its requires large numbers of clean eggs to conduct the test and its validation for field diagnosis of anthelmintic using eggs from faeces remains to be performed.

1.12 Aims and Objectives

The aim of this study was to:

1. Evaluate and compare three diagnostic tests, composite faecal egg count reduction test (cFECRT), coproantigen ELISA (cELISA) and PCR assay, for their ability to detect *F. hepatica* infection and determine TCBZ efficacy in sheep experimentally infected with a TCBZ-S isolate.

2. Improve the design of the composite FECRT (cFECRT) to detect TCBZ resistance in fluke populations.

3. Determine the prevalence of TCBZ resistance in sheep farms in the UK using the Composite Faecal Egg Count Reduction Test (cFECRT).

Table 1.6 Summary of coproantigen ELISA studies.

Test used/Antibody	Experimental procedures	Faecal sampling	Parasite	Results	References
Coproantigen ELISA *Mab-ES78, mouse IgG2a*	Rats (n=20), experimentally infected with 25 metacercariae	Weekly from day of infection until 16 wpi	*F. hepatica*	-Coproantigen was first detected at 4wpi in 45% of animals. 100% tested positive by 6 wpi. - Eggs began to appear in faeces at 8 wpi.	(Espino et al., 1997)
Coproantigen ELISA *Mab-ES78*	Sheep (n=10) experimentally infected with 200 metacercariae	From day of infection until 14 wpi	*F. hepatica*	Coproantigen first detected at 4wpi in 5 animals. All animals were positive by 6 wpi. -Eggs began to appear in faeces between 10 and 12 wpi.	(Dumenigo et al., 2000)
Coproantigen ELISA *E/S antigens*	-experimental infected sheep (n=20) with 50 metacercariae (Group 1), 100 metacercariae (Group 2), 200 metacercariae (Group 3) and control group	From day of infection until 12 wpi	*F. hepatica*	Coproantigen detected from 4wpi and at the 9th wpi, 14 out of 15 infected animals were positive to coproantigens. The sensitivity was 93.3%. Eggs began to appear in faeces after 8 wpi.	(Almazan et al., 2001)
Coproantigen ELISA *IgG*	Rats (n=36) experimentally infected with 20 metacercariae	From day of infection until 21 wpi	*F. hepatica*	- For coproantigen detection, the infection was firstly detected 1 or 2 weeks before the appreance of *F.hepatica* eggs in the faeces. - Eggs began to appear in faeces at 7 wpi.	(Paz-Silva et al., 2002)

Coproantigen ELISA *Mab MM3*	Sheep, experimentally infected with 5, 10, 20 and 40 metacercariae	-From day of infection until 18 wpi - 6 lambs were treated with TCBZ on week 14 pi - 15 lambs were untreated -lambs were slaughtered at 18 wpi to determine fluke burden	*F. hepatica*	-all treated animals (n=6) had no fluke whereas untreated animals (n=15) were found to be infected with between1 and36 flukes. - Comparison of coproantigen levels and egg counts indicated that infected sheep had detectable amounts of coproantigens 1-5 weeks before patency - in treated animals, coproantigen became undetectable from 1 - 3 weeks after treatment; however eggs continued to be intermittently shed in faeces until 4 weeks after treatment - in untreated animals, coproantigen remained detectable until at least 18 wpi	(Mezo et al., 2004)
Coproantigen ELISA *Mab ES78*	Sheep, (n=7) experimentally infected with 100 metacercariae	From day of infection until 25 wpi At week 18, sheep were drenched with TCBZ	*F. gigantica*	Coproantigen was first detected within 5 to 9 wpi (57-71% of animals were positive) and reached 100% by 11 wpi. Eggs began to appear in faeces from 15 to 17 wpi.	(Endah Estuningsih et al., 2004)
Coproantigen ELISA *Mab MM3*	Infected sheep with 200 metacercariae	from day of infection and once a week, starting at 5wpi - until 32 wpi	*F. hepatica* *F. gigantica*	<u>*F. hepatica*-infected sheep</u> i) Eggs began to appear in faeces from 10 to 14 wpi. ii) coproantigen levels and egg counts indicated that sheep had detectable amounts of coproantigens 4-7 weeks before patency and coproantigen level increased above the cut-off value from 6 to 9 wpi	(Valero et al., 2009)

				F. gigantica-infected sheep i) The eggs began to appear in faeces from weeks 11 to 16 wpi. ii) coproantigen levels and egg count indicated that sheep had detectable amounts of coproantigens 3-6 weeks before patency and coproantigen levels increased above the cut-off value between 7 to 11 wpi	
Coproantigen ELISA *Mab MM3*	Infected sheep (n=38) with 250 metacercariae	Faecal samples were collected twice-weekly throughout the trial period -CRT as a diagnostic marker	*F. hepatica*	-For coproantigen detection, the infection was firstly detected at 5 to 6 wpi and onwards. -The eggs began to appear in faeces from weeks 10 wpi.	(Flanagan et al., 2011b)
Coproantigen ELISA *Mab MM3*	Infected sheep (n=49) with 200 metacercariae of 1 of 4 *F.hepatica* isolates	Faecal samples were collected twice-weekly throughout the trial period CRT as a diagnostic marker	*F. hepatica*	Faecal samples tested positive by coproantigen and egg count respectively, at: i) Cullompton isolates – 50 and 77 dpi ii) Leon isolates – 62 and 75 dpi iii) Fairhurst isolates – 53 and 70 dpi iv) Oberon isolates – 47 and 59 dpi All sheep, regardless of fluke isolate, were positive for coproantigens at 12 wpi. Eggs were detected in all the Oberon *F. hepatica*-infected sheep at 12 wpi, but eggs were only detected in 90% (9/10), 69% (9/13) and 77% (10/13) of the	(Flanagan et al., 2011a)

				Cullompton, Leon and Fairhurst *F. hepatica*-infected sheep groups at 12 wpi.	
Coproantigen ELISA *Mab MM3*	-experimental serology positive sheep (n=24)	Faecal samples collected on day 0, 7, 24, 46 and 74. -CRT as a diagnostic marker - Group A (5mg/kg ABZ) -Group B (10mg/kg TCBZ) -Group C (control) At day 24: -Group B (10mg/kg TCBZ) -Group C (10mg/kg TCBZ)	*F. hepatica*	-In TCBZ treated animals, coproantigen and eggs were no longer detected in faeces 7 days after application -ABZ treatment had no effect on either egg or coproantigen detection -In all groups, a greater number of egg positive than coproantigen positive animals were detected. -all coproantigen positive animals had fluke eggs in their faeces - False negative results were detected in 2 animals in Group C (positive for egg count on day 0, 7 and 24), but were negative for the presence of coproantigen.	(Novobilsky et al., 2012)
Coproantigen ELISA *Mab MM3*	-naturally infected sheep (n=45) -experimental infected sheep (n=7) with 200 metacercariae	Experiment group: Faecal samples were performed weekly from 6wpi to 12 wpi.	*F. hepatica*	<u>natural infection</u> -only 24 out of 45 sheep had positive egg counts -All animals were positive by coproantigen detection <u>experimental infection</u> -Eggs were detected by 9wpi. -Coproantigen was first detected by 4wpi (57.1% of animals were positive) and reached 100% by 8wpi.	(Martinez-Perez et al., 2012)

Coproantigen ELISA *Mab MM3*	-naturally infected sheep (2 farms)	CRT as a diagnostic marker Longitudinal study from June to November	*F. hepatica*	Coproantigen ELISA and FEC became positive at the same time point (in July). *The coproantigen ELISA was negative before FEC and this could be due to the low fluke burdens (5 or lower) in the livers.	(Gordon et al., 2012b)
Coproantigen ELISA *Mab MM3*	naturally infected sheep (Northern Ireland)	Treated: TCBZ, nitroxynil and closantel, (Faeces: at day 0 and day 21	*F. hepatica*	Nitroxynil and closantel reduced the FECs and was supported by the results of CRT. However, results of CRT remained positive after 21 days. TCBZ was ineffective; confirmed by FECRT and CRT results.	(Hanna et al., 2015)

E/S excretory/secretory; Mab monoclonal antibody

CHAPTER 2

Evaluation of diagnostic tests for the detection of *Fasciola hepatica* infection and determination of triclabendazole efficacy in sheep experimentally infected with a triclabendazole susceptible field isolate

2.1 INTRODUCTION

The availability of effective and sensitive methods for the accurate detection of liver fluke infection under field conditions is essential if *Fasciola hepatica* is to be effectively controlled and has an important role in monitoring the efficacy of drug treatment. The ability to detect infection at the early stage as parasites migrate through the liver is of particular importance. To date a number of different techniques for the detection of *F. hepatica* infection have been described in the literature; FEC, coproantigen ELISA (cELISA) and polymerase chain reaction (PCR) based assays.

2.1.1 Detection of *Fasciola hepatica* infection in sheep

Historically the diagnosis of fasciolosis was made using coprological methods to detect fluke eggs in the faeces of infected sheep (Anderson et al., 1999). FEC has become a common diagnostic and research tool worldwide due it being both simple to conduct and inexpensive. However, there is a long timeframe before infections become patent approximately 8-12 week (Andrews, 1999) and in experimental studies in sheep observation of *F. hepatica* eggs under the microscope does not occur prior to 8 weeks post infection (Zimmerman et al., 1982). Furthermore, poor sensitivity of the FEC (estimated to be only 30%) can lead to false negative results (Happich and Boray, 1969a). Therefore, in order to detect early stages of liver fluke infection, effort has been made to develop alternative robust and sensitive diagnostic tools for *F. hepatica* infection in sheep. A number of diagnostic tests have been developed for detecting infection of liver flukes including the cELISA (Brockwell et

al., 2013; Flanagan et al., 2011a; Flanagan et al., 2011b; Gordon et al., 2012b; Hanna et al., 2015; Kajugu et al., 2015) and PCR assay (Martinez-Perez et al., 2012; Robles-Pérez et al., 2013).

Traditionally enzyme-linked immunosorbent assay or ELISA has been used to detect antibodies that are produced in response to liver fluke infection in blood or milk samples (Salimi-Bejestani et al., 2005b). A commercial coproantigen ELISA (cELISA, BIO K 201, Bio-X Diagnostics, Jemelle, Belgium) test for fasciolosis, based on the use of MM3 monoclonal antibody for antigen capture is available and detects fluke coproantigens in faeces. The test was used as an alternative method to FEC for detecting fluke infection in the faeces of cattle and sheep. In the antigen-capture ELISA, polyclonal antibody is coated on a 96-well microplate and the faecal samples added; if the fluke specific coproantigen is present in the faecal samples it can bind to the antibody. A first conjugate (MM3 monoclonal, biotin conjugated) is added to bind to the antibody to form a Y structure (Fig. 2.1) and then the second conjugate (avidin peroxidase) is added. Finally TMB chromogen (3,3',5,5'-tetramethylbenzidine) is added to form a colour development – yielding a blue colour which changes to yellow upon addition of a sulfuric or phosphoric acid stop solution. Coproantigen values are expressed as the percentage positivity (PP) according to the formula; % = (Mean OD of the sample / Mean OD of positive control) x 100. If the value of PP (%) falls above the threshold (according to the manufacturer's protocol) positive reactions are reported.

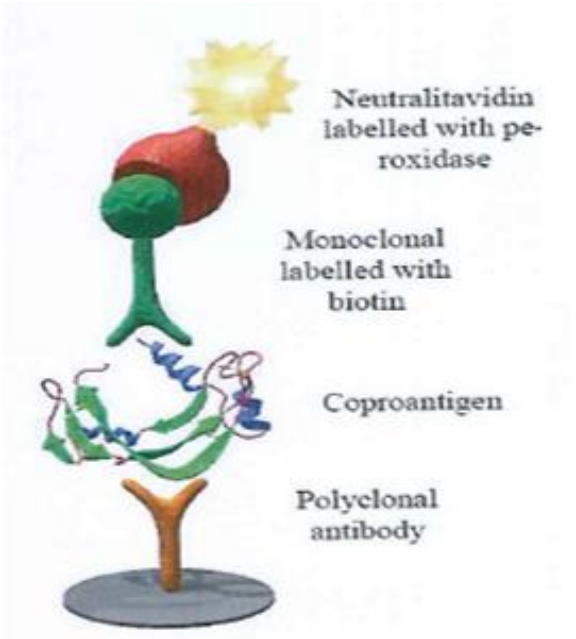


Figure 2.1 Coproantigen ELISA (BIO K 201, Bio-X Diagnostics, Jemelle, Belgium).

In experimental infection in sheep, the early detection of *F. hepatica* infection has been confirmed by cELISA due to coproantigens present in faeces during the pre-patent period of infection. Infections can be detected as early as 5 weeks post infection (wpi); earlier than FEC (Flanagan et al., 2011a; Flanagan et al., 2011b). Furthermore, other tests on MM3 capture ELISA have reported 100% sensitivity and 100 % specificity with the ability to detect infection even when sheep were infected with the lowest infective dose of metacercariae (ranging from 10 to 40 metacercariae, (Mezo et al., 2004). These authors reported that this test can detect infection as early as 5 wpi. However, this is somewhat limited by the unpredictable release of coproantigen in the faeces and false negative results can be reported (Flanagan et al., 2011b). The cELISA is a rapid, specific and easy diagnostic tool to use in the laboratory. The manufacturers report that it can be used for large number of samples in routine diagnosis which can be finished within 5 hours (BIO K 201 manufacturer's guidelines). The assay is therefore considered a sensitive method for detecting early diagnosis of liver fluke infections in sheep faeces.

Recently, PCR assays have showed promise as a sensitive diagnostic tool for the detection of liver fluke infection in sheep. Amplification of *F. hepatica* DNA from faecal samples by PCR assay was first achieved by Martinez-Perez et al. (2012). In

that study, they conducted two different PCR assays to detect the cytochrome C oxidase 1 gene (Cox-1); standard PCR and nested-PCR. For standard PCR, specific PCR primers were designed based on *F. hepatica* mitochondrial DNA (mtDNA) Cox-1 sequence (Cox1 Forward/Reverse) (Martinez-Perez et al., 2012). Standard PCR analysis from faecal samples was carried out from 0 to 8 wpi and showed that the detection of *F. hepatica* infection was positive from three weeks post infection. An alternative nested-PCR utilizes two consecutive PCRs and is carried out the same way as the standard PCR but the larger fragment produced by the first reaction is used as the template for the second PCR reaction. Using this method, the sensitivity and specificity of DNA amplification was higher than the standard PCR as results showed that infection with *F. hepatica* can be detected as early as 2 wpi (Martinez-Perez et al., 2012). Most recently, another PCR assay was developed to detect liver fluke infection in faecal samples from sheep. This PCR was designed to specifically amplify the *F. hepatica* internal transcribed spacer 2 (ITS2) region on faecal samples from infected sheep (Robles-Perez et al., 2013). The infection can be detected as early as 2 wpi (Robles-Pérez et al., 2013). On the basis of these studies PCR offers much higher sensitivity than the other methods (Robles-Perez et al., 2013).

Further to detecting *F. hepatica* infection a sensitive, specific diagnostic test is needed to confirm the efficacy of triclabendazole (TCBZ) treatment and the presence of TCBZ resistance. The use of the simpler and less expensive FEC in which only pre- and post-treatment samples are required is a widely used test; however, *F. hepatica* egg shedding is intermittent and infected sheep only consistently shed eggs in the faeces from 10 to 12 wpi (Andrews, 1999). While this is the most technically correct procedure for detecting fluke eggs from infected sheep, a particular problem with this test is that its low sensitivity can result in false negative post-treatment FEC results. Alternatively, fluke eggs can be stored or remain in the gall bladder of the animal following drug treatment, even when sheep have been successfully treated, which makes interpretation of post treatment counts difficult (Flanagan et al., 2011a; Flanagan et al., 2011b; Mitchell et al., 1998). Furthermore, sedimentation methods are laborious techniques and are time consuming, requiring each sample to be examined by trained staff, suggesting it is not suitable for analysing samples from large flocks.

The controlled slaughter test is the 'gold standard' to establish anthelmintic resistance in liver fluke populations (Gordon et al., 2012a) as suggested by WAAVP guideline to obtain full confirmation of true parasite resistance (Coles et al., 2006). In this test, animals in both of control and treated groups are slaughtered following treatment and sheep fluke burdens counted. However, this test is expensive and is of limited diagnostic value as it requires sacrificing of animals, thus it is not recommended for the routine diagnosis of anthelmintic resistance. Other tests are used to monitor susceptibility of *F. hepatica* to TCBZ are post-treatment fluke histology (Hanna et al., 2015) and egg hatch assay (Alvarez et al., 2009; Canevari et al., 2014; Robles-Perez et al., 2014).

The cELISA has been applied in a number of experimental and field studies reporting cases of TCBZ resistance (see Table 1.5). However, the nature of the release of coproantigen may cause this test to give false negative results (Novobilsky et al., 2012) and cases of naturally infected sheep recording negative cELISA results but positive by FEC raises questions over the performance of the test in detecting TCBZ resistance (Gordon et al., 2012b; Robles-Perez et al., 2013). Similarly the sensitivity and specificity of detection of *F. hepatica* DNA by PCR has identified this as a diagnostic tool for monitoring the efficacy of TCBZ treatment and detect cases of TCBZ resistance, although it remains to be evaluated.

Aim of the study

A number of diagnostic tools have been developed for detecting *F. hepatica* infection in sheep, but each suffers to some degree from sensitivity, reliability, reproducibility and ease of interpretation. In this section, the research is therefore focused on evaluating the currently available diagnostic tests and their ability to detect *F. hepatica* infection in experimentally infected sheep. The study was carried out on sheep infected with 200-215 metacercariae of a TCBZ susceptible field isolate of *F. hepatica*. Infected sheep were maintained until patent infections were well established (until 14 wpi). Faecal samples were taken weekly from 0 to 14 weeks wpi and subjected to cELISA, FEC and PCR analysis. At 14 wpi sheep were treated with TCBZ at 10 mg/kg bodyweight. Daily faecal samples were taken from day 0 to

day 10 post treatment (pt) and subjected to cELISA and FEC analysis. Ten days post treatment (dpt) sheep were slaughtered and the number of fluke present were counted.

2.2 MATERIALS AND METHODS

2.2.1 Experimental design for the infection of sheep with a triclabendazole susceptible isolate of *Fasciola hepatica*

Twelve lambs were infected on day 0 with a range of 200-215 metacercariae using an oral dosing syringe. ~Two hundred metacercariae were counted using a magnification of 4 x under a dissecting microscope and viability was determined by observing movement within the metacercarial cyst. The visking tubing (Sigma-Aldrich Company Ltd, Dorset, UK) is an artificial permeable membrane and it was used in the study. The visking tubing was cut out and inserted into the end of a syringe loaded with water, which was then used to flush the sheep's mouth. Faecal samples were taken per rectum at weekly intervals up to 14 weeks post infection (wpi). Faecal samples were subjected to coproantigen detection, PCR faecal assay and egg detection via sedimentation to detect the presence of *F. hepatica* infection. At 14 wpi, sheep were divided into two groups. Group 1 was allocated as the untreated control group whereas Group 2 animals were treated with TCBZ (Fasinex®; 10 mg/kg) per os. Faecal samples were collected daily until 10 day post treatment (dpt). Again, individual faecal samples were assessed using sedimentation and cELISA. Faecal egg counts were carried out weekly from week 0 to 14 wpi and daily post treatment from day 0 to 10 dpt. Similarly the cELISA was conducted on weekly samples taken from 1 to 14 wpi and daily from day 0 to 10 dpt. At 10 dpt, all sheep were euthanized and post mortem exam was carried out for liver fluke recovery and enumeration.

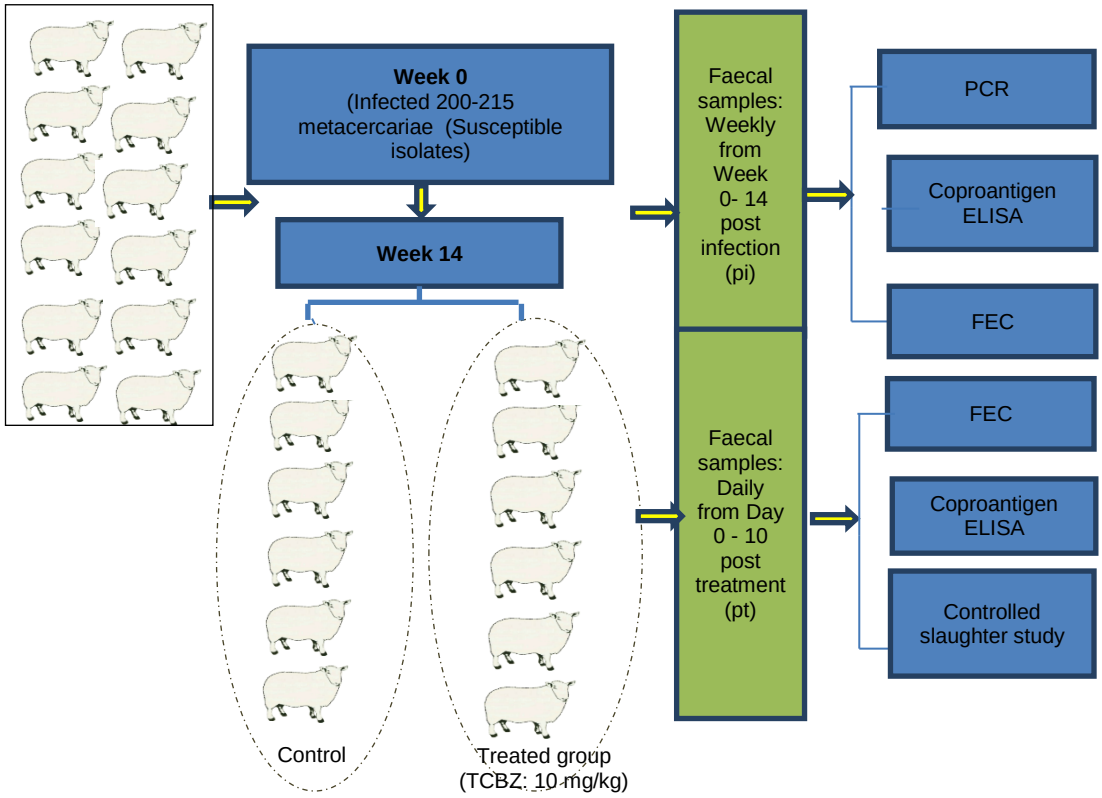


Figure 2.2.1 Experimental design for the infection of sheep with a triclabendazole susceptible *Fasciola hepatica* isolate and comparison of diagnostic methods for the detection of *F. hepatica* infection and triclabendazole efficacy.

2.2.1.1 Source of *metacercariae*

The maintenance and infection of snails and production of metacercariae was carried out by Katherine Allen, in the Department of Infection Biology, University of Liverpool. A field isolate of *F. hepatica* from an organic farm with no history of TCBZ treatment was sourced from naturally infected sheep. Eggs were isolated from faecal samples using standard parasitological techniques and used to infect *Galba truncatula* snails from a colony of snails maintained in house on pans of clay mud and fed on a diet of *Oscillatoria* algae. Both the snails and algae were maintained at a controlled temperature of 22^{o}C. For experimental infection miracidia were released from *F. hepatica* eggs following embryonation at 27^{o}C in the dark for 14 days followed by exposure to a direct light source to stimulate hatching. Following infection, snails were maintained on mud pans and fed every two to three days until

six wpi. Prior to being shed snails were sealed in Visking tubing containing water in individual wells of a six well plate. Shedding of cercariae was triggered by exposing to a drop in temperature of 8-12^{o}C for 30 min followed by a slow return to room temperature (RT) under a light source over a period several of hours. Snails were left overnight for the emerged cercariae to encyst as metacercariae on the sides of the Visking tubing after which they were washed and stored at 4^{o}C until use.

2.2.1.2 Experimental infection of lambs

All experimental procedures described were conducted at the University of Liverpool, subject to the Institute's Experiments and Ethics Committee approval, and were conducted under approved British Home Office license no PPL4003621 in accordance with the Animals (Scientific Procedures) Act of 1986. Lambs were purchased and housed in indoor pens throughout the study and for a minimum period of four weeks prior to infection. On housing and immediately prior to infection they were subjected to both faecal analysis and serum antibody ELISA testing (Salimi-Bejestani et al., 2005b) to confirm fluke-free status prior to infection. Whilst housed they were fed with ad libitum access to hay and a 17% protein pellet at 0.5 kilo per day and water.

2.2.2 Parasitology Techniques

2.2.2.1 Faecal egg count by sedimentation

Faecal egg counts were performed from each animal using 5g of faecal sample mixed with water to make a faecal slurry. The faecal slurry was washed through a stack of three difference sized sieves with the 710 μm sieve on top, followed by the 150 μm and 38 μm mesh size sieves (Figure 2.2.2 a). The stack of sieves was washed under the running tap water until the water ran clear from the bottom sieve. At which point the top sieve was removed and the lower two sieves were washed through by repeating the same washing procedure as above. The 150 μm sieve was removed and the sediment on the surface of the 38 μm sieve was backwashed into a

500 ml beaker. The beaker was topped up with tap water and allowed to settle for 4 min (Figure 2.2.2 b). The supernatant was discarded and to leave approximately 100 ml at the bottom of the beaker which was then refilled with tap water and left to stand for another 4 min. The process was repeated as many times as necessary until the supernatant was clear (Figure 2.2.2 c). The supernatant was then removed until ~50ml remained in the bottom of the beaker and the entire volume was transferred to a viewing chamber petri dish and two drops of methylene blue were added. The petri dish was placed under a microscope (Motic®) with a minimum magnification of 4 x to screen the presence of the fluke eggs. All eggs were counted in the 5 g of faeces and adjusted to provide an egg per gram (epg) count. The procedure for recovering eggs from faecal samples is described in detail in Appendix 2.1.

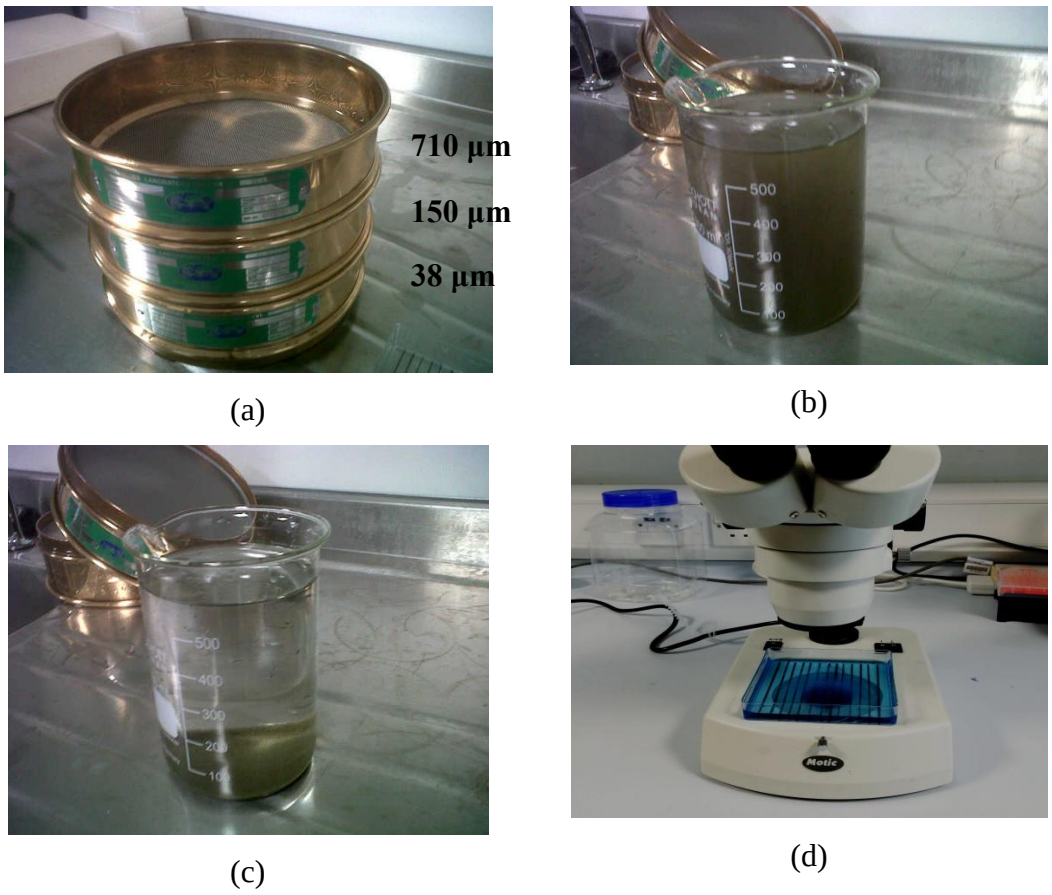


Figure 2.2.2 (a). A stack of three different sized sieves (710, 150, and 38 µm). (b) The beaker was filled with tap water and allowed to sediment for 4 min. (c). The process was repeated several times until the supernatant was clear of debris. (d) Methylene blue was added and the solution was viewed under the dissecting microscope.

2.2.3 Liver fluke recovery

At 10 dpt, all 12 sheep were euthanized with a captive bolt and the liver was removed soon after slaughter. Approximately 5 g of faecal sample was removed from the rectum and stored for subsequent FEC, coproantigen and PCR analysis. At necropsy, all bile ducts were dissected and adult flukes were removed and counted. All flukes were collected into a petri dish containing phosphate buffered saline (PBS) warmed to 37°C. The parasites were again rinsed in warm PBS and incubated individually in 1 ml of RPMI media (Gibco®) at 37°C for 2 hr. The liver was sectioned at 1 cm intervals, soaked in sterile PBS (or saline) and was incubated at 37°C for 2 hr to allow any immature flukes to emerge. Prior to counting, liver slices and sediment were passed through a 38 µm sieve and examined grossly for immature flukes. The flukes were stored in PBS for transfer to the laboratory. The parasites were then removed from the petri dish and stored frozen ahead of further tests (See Appendix 2.2).

2.2.4 Coproantigen ELISA

The commercially available cELISA kit (BIO K 201, BIO-X Diagnostics, Belgium) was developed for the diagnosis of *F. hepatica* infection in sheep. The plate has four rows (rows A, C, E and G), which include polyclonal antibody specific to *F. hepatica* and another four rows (rows B, D, F and H) that have been sensitised with a polyclonal antibody that is not specific for *F. hepatica.*

Fresh faecal samples were used. The faecal samples were weighed out to 0.5 g and then mixed up with 2 ml of the dilution buffer in a 15 ml tube. Each sample was homogenized for 10 s using the vortex and the tubes were then centrifuged at 1,000 x g for 10 min. Following centrifugation, the supernatant was collected and stored in 1.5 ml Eppendorf tubes at -20°C until needed. The samples were not stored for longer than 6 weeks after collection as per recommendation (Gordon et al., 2012b).

The BIO K 201 cELISA was performed according to the manufacturer's specifications. Briefly, plates were prepared by dispensing 110 µl of the diluted

samples into each well in duplicate except in wells G1 and H1 as these two wells acts as a controls. The plate was sealed and placed on a plate agitator at 21ºC for 2 hours. All the solution was then removed and the plate was washed with the washing solution three times. One hundred µl of diluted biotin-linked anti- *F. hepatica* antibody conjugate was dispensed into each well and the plate was sealed and incubated at 21ºC for 1 hr. The plate was again washed three times with the washing solution following which 100 µl of diluted avidine-peroxidise-linked conjugate was added to each well and the plate was incubated at 21ºC for 1 hr. At which point, 100 µl of chromogen solution was pipetted to each well. The plate was then incubated at 21ºC for 10 min in the dark and followed by adding 50 µl of stop solution to each well. A colour change from blue to yellow was read using an ELISA plate reader set to 450 nm (Infinite® F50, Tecan Ltd, Reading, UK) after the stop solution was added (See Appendix 2.3). Coproantigen values are expressed as the percentage positivity (PP) according to the formula; % = (Mean OD of the sample / Mean OD of positive control) x 100. As per the manufacturer's instructions the cut-off value for and percentage positive (PP) value was 6.65 and 9.32%, respectively.

2.2.5 PCR of faecal samples

2.2.5.1 Faecal DNA samples

Following collection per rectum, all faecal samples were stored at 4°C until testing. DNA was extracted from individual faecal samples using 0.5 g from each sheep.

2.2.5.2 Extraction of DNA from faeces

Two commonly used DNA extraction procedures, Qiagen kit (Qiagen Ltd, Manchester, UK) and Nucleospin® Tissue (Fisher Scientific UK Ltd, Loughborough, UK) kit were used according to manufacturer's instructions but only the Qiagen kit was used for the majority of samples. DNA was extracted from faeces using the QIAamp DNA Mini Stool Kit (Qiagen Ltd, Manchester, UK) according to the manufacturer's instructions, with only minor modification. A total of 0.5 g of fresh sheep faeces was placed into a 15 ml tube and 2.8 ml of stool lysis buffer (buffer ASL) was added. Each tube was homogenized for 1 min using a vortex, and the

suspension was incubated at 95°C for 5 min. Following incubation, all samples were vortexed for 20 s and centrifuged at 2500 x g (MSE Mistral 3000*i*) for 8 min. A 1.5 ml volume of the supernatant was transferred into a new 15 ml tube and the pellet was removed. A tablet of InhibitEX (Qiagen Ltd, Manchester, UK) was added to the sample in order to absorb DNA-degrading substances and PCR inhibitors from the faecal samples. The mixture was vortexed thoroughly until the tablet was completely re-suspended. The sample was then incubated for 3 min at RT to allow inhibitors to adsorb to the InhibitEX® matrix and then centrifuged at 2500 x g for 6 min to pellet the inhibitor:InhibitEX® matrix. Four hundred microliters were transferred into a new 1.5 ml microcentrifuge tube and the tube was centrifuged at 2500 x g for 3 min. Next, 200 µl of the supernatant was transferred into another 1.5 ml microcentrifuge tube and 15 µl of proteinase K, was added followed by 200 µl of Buffer AL from the QIAamp DNA Mini Stool Kit and the mixture vortexed for 15 s. The suspension was processed further by incubation at 70°C for 10 min. To precipitate the DNA, 200 µl of absolute ethanol (ABS ethanol) was added and mixed by vortex, followed by centrifuging for 1 min at 16 000 x g (microliter centrifudge; Heraeus® Biofuge Pico). To complete the purification process, a QIAamp® spin column was used. The spin column was placed into a new 2 ml collection tube and the entire sample was transferred onto the column and centrifuged for 1 min at 12, 850 x g. The DNA bound to the silica membrane of the spin column was washed by adding 500 µl of washing buffer (buffer AW1) and then centrifuged at 12, 850 x g for 1 min. The column was washed twice by adding 500 µl of washing buffer AW2 buffer to the spin column and centrifugation at 12, 850 x g for 3 min, each time. A final centrifugation for 1 min at 12, 850 x g was carried out in order to completely remove residual traces of washing buffer AW2 and to dry the column. Bound DNA was eluted into a new labelled 1.5 ml microcentrifuge tube by adding 60 µl of Buffer AE onto the QIAamp® membrane, incubatation at RT for 5 min and centrifugation for 1 min at 12,850 x g. The eluted DNA was stored at -20°C until further use as template for PCR (See Appendix 2.4).

2.2.5.3 Optimizing the PCR assay

A list of available PCR assays for *F. hepatica* is shown in Table 2.2.1. Comparison of the tests was made using DNA extracted from a *F. hepatica* FEC-positive faecal sample from sheep 14 and 18 wpi, collected as part of concurrent experimental studies in the Veterinary Parasitology group. Comparison was also performed with DNA extracted from adult *F. hepatica* recovered from the liver of infected sheep and DNA extracted from purified *F. hepatica* eggs recovered from the gall bladder. The 18 wpi sheep faecal samples were also used to determine the optimal dilution factor of the extracted faecal DNA; undiluted; 1:10; 1:20 and 1:50 dilutions were prepared and tested by PCR. For all dilutions, 1 µl of template DNA was added to the PCR reaction, except for 1:20 and 1:50 dilution where 4 µl of template DNA was used. A negative control (water) and positive control (adult *F. hepatica* DNA) was included. Based on these PCR results, ITS2 was identified as the most sensitive PCR and was used in all subsequent analyses. Following optimisation the PCR conditions used to test all 12 experimentally infected sheep were as follows: ITS2 PCR, 1:50 dilution and 4 µl template DNA.

Table 2.2.1 Published PCR methods for the diagnosis of liver fluke infection in sheep.

PCR assay	Primer name	Primer sequence	Size of PCR product (bp)	Reported sensitivity (wpi)	Reference
RAPD	Fhep F / Fhep R	5´-GCG GCCAAA TAT GAG TCA-3´ 5´-CTG GAGATTCCGGTTACC AA-3´	568	ND	(McGarry et al., 2007)
Cox: Standard-PCR	Standard: Cox1 F / Cox1 R	5´-GTTGGCATATTGCGGCTTAG-3´ 5´-AGGGATCTGCACCTCAACTC-3´	423[1]	3	(Martinez-Perez et al., 2012)
Nested-PCR	Nested:				
	1° Primer: Cox2 F / LrRNA R	5´-TNTGTTTTTTKCCKATGCAYTA-3´ 5´--TCYYRGGGTCTTTCCGTC-3´	1° PCR =1045 2° PCR =423[1]	2	
	2° Primer: Cox1 F / Cox1 R	5´-GTTGGCATATTGCGGCTTAG-3´ 5´-AGGGATCTGCACCTCAACTC-3´			
ITS2	ITS 2 F / ITS 2 R	5´-GTGCCAGATCTATGGCGTTT-3´ 5´-ACCGAGGTCAGGAAGACAGA-3´	292	2	(Robles-Pérez et al., 2013)

[1]Primers for standard PCR and final product of the nested PCR are identical; ND not determined.

The 292 bp fragment of ribosomal ITS2 was amplified using the forward primer ITS2; 5'- GTGCCAGATCTATGGCGTTT-3' and the reverse primer ITS2; 5'-ACCGAGTCAGGAAGACAGA-3' (Robles-Perez et al., 2013). These primers were based on the ITS2 sequence of *F. hepatica* (GenBank accession number GQ231547.1). The PCR reaction were performed in a total reaction volume of 25 µl containing 12.5 µl of Taq Polymerase Master Mix (2.0 mM $MgCl_2$, 0.4 mM dNTPs and 0.05 units/ µl Ampliqon Taq polymerase), 0.5 µl of each ITS2 primer and 4 µl of DNA diluted 50 times.

The following temperature programme was used: after an initial denaturation step for 2 min at 95°C, a set of 40 cycles was run, each one including 30 s at 95°C, 30 s at 63°C and 45 s at 72°C followed by a final extension step of 10 min at 72°C. Reactions were performed using a thermal cycle (T3 Thermocycler, Biometra®, Goettingen, Germany). Five microliters of the PCR product were electrophoresed on

a 1.2% agarose gel and stained with 10 µl SYBR® Green safe (Roche Diagnostics GmbH, Mannheim, Germany) to determine the amplified fragments. The obtained PCR product was analysed using agarose gel (1.5%) electrophoresis and a 100 bp DNA ladder (Invitrogen, Taastrup, Denmark) was used as a marker. The gel was later run at 100V, 120 mA (PowerPac™, Bio-Rad Laboratories Ltd, Hertfordshire, UK) for 30 min and then the amplified fragment was visualized into ultraviolet light imaging chamber.

Statistical analysis

The percentage efficacy of the TCBZ treatment in sheep was calculated using the following equation according to the WAAVP guidelines (Wood et al., 1995):

$$\text{Efficacy} = \left(\frac{\text{Mean of } \textit{F. hepatica} \text{ in control group} - \text{mean of } \textit{F. hepatica} \text{ in treated group}}{\text{Mean of } \textit{F. hepatica} \text{ in control group}} \right) \times 100$$

The number of parasites is presented as an arithmetic mean and was analysed using a Student's "*t*" test.

All parameters for epg and PP values, means ± SDs are given. A *P* value for epg and PP were compared with the Mann-Whitney test. For linear correlation analysis, the Spearman rank correlation coefficient *R* was computed by using SPSS anaylsis software (SPSS, release V.20.0; SPSS Inc, USA). A *P* value of $P \geq 0.05$ was considered to indicate a statistically significant difference at the 5% level of significance. Tables and graphs of the data were produces using Microsoft Excel (Microsoft Office 2010).

2.3 RESULTS

2.3.1 Detection of *Fasicola hepatica* infection by coproantigen ELISA and FEC

Fasciola hepatica infection was first detected by the cELISA at 5 wpi in two animals and all sheep tested positive by 8 wpi (Figure 2.3.1). Eggs of *F. hepatica* were first

detected in the faeces of one animal at 7 wpi and all animals had a positive FEC by 11 wpi (Figure 2.3.1). For some sheep, there was insufficient faecal sample to allow evaluation of FEC at every time point post infection: week 7 (8 out of 12 sheep tested), week 8 (9 out of 12 sheep tested), week 10 (11 out of 12 sheep tested), week 12 (10 out of 12 sheep tested), week 13 (11 out of 12 sheep tested) and week 14 (11 out of 12 sheep tested). With the exception of the one animal positive by FEC at 7 wpi, individual sheep remained positive from the first test-positive result until they were divided into control and treatment groups at 14 wpi (Figure 2.3.1). Results of the cELISA for the 12 individual sheep tested weekly until 14 weeks post infection showed a steady rise in percentage positivity (PP) over time until ~11 wpi (Figure 2.3.2). Mean PP values (± SD) for the group of 12 sheep are shown and reflects the rise in cELISA values until they plateau at 11-12 wpi (Figure 2.3.3). In Figure 2.3.4, the same pattern was shown in the rising of FEC for the 12 individual sheep tested weekly until 14 wpi. Results of mean FEC (± SD) are presented in Figure 2.3.5 and reflect that, unlike the cELISA the mean FEC value continues to rise until 14 wpi. There was no correlation between the cELISA and FEC values for all sheep, R^2 = 0.031 (Spearman's test p = 0.21) at 14 wpi (Figure 2.3.6).

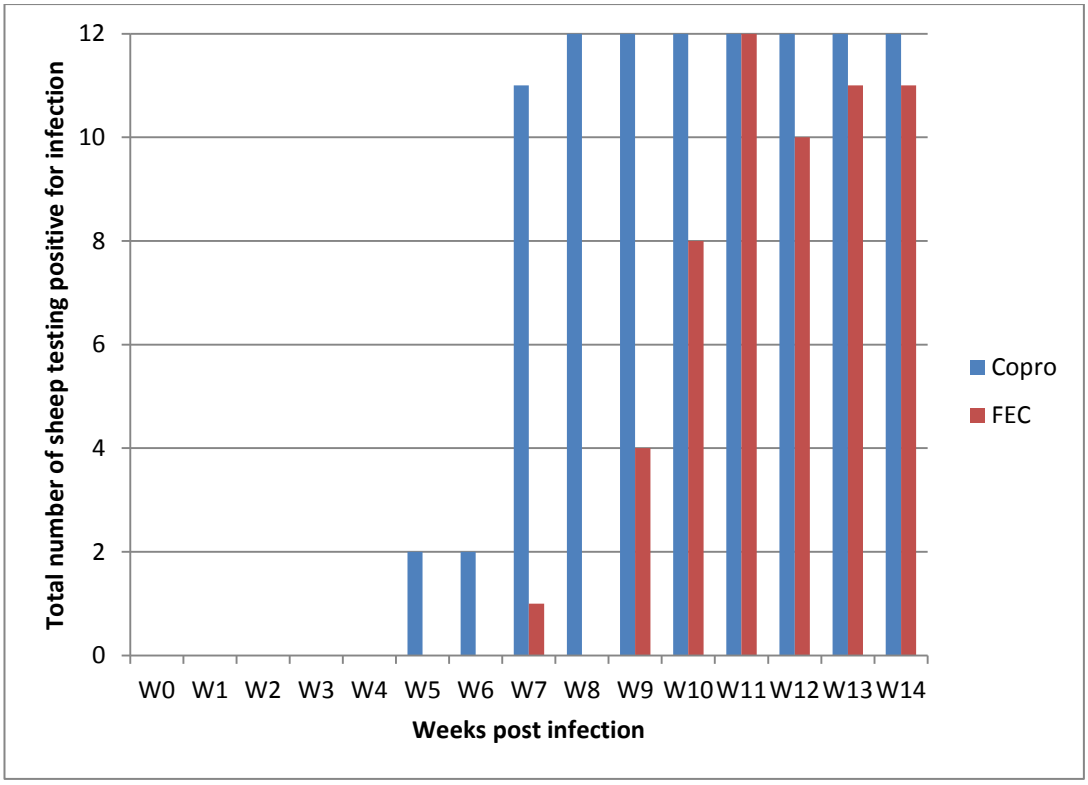


Figure 2.3.1 The total number of individual sheep testing positive for infection with *Fasciola hepatica* by i) the coproantigen ELISA detection kit-BIO-X K 201 (blue bars) and ii) the FEC (red bars).

Note that due to insufficient faecal sample availability for FEC testing only a proportion of samples were tested at the following timepoints: W7 (n=8 samples tested), W8 (n=9 samples tested), W10 (n=11 samples tested), W12 (n=10 samples tested), W13 and 14 (n=11 samples tested). For coproantigen testing, the test was performed on all samples at all time points expect: W6 (n=11 samples tested).

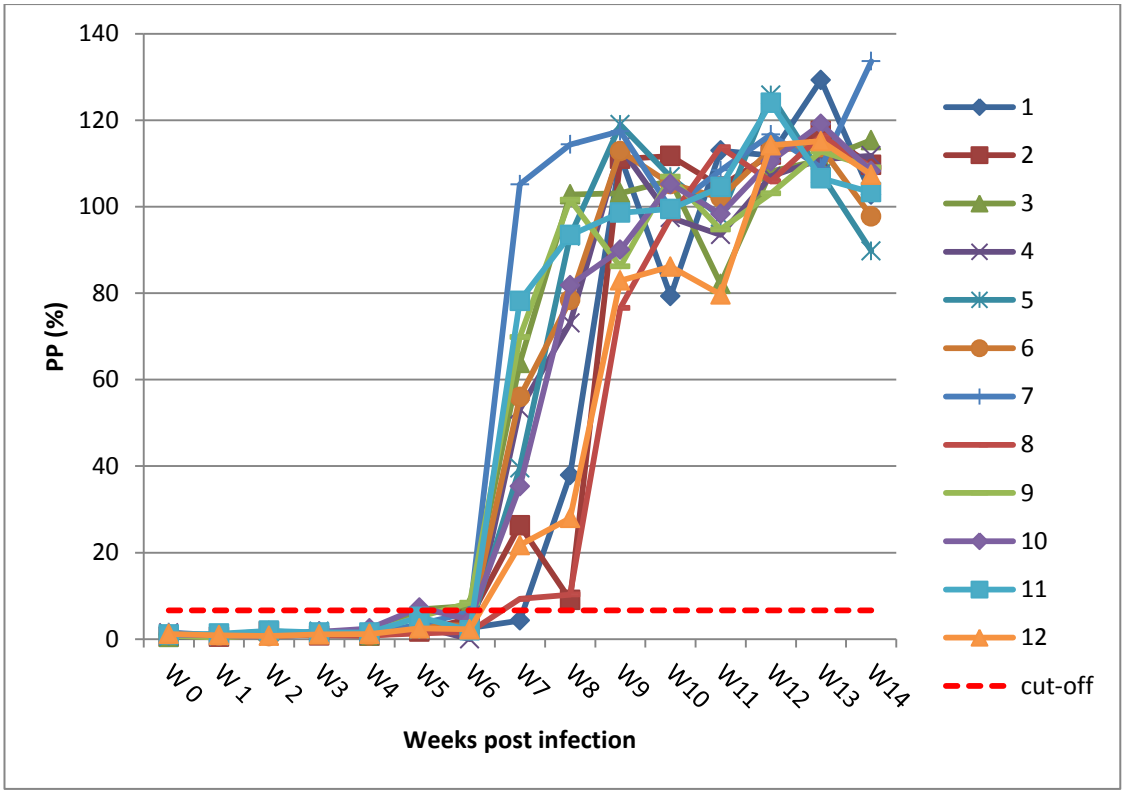


Figure 2.3.2 Coproantigen ELISA values (PP, percentage positivity) for 12 individual sheep (1-12) tested weekly for 14 weeks post infection.

The red dashed line represent the cut-off value (PP > 6.65%) for a positive result. Note that the test was not all performed on W6 (n=11 samples tested).

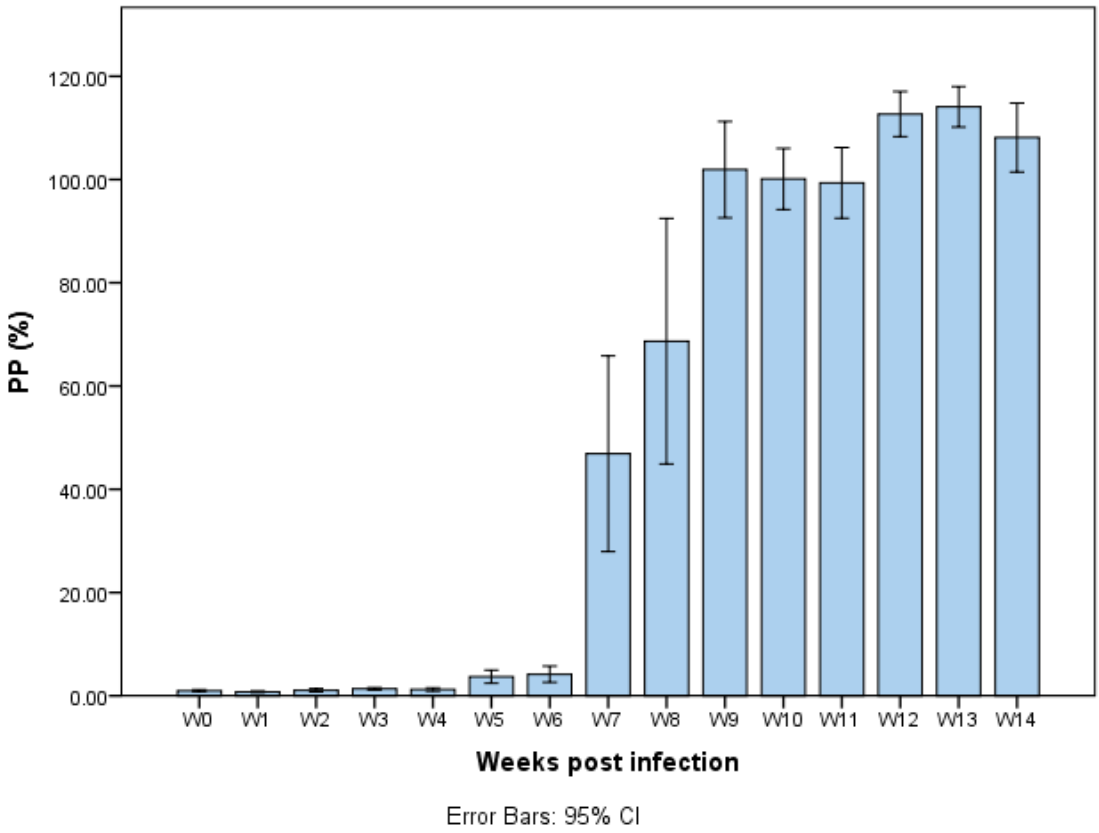


Figure 2.3.3 Mean coproantigen levels (PP, percentage positivity, ± SD) for the group of 12 sheep tested weekly for 14 weeks post infection. Bars represent the 95 percent confidence interval.

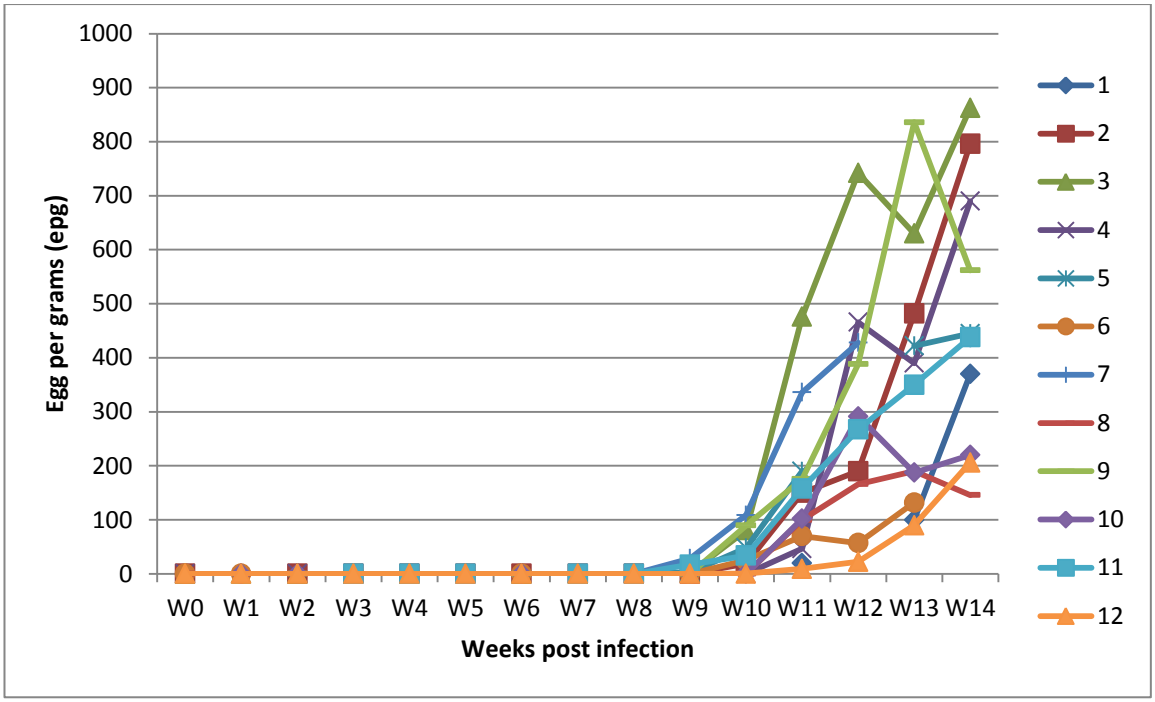


Figure 2.3.4 Fluke faecal egg counts in eggs per gram (epg) for 12 individual sheep (1-12) tested weekly for 14 weeks post infection.

Note that due to insufficient faecal sample availability for FEC testing only a proportion of samples were tested at the following time points: W7 (n=8 samples tested), W8 (n=9 samples tested), W10 (n=11 samples tested), W12 (n=10 samples tested), W13 and 14 (n=11 samples tested).

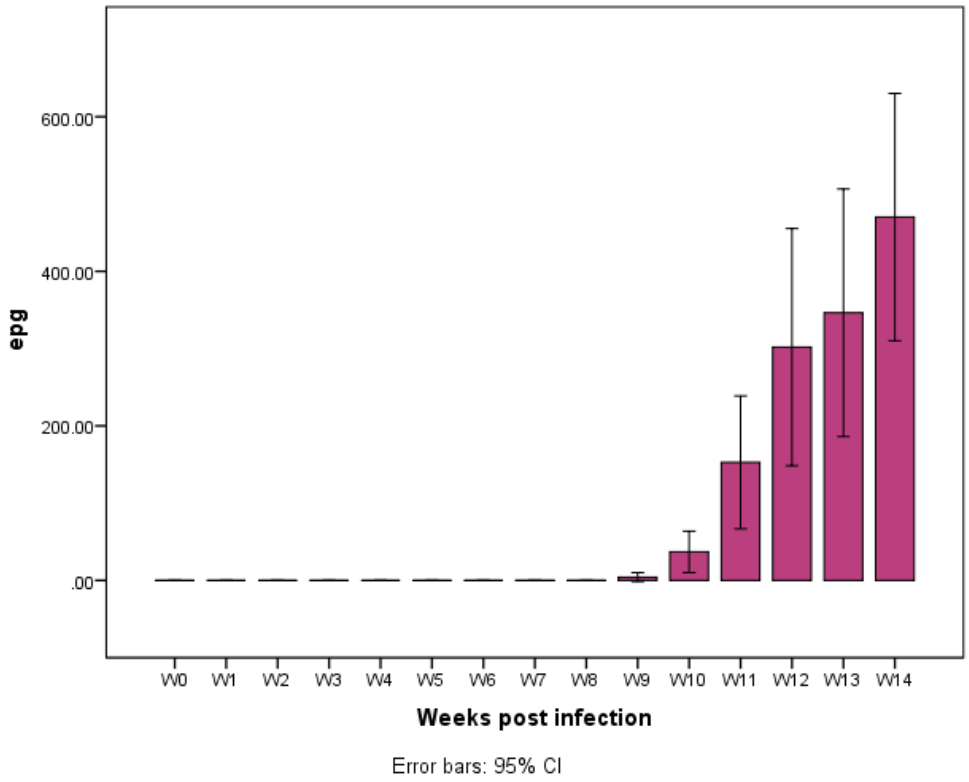


Figure 2.3.5 Mean fluke faecal egg counts (± SD) in eggs per gram (epg) for the group of 12 sheep tested weekly for 14 weeks post infection. Bars represent the 95 percent confidence interval.

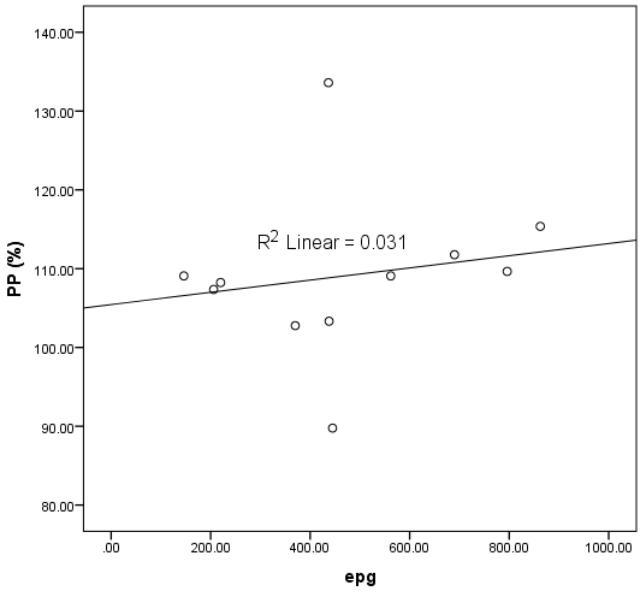


Figure 2.3.6 The correlation between FEC (epg) and PP (%) values on 14 wpi (n=11 samples tested).

2.3.2 Evaluation of coproantigen ELISA and FEC to detect efficacy of triclabendazole

Following TCBZ treatment, four out of six treated sheep (numbers 2, 3, 7 and 11) remained persistently positive by cELISA from 0 to 10 dpt (Table 2.3.1). At 5 dpt, one treated sheep recorded a negative cELISA result and the PP value remained below the cut-off value over the remainder of the sampling timeframe (ranged PP = 0 to 4.21). The remaining treated sheep tested negative by cELISA at 8 dpt recording a PP value of 5.67, below the 9.32% cut-off but recorded a PP value of 8.31 at 9 dpt prior to recording another negative value of 3.29 at 10 dpt. When the FEC test was used to detect infection two out of six treated sheep were negative when sampled on 7 dpt but one sheep recorded a positive FEC again at 8 dpt and the other at 10 dpt (Table 2.3.2). The daily mean PP values and mean FEC (± SD and the 95% CI levels) over the 10 dpt sampling timeframe are shown in Table 2.3.3. Comparing control and treated groups of sheep revealed statistically significant differences in the cELISA PP values from 4 to 9 dpt but no statistically significant difference was observed at day 10 post treatment (see Table 2.3.3 and Figure 2.3.7). Although, treated sheep continued to shed fluke eggs right up until 10 dpt FEC were statistically significantly lower from 5 dpt, when compared to control sheep (see Table 2.3.3 and Figure 2.3.8). There was a statistically significant difference between the two groups, however, on the first 3 dpt, there was an overlap between the standard errors of coproantigen levels for treatment and control groups (Figure 2.3.8).

Table 2.3.1 *Fasciola hepatica* coproantigen ELISA values (PP, percentage positivity) from 0-10 dpt for sheep treated with triclabendazole (10 mg/kg) per os compared to untreated controls. The cut-off value (PP > 9.32%) for a positive result.

Sheep ID	**Days post treatment**										
Control	**PP (%)**										
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
1	**112.6**	**105.16**	**111.48**	**104.19**	**104.69**	**109.19**	**100.51**	**104.74**	**97.79**	**98.26**	**104.6**
4	**93.7**	**113.77**	**119.2**	*	**78.60**	**110.4**	**46.77**	**120.05**	**154.85**	**124.91**	**119.72**
6	**122.93**	**110.68**	**98.49**	**105.28**	**87.08**	**93.42**	**92.82**	**94.99**	**91.68**	**89.06**	**118.63**
8	**137.68**	**113.88**	**122.30**	**116.44**	**119.05**	**110.53**	**109.34**	**119.54**	**101.67**	**113.80**	**83.61**
10	**132.49**	**115.57**	**113.86**	*	**98.62**	**106.81**	**84.71**	**104.91**	**95.95**	**93.52**	**71.54**
12	**131.08**	**110.4**	**124.87**	**113.13**	**95.09**	**101.89**	**101.2**	**123.71**	**107.2**	**129.42**	**76.79**
Treated	**PP (%)**										
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
2	**115.74**	**113.17**	**120.12**	**91.30**	**63.87**	**61.71**	**59.92**	**75.95**	**46.40**	**72.48**	**87.36**
3	**115.72**	**115.97**	**115.26**	**78.99**	**42.49**	**38.52**	**55.02**	**43.34**	**29.68**	**25.97**	**45.85**
5	**117.87**	**94.01**	**99.07**	**56.71**	**26.95**	**42.44**	**18.28**	**17.02**	5.67	8.31	3.29
7	**126.06**	**117.30**	**120.14**	**90.84**	**63.27**	**72.48**	**91.74**	**83.18**	**66.62**	**76.23**	**108.78**
9	**98.23**	**59.67**	**101.96**	**48.18**	**12.49**	4.21	1.14	0.57	0	0.62	0.53
11	**124.46**	**121.86**	**121.47**	**88.12**	**60.92**	**53.89**	**52.66**	**31.70**	**44.16**	**19.24**	**19.12**

* Missing data; Note that **bold values** are representing positive coproantigen results

Table 2.3.2 *Fasciola hepatica* faecal egg counts (epg) from 0-10 dpt for sheep treated with triclabendazole (10 mg/kg) per os compared to untreated controls.

Sheep ID	**Days post treatment**										
Control	**FEC (epg)**										
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
1	357.5	37.8	79.1	90.6	107.2	309.8	79.0	160.0	325.6	325.4	168.8
4	814.0	245.3	494.8	*	667.0	343.6	160.5	432.2	460.5	287.6	233.0
6	282.0	74.0	305.2	90.8	133.2	411.4	54.0	284.3	425.6	290.3	106.4
8	535.6	*	129.8	97.2	692.8	422.0	388.6	392.0	450.6	276.0	93.2
10	501.7	200.5	143.0	*	110.8	517.4	129.6	245.0	179.8	527.6	113.8
12	360.0	39.0	116.0	135.6	140.0	250.0	131.2	285.6	198.8	251.6	48.8
Treated	**FEC (epg)**										
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
2	712.0	697.2	14.0	99.0	87.6	29.8	16.0	13.2	*	29.2	15.4
3	397.5	277.2	283.0	79.6	194.2	21.8	13.2	7.2	7.0	1.4	1.2
5	633.3	336.6	28.3	9.0	*	3.6	0.4	1.5	4.2	1.4	0.8
7	391.8	84.5	44.8	31.2	*	29.6	45.2	16.6	23.25	17.4	1.2
9	721.0	277.2	130.6	12.2	5.6	3.4	0.8	0	0	0	0.2
11	479.0	217.6	93.6	3.0	3.6	1.2	2.0	0	0.6	0.2	0.6

* Missing data

Table 2.3.3 Fluke egg counts (epg) and *Fasciola hepatica* coproantigen ELISA (PP, percentage positivity) from 0-10 dpt for sheep treated with triclabendazole (10 mg/kg) per os compared to untreated controls.

Group	**Days post treatment**										
	epg (Mean ± SD)										
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Control	475.1 ± 191.6	119.3 ± 100.0	211.4 ± 159.3	103.6 ± 21.6	308. 5 ± 288.1	375.7 ± 94.5	157.2 ± 119.8	299.8 ± 99.0	340.2 ± 126.4	326.4 ± 101.4	127.3 ± 64.6
Treated	555.8 ± 152.0	315.1 ± 205.9	99.1 ± 100.0	39.0 ± 40.6	72.8 ± 89.9	14.9 ± 13.7	12.9 ± 17.2	6.4 ± 7.2	7.0 ± 9.5	8.3 ± 12.2	3.2 ± 6.0
p-value	p=0.337	p=0.028	p=0.109	p=0.273	p=0.088	p=0.004	p=0.004	p=0.004	p=0.006	p=0.004	p=0.004
	PP (Mean ± SD)										
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Control	121.7 ± 16.3	111.6± 3.7	115.0 ± 9.5	109.8 ± 5.9	97.2 ± 14.1	105.3 ± 6.7	89.2 ± 22.4	111.3 ± 11.4	108.2 ± 23.5	108.2 ± 17.0	95.8 ± 21.3
Treated	116.3 ± 10.0	103.7 ± 23.6	113.0 ± 10.0	75.7 ± 18.7	45.0 ± 21.6	45.5 ± 23.8	46.5 ± 32.2	42.0 ± 32.5	32.0 ± 25.6	33.8 ± 32.6	44.1 ± 45.3
p-value	p=0.423	p=0.631	p=0.873	p=0.201	p=0.004	p=0.004	p=0.037	p=0.004	p=0.004	p=0.004	p=0.078

The Mann-Whitney test statistics on both fluke egg count and coproantigen level data for each group (n=6). Values shown represent the mean and standard deviation.

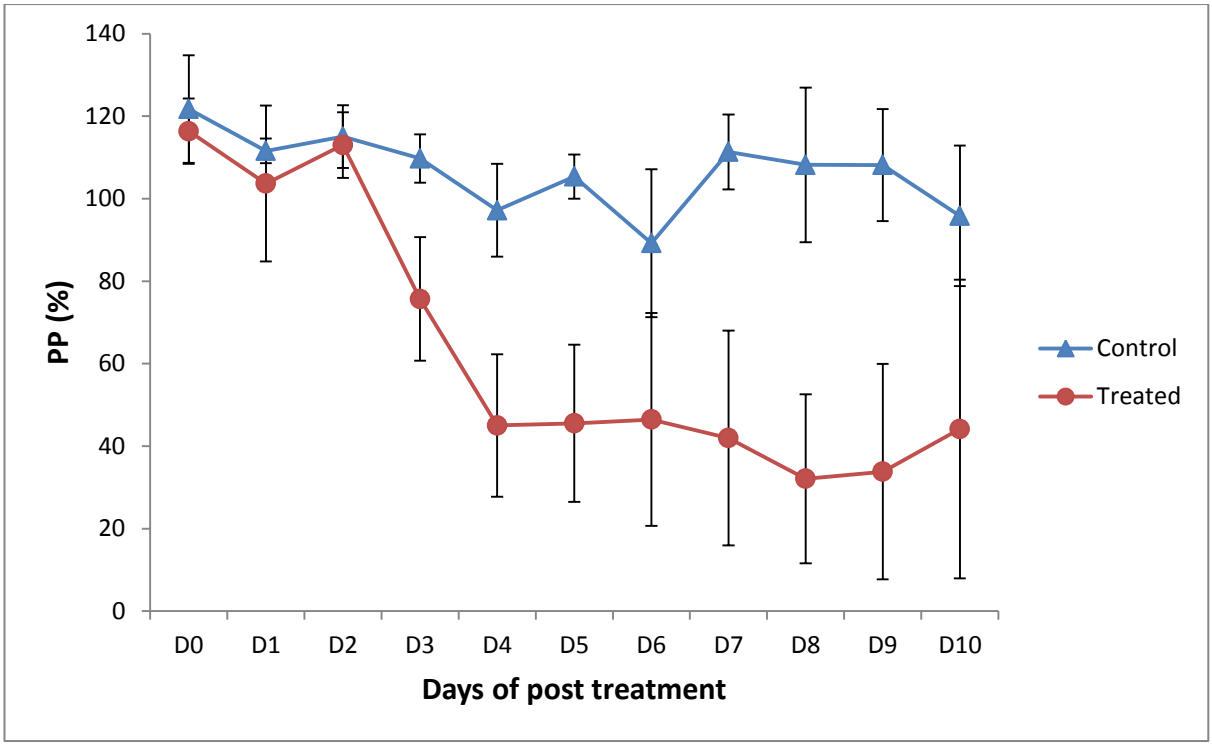


Figure 2.3.7 Mean coproantigen ELISA levels (PP, percentage positivity) and bars represent the 95 per-cent confidence interval.

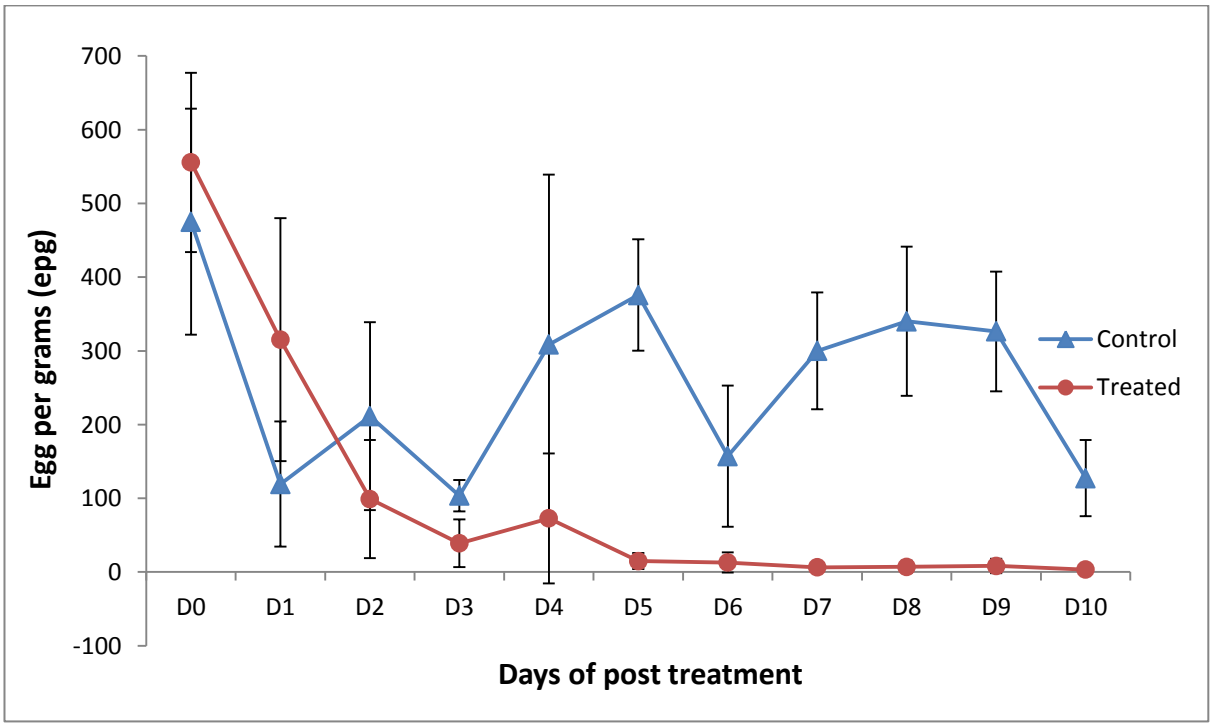


Figure 2.3.8 Mean fluke egg counts in eggs per gram (epg) and bars represent the 95 per-cent confidence interval.

2.3.3 Fluke recovery

An infective dose ranging from 200-215 metacercariae was used to infect each of the 12 sheep (Table 2.3.4). A total of 21 live adult fluke were recovered from the six TCBZ-treated sheep (one animal was negative) whereas 760 live adult fluke were recovered from the livers of the control *F. hepatica*-infected sheep (Table 2.3.4). This difference was shown to be highly significant by using the T-tests ($p < 0.001$). The efficacy of TCBZ was determined by percentage reduction in live adult fluke recovered post mortem in TCBZ-treated sheep compared to untreated controls. This represented a significant reduction of 97%.

Table 2.3.4 *Fasciola hepatica* cELISA (PP, percentage positivity), faecal egg counts (epg) and number of adult fluke recovered post mortem on 10 dpt for the six untreated control and six treated sheep.

Sheep ID	**Untreated control Sheep**			
	No. metacercariae given	**PP (%)**	**FEC (epg)**	**Fluke recovery**
1	203	**104.60**	168.8	129
4	209	**119.72**	233.0	136
6	200	**118.63**	106.4	78
8	208	**83.61**	93.20	145
10	213	**71.54**	113.8	122
12	215	**76.79**	48.8	150
	Treated Sheep			
		PP (%)	**FEC (epg)**	**Fluke recovery**
2	212	**87.36**	15.4	5
3	210	**45.85**	1.2	1
5	208	3.29	0.8	1
7	203	**108.78**	1.2	13
9	200	0.53	0.2	0
11	208	**19.12**	0.6	1

(Note that **bold values** are representing positive coproantigen results)

2.3.4 Detection of *Fasciola hepatica* infection by PCR from faeces

A comparison of the three published PCR assays (see Table 2.2.1) was performed. All PCR assays reliably detected *F. hepatica* DNA when the template was extracted from adult parasites or purified eggs from the gall bladder, however, only the ITS2 PCR gave a reliable positive result when DNA from a known *F. hepatica* positive faecal sample was used (Figure 2.3.9). Using DNA extracted from two 18 wpi sheep faecal samples the optimal dilution factor of the extracted faecal DNA was identified as a 1:50 dilution (Figure 2.3.10), although typically both 1:10 and 1:50 dilutions were subjected to PCR.

When the optimised ITS2 PCR was applied to the faecal samples from the sheep experimentally infected with *F. hepatica* no consistent amplification was seen for any individual sheep at each timepoint from 0 to 4 wpi, 8 and 10 wpi (Figure 2.3.11). The PCR reaction did appear to amplify a product of the correct size from individuals 1, 3, 5, 7, 8 and 11 at 0 wpi (Figure 2.3.11 A) which was prior to infection but faeces for these animals did not record a positive result at later timepoints (Figure 2.3.11 C and D). The ITS2 PCR recorded a few samples as fluke-DNA positive at later timepoints (Figure 2.3.11 F and G) but did not detect fluke DNA in faecal samples from all sheep at 8 and 10 wpi even though the majority of these faecal samples were confirmed positive by FEC and cELISA (Figure 2.3.1).

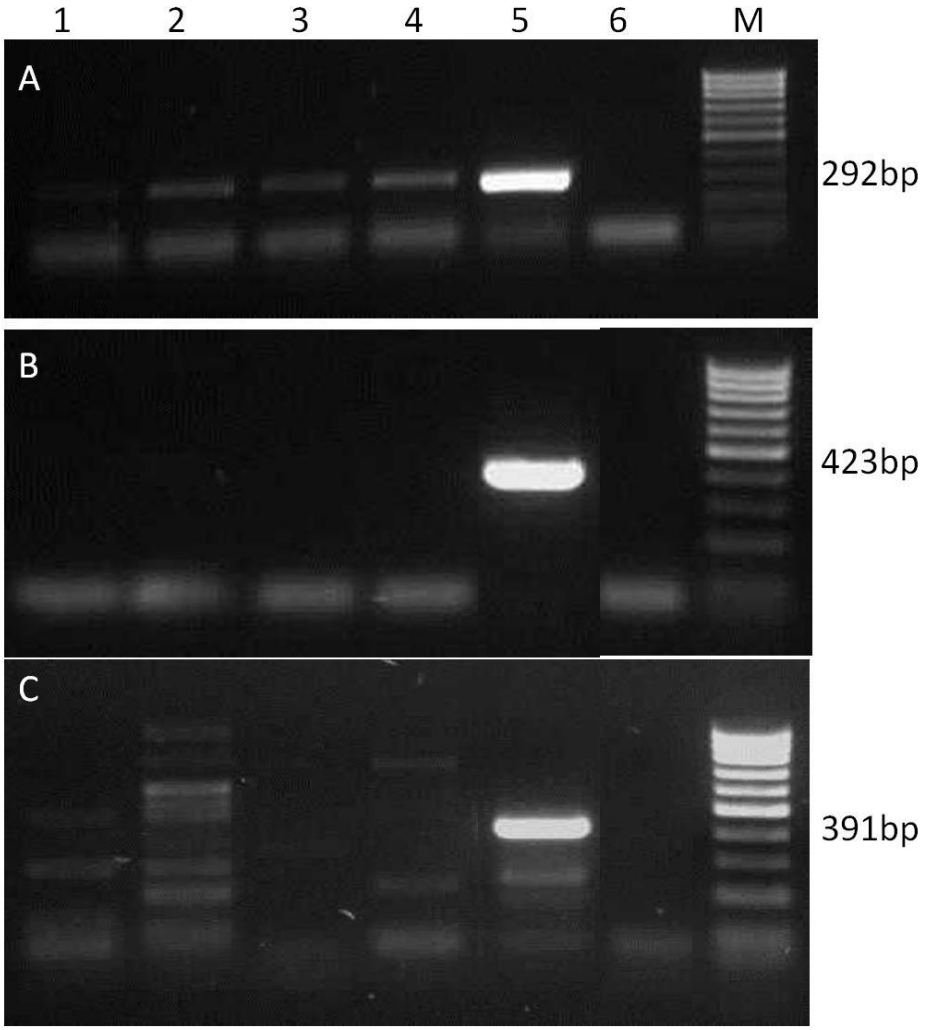


Figure 2.3.9 Composite agarose gel showing the products obtained by ITS2, Cox-1 (nested) and RAPD PCR using DNA extracted from faecal samples from two sheep 18 wpi (duplicate samples of sheep A and sheep B).

PCR methods used: A = ITS2 PCR, B = Cox-1 nested PCR and C = RAPD PCR. Lanes 1-6 – 1. Sheep A_1, 2. Sheep A_2, 3. Sheep B_1, 4. Sheep B_2, 5. positive control and 6. negative control. A 100bp ladder (M) was used as a molecular size marker.

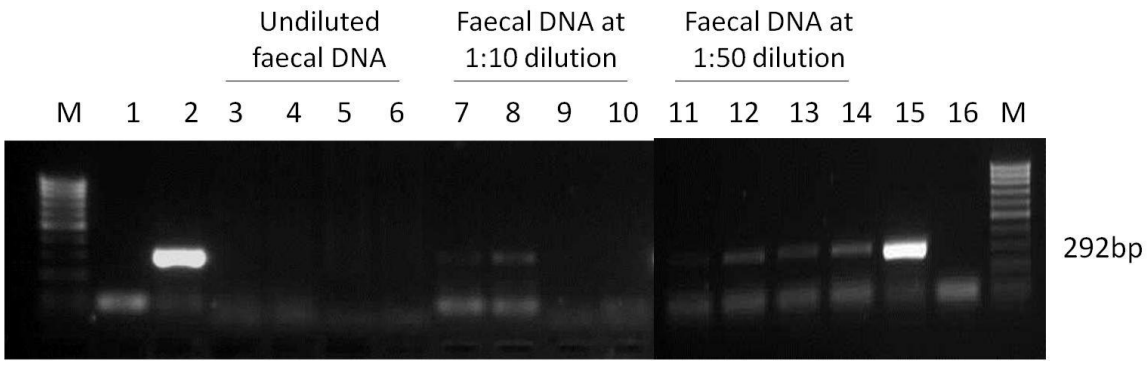


Figure 2.3.10 Composite agarose gel showing the products obtained by ITS2 PCR using DNA extracted from faecal samples from two sheep 18 wpi at three dilutions: undiluted, 1:10 dilution and 1:50 dilution.

Lanes 1-16 – 1. negative control, 2. positive control, 3. Sheep A_1 undiluted, 4. Sheep A_2 undiluted, 5. Sheep B_1 undiluted, 6. Sheep B_2 undiluted, 7. Sheep A_1 1:10, 8. Sheep A_2 1:10, 9. Sheep B_1 1:10, 10. Sheep B_2 1:10, 11. Sheep A_1 1:50, 12. Sheep A_2 1:50, 13. Sheep B_1 1:50, 14. Sheep B_2 1:50, 15. positive control and 16. negative control. A 100bp ladder (M) was used as a molecular size marker.

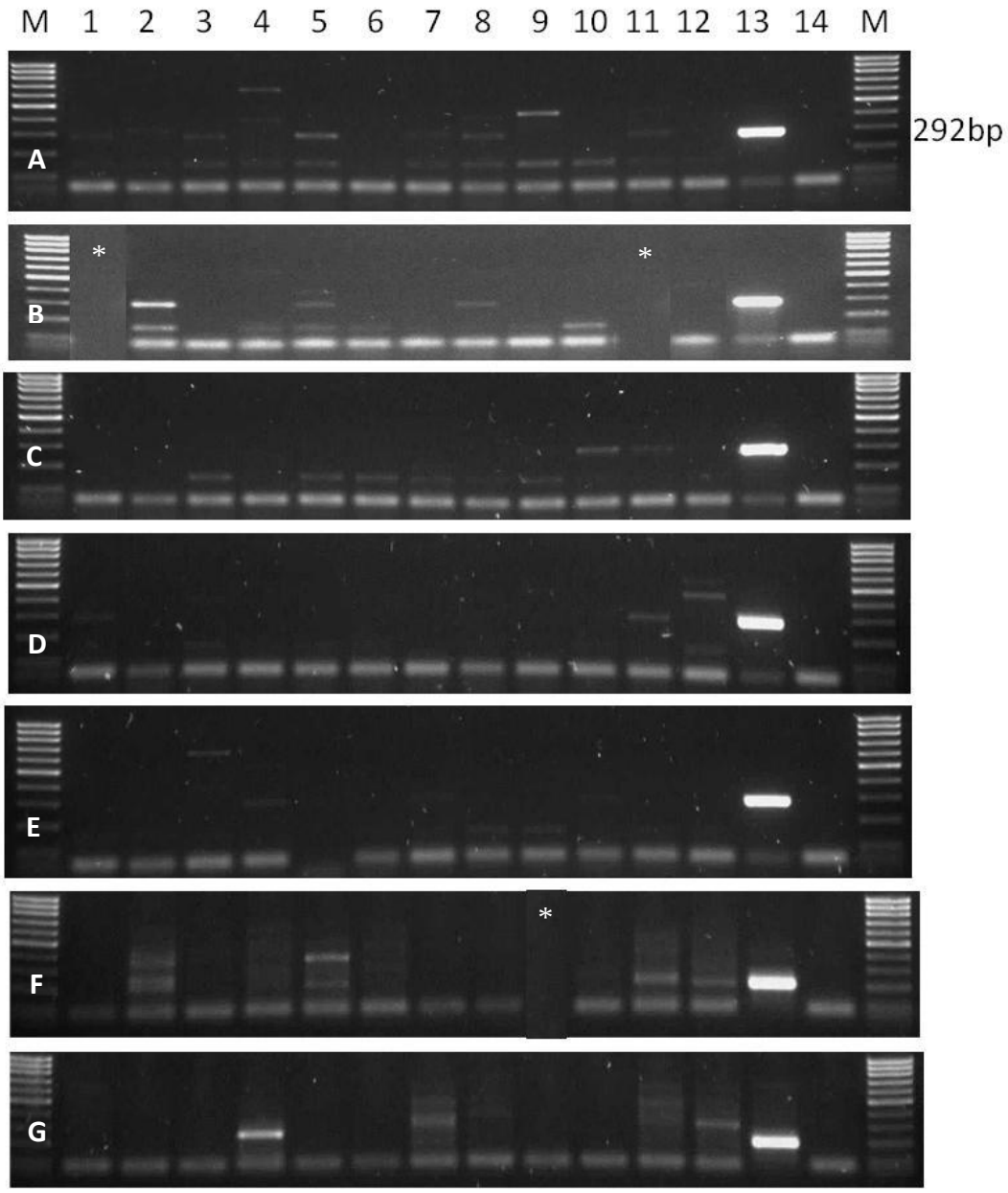


Figure 2.3.11 Composite agarose gel showing the products obtained by ITS2 PCR using DNA extracted from faecal samples from the 12 sheep experimentally infected with *Fasciola hepatica*.

Each of the 12 animals was subjected to faecal sampling and PCR at several time-points throughout infection: A = 0 wpi, B = 1 wpi, C = 2 wpi, D = 3 wpi, E = 4 wpi, F = 8 wpi, G = 10 wpi. Lanes 1-12. Sheep 1-12, 13. Positive control and 14. Negative control. A 100bp ladder (M) was used as a molecular size marker. *denotes samples not run in PCR as insufficient faecal material was available.

2.4 DISCUSSION

In vivo and *in vitro* tests, incorporating both experimental and natural infection studies in sheep, have been developed for the detection of *F. hepatica* infection and to test for populations resistant to TCBZ (Flanagan et al., 2011b; Gordon et al., 2012b; Hanna et al., 2015; Novobilsky et al., 2012; Robles-Pérez et al., 2013). Despite their availability few studies compare the three diagnostic tools; FEC, cELISA and new PCR assays for detecting early infection of liver fluke in experimentally infected sheep, hence the study reported here.

2.4.1 Sensitivity of the coproantigen ELISA

This experimental study confirmed that the cELISA is sufficiently sensitive to detect early fluke infection between 5 wpi to 8 wpi (Figure 2.3.1). Several studies using the BIO K 201 cELISA report detection of *F. hepatica* by 5 wpi (Flanagan et al., 2011b) and between 7 to 9 wpi (Flanagan et al., 2011a) in experimentally infected sheep. Martinez-Perez et al. (2012) also confirmed that the infection of liver fluke were detected by 4 wpi in experimentally infected sheep. In cattle, infection of liver fluke was identified by cELISA between 6 to 8 wpi (Brockwell et al., 2013). Other studies, using the same ELISA as in the present experiment, have shown that coproantigen were found as early as 5 wpi (Mezo et al., 2004) and after 4 to 7 wpi before fluke egg shedding in *F. hepatica*-infected sheep (Valero et al., 2009). Similar findings were observed with naturally infected sheep which show the sensitivity of this commercial ELISA kit (Hanna et al., 2015).

In our study, following metacercarial challenge, the cELISA increased steadily from 6 wpi and reached a peak for individual animals and as a group mean at 11-12 wpi (Figure 2.3.2 and 2.3.3); at which point cELISA values reached a plateau and did not continue to rise between 12 and 14 wpi (Figure 2.3.3) suggesting once patency is reached the amount of coproantigen released remains relatively stable. It is suggested that immature fluke may produce lower amounts of excretory-secretory antigens (Mezo et al., 2004) presumably due to their smaller size but once patency is achieved it appears that levels of coproantigen remain the same. In the present

study, the percentage positivity (PP %) cut-off value for a positive result was calculated according to the manufacturer's recommendations but the actual cut off value varied between batches of plates, ranging from 6.55% to 9.32% as recommended by the manufacturer.

Following treatment the cELISA values between TCBZ treated and control groups were statistically significantly different (Figure 2.3.7). This is in agreement with results from a cELISA in cattle, infected with a TCBZ-susceptible isolate of *F. hepatica*, where all TCBZ-treated animals produced negative cELISA results by 7 dpt (Brockwell et al., 2013). Following treatment of this TCBZ susceptible isolate of *F. hepatica* at the recommended dose of TCBZ four out of six treated sheep remained positive by cELISA for the duration of the trial (except for sheep 11; negative FEC but positive cELISA on day 7 pt). For treated sheep 9 and 5, a negative result was reported for cELISA however they were positive for FEC (Table 2.3.3). We can conclude from our findings for these two animals that coproantigen negative sheep contained fluke eggs in their faeces whilst the other four sheep recorded both positive cELISA and FEC values from day 0 to 10 pt (except for treated-sheep 11; zero fluke egg but 31.7% PP value on day 7 pt; Table 2.3.3). This is in agreement to previous studies where evidence of false negative results was reported (Gordon et al., 2012b; Novobilsky et al., 2012). These false negative results probably reflect the sensitivity of cELISA when using this test on natural infected sheep. The reason for the discrepancy in their findings (Gordon et al., 2012b; Novobilsky et al., 2012) and our findings with experimental infected sheep remains to be determined.

Ten dpt the highest PP value amongst treated sheep was reported for the animal that was infected with 13 parasites, which was the general trend through the 0-10 dpt timeframe (Table 2.3.1) but sheep found to have 5 and 1 parasite at post mortem also had values well above the PP positive cut-off value. Similar results have been reported by other studies (Brockwell et al., 2014; Mezo et al., 2004). This shows that the cELISA is able to detect very low fluke infections even with one fluke (Mezo et al., 2004). The present study proved that even as few as one fluke that survived drug treatment, as with sheep 3 and 11, can record a high level of coproantigen in the faeces. However, one sheep with 1 parasite at slaughter was negative by cELISA but positive by FEC, thus indicating that BIO K201 cELISA

may give false negative results. Due to inconsistent results found in this study, this diagnostic test requires further investigation. Given that a susceptible isolate of *F. hepatica* was used to infect the sheep in this study, it raises questions over how to interpret post treatment and cELISA values. It has been suggested that the reason for continued release of coproantigen is due to disintegrating fragments of flukes that may still be present by 10 days post TCBZ treatment (Flanagan et al., 2011b). Flanagan et al. (2011b) also confirmed that cELISA results remained positive in the control Cullompton sheep group and were largely absent at 2 wpt for the TCBZ-treated Cullompton (TCBZ susceptible) sheep groups whereas for TCBZ-treated Sligo (TCBZ resistant) sheep groups, the cELISA results remained positive. In these studies live flukes were recovered from animals infected with the Sligo isolate, and dead fluke from those sheep infected with the Cullompton isolate (Flanagan et al., 2011b) which is in contrast to our findings where we recovered liver fluke from the host even though a TCBZ susceptible isolate was used.

2.4.2 Sensitivity of the FEC

Eggs were first recovered from one sheep infected with 200-215 metacercariae as early at 7 wpi (Figure 2.3.1). These findings are in agreement with another study, which examined fluke eggs at 7 wpi, but this was in rats that have a shorter pre-patent period (Paz-Silva et al., 2002). By 11 wpi all sheep tested positive, indicating the presence of mature liver fluke (Figure 2.3.1). Other studies have shown that *F. hepatica* eggs are first detected a bit later between 8 to 10 wpi in mice (O'Neill et al., 2000), and in sheep from weeks 10 and 12 pi (Dumenigo and Mezo, 1999) or even later, between weeks 11 to 16 pi (Rodriguez-Perez and Hillyer, 1995; Zimmerman et al., 1982). More recent studies reported that sheep were positive for the first time by egg count sedimentation method at 10 wpi (Flanagan et al., 2011b), at 9 wpi (Martinez-Perez et al., 2012) and 12 wpi (Flanagan et al., 2011a). In the present study, after metacercarial challenge, the number of eggs shed in faeces increased from 9 or 10 wpi and reached a peak for individual animals and as a group mean at 14 wpi (Figure 2.3.4 and 2.3.5); reflecting the time taken to reach patency of infection as described in other studies (Flanagan et al., 2011b; Mezo et al., 2004) but also suggests that, in contrast to coproantigen, the numbers of eggs shed continues to

increase even once patency has been reached. Our findings clearly demonstrate the relationship between egg shedding in sheep faeces from 7 to 11 wpi and the period of development from ingestion of metacercariae by sheep and development to the adult fluke that sheds eggs onto the pasture between 10 to 12 weeks (Andrews, 1999).

For both treated and control groups, prior to treatment (Week 0 to 14 pi) FEC showed variation within individual animals (Figure 2.3.4). Post-treatment, *F. hepatica* egg counts fluctuated greatly in both the treated and control group sheep (Table 2.3.2). Fluctuations of FECs were observed in the present study, where animals changed from positive to negative to positive again. Two treated sheep 9 and 11 have shown that at 7 dpt, individual epg was zero count. No eggs were seen for two consecutive days post treatment for sheep 9 but became positive again at 10 dpt. As for sheep 11, it consistently became positive again at 8, 9 and 10 dpt. These observations are in accordance with the results from other studies as discussed above (Brockwell et al., 2013; Flanagan et al., 2011a). Similar results have been confirmed by others that natural fluctuations in epg occurred for *F. hepatica* and identified this as a limitation of this test (Honer, 1965; Mezo et al., 2004). Observation of variation in FEC and possible reasons why are discussed in Chapter 3 but this does not detract from the overall FECRT result. In this study sheep were slaughtered at 10 dpt which is a little earlier than in other studies so there is the possibility that negative FEC would have been reported if the experiment was extended and necropsy had taken place slightly later.

Only one out of six of the treated animals was FEC negative at 10 dpt and the presence of a total of 21 parasites at 10 dpt is consistent with the shedding of low numbers of eggs at this timepoint. These observations are in accordance with the results from other studies (Brockwell et al., 2014; Mezo et al., 2004).

2.4.3 Comparison of the cELISA and FEC

In contrast, the study reported here used an experimental approach involving the infection of multiple sheep to determine the TCBZ susceptibility of the isolate

because it is known that there can be sheep to sheep variation in drug efficacy and that parasites exist as populations of genetically distinct individuals. Results of this study confirm that FEC was less sensitive at detecting liver fluke infection compared to the cELISA in these 12 individual sheep (Figure 2.3.1). The first detection of fluke eggs in faeces at 7 wpi in one animal is two weeks later than the first positive by cELISA. Several studies in experimentally infected sheep using the cELISA BIO K 201 have shown that the infection is detected by 5 wpi, 5 weeks earlier than eggs (Flanagan et al., 2011b) and 2.5 and 3 weeks earlier, respectively (Flanagan et al., 2011a). Martinez-Perez et al. (2012) also confirmed in experimentally infected sheep that detection of liver fluke by cELISA was possible at 4 wpi, five weeks earlier than detection of eggs by FEC. In the present study, after metacercarial challenge, coproantigen levels increased gradually from 6 wpi (Figure 2.3.2) whereas FEC did not increase until 9 or 10 wpi (Figure 2.3.4) reflecting that the cELISA detects infection in the prepatent period (Flanagan et al., 2011b; Mezo et al., 2004). Several studies have focused on correlation on fluke burden between cELISA and FEC (Brockwell et al., 2014; Brockwell et al., 2013). In this study there is a clear lack of correlation between FEC (epg) and PP (%) values (Figure 2.3.6) which may be due to the fact that the cELISA is less quantitative and that PP levels reach a peak even when FEC values continue to rise (Figure 2.3.3 and 2.3.5), probably as peak egg production / fertility may not be achieved until a certain age. It is likely that there is variation in the amount of coproantigen released as flukes mature in post infection period, alternatively it could be argued that the cELISA gives a better indication of the number of adult parasites present as the peak in coproantigen coincides with the 10-12 week patency window. At a practical level, a limitation of the FEC method is that a larger weight of sample is required and it was not always possible to collect a sample of sufficient weight which meant that it was not possible to test all animals at every timepoint. On the other hand the reduced volume for the cELISA meant that it was possible to test every animal at each timepoint.

2.4.4 Fluke burden in treated and control groups at postmortem

This current study confirmed that the reduction in fluke burden after treatment with TCBZ was 97%. Thus confirming that this was a susceptible isolate of *F. hepatica*

according to published criteria (Gordon et al., 2012a). Researchers from The Netherlands working on resistant isolates of *F. hepatica* from mixed cattle and sheep farms experimentally infected sheep with susceptible and resistant isolates and found high efficacies of TCBZ against these strains; 99.8% whereas 10.8% efficacy was reported for resistant strains of *F. hepatica* (Gaasenbeek et al., 2001). Our results showed that treated sheep continued to shed eggs on the day of slaughter and showed very low numbers of eggs (range of 0.2 to 15.4 epg) at the end of the trial (Table 2.3.4). The presence of a positive FEC in all six treated sheep and the presence of adult fluke at post mortem reflects that parasite populations have genetic variation meaning that some parasites within a drug susceptible population survive treatment and that drugs are never 100% effective.

Correlation between fluke burden at necropsy and cELISA and FEC is shown in the Appendix 2.5. Our data showed good correlations between fluke burden when 14 wpi coproantigen levels were used (R^2 = 0.642; p = 0.32) but very weak correlation for FEC (R^2 = 0.045; p = 0.39). However, on the day of slaughter (10 dpt), both coproantigen levels and FEC for the six control sheep correlated weakly with fluke burden, R^2 = 0.265 (p=0.70) and R^2 = 0.001 (p=0.39), respectively. This agrees with other studies that confirmed weak correlation was found between FEC and fluke burden (Charlier et al., 2008; McConville et al., 2009). Abdel-Rahman et al. (1998) also detected low correlation between fluke burden and egg production (R^2 = 0.39) in early studies in cattle. However, unexpected results were reported by Brockwell et al. (2013) that detected strong correlation (R^2 = 0.8368) between FEC and number of flukes recovered at the day of slaughter. A strong correlation between coproantigen and fluke burden at slaughter was reported in several studies: in cattle (R^2 = 0.96) (Abdel-Rahman et al., 1998), R^2 = 0.87 in cattle (Brockwell et al., 2013), R^2 = 0.79 in lambs (Mezo et al., 2004) and R^2 = 0.69 in cattle (Charlier et al., 2008). However this is on contrast to our findings and given the inherent variation in cELISA values, even in the same animal over time (Table 2.3.1), it is difficult to draw conclusions as the correlation is different depending on which dataset within the experiment is used (Appendix 2.5).

2.4.5 PCR

PCR-based technique is an extremely sensitive diagnostic tool that may simplify the diagnosis of parasitic infection. This method successfully detects even a very small amount of DNA. However, despite this superior sensitivity, our experiments have shown limitations in detecting *F. hepatica* infection. One main challenge when preparing samples is that faecal specimens contain mixtures of host cells, microflora, complex polysaccharides, bile salts and other materials which can act of inhibitors to PCR (Monteiro et al., 1997; Radstrom et al., 2003) by inactivating Taq polymerase and, may also degrade nucleic acids and reduce efficiency of the test (Radstrom et al., 2004).

In the current study, sheep which had been confirmed to have infection with liver fluke by cELISA and FEC were negative by PCR, this PCR negative result likely indicates unsuccessful amplification of fluke DNA from infected faecal samples with the ribosomal ITS2 primers (Robles-Pérez et al., 2013) or inhibition of PCR due to chemicals within faeces. However this finding contradicts the findings of studies conducted in Spain (Martinez-Perez et al., 2012; Robles-Pérez et al., 2013). Martinez-Perez et al. (2012) showed that PCR to amplify a 423 bp fragment of mitochondrial DNA from faecal samples was able to detect *F. hepatica* infection in sheep as early as 2 wpi with a nested-PCR (Cox2_F and LrRNA_R primers) and from 3 wpi using a standard PCR (Cox1_F and Cox1_R primers). This means that infection has been detected during the pre-patent period of *F. hepatica* and so the PCR techniques were more sensitive than cELISA and FEC. Subsequently this finding was confirmed by Robles-Pérez et al. (2013) who also detected *F. hepatica* infection in the second wpi in sheep from the faecal samples. This earlier detection, using a standard/nested PCR, was based on amplification of a 292 bp fragment of the ITS2 rather than a 423 bp fragment of mitochondrial DNA.

The majority of work conducted for this project was on optimisation of the two PCR assays (standard and nested) developed by Martinez-Perez et al. (2012) and later the ITS2 PCR by Robles-Pérez et al. (2013). The PCRs were initially trialled using known *F. hepatica* positive samples: adult *F. hepatica* DNA, DNA recovered from infected sheep faecal samples from 14 or 18 wpi and DNA recovered from *F. hepatica* eggs recovered from either the gall bladder or faecal samples. These

samples were confirmed positive to *F. hepatica* using the RAPD PCR (McGarry et al., 2007), however results from these trials were inconsistent so troubleshooting and further optimisation was performed.

Four areas of interest were explored: (i) quantity of faeces used for DNA extraction (ii) process of DNA extraction – two different commercially available kits – Qiagen and Nucleospin (iii) dilution factor of the extracted faecal DNA and (iv) optimization of the annealing temperature. Each variable was taken in turn using the *F. hepatica* positive samples mentioned above.

i. Quantity of faecal DNA. In trials conducted by Martinez-Perez et al. (2012), DNA extraction appears to have been conducted using 2 g of sheep stool samples. However, when we attempted to replicate this we found the size of the sample difficult to handle. Therefore we used 250 mg of faeces as recommended from the QIAamp DNA Mini Stool Kit. Attempts to quantifying 250 mg from *F. hepatica* positive samples from infected sheep on 14 wpi, however, gave us low yields of DNA and inconsistent bands on standard and ITS2 PCR as well as a negative RAPD and nested PCR result (data not shown). Therefore the quantity of DNA was increased to 0.5 g of faeces as described by Robles-Perez et al. (2013). Following quantification it was found that by increasing the faecal sample from 250 mg to 0.5 g we had increased the quantity of DNA extracted from 1.53 ng/µl to 25.77 ng/µl; faecal samples from infected sheep on 14 wpi and 18 wpi, respectively. On this basis, we might expect to amplify *F. hepatica* DNA during PCR but this was not successful. We suggest that this is due to increasing the level of PCR inhibitors in the samples due to the larger sample volume.
ii. DNA extraction kit. DNA extraction was performed using a QIAamp® DNA Mini Stool Kit (Qiagen Ltd, UK) and Nucleospin® Tissue (Macherey-Nagel) and the DNA yield was measured. Based on extraction of DNA from 250 mg of faeces taken from a sheep 14 wpi that was positive for *F. hepatica* showed that the Qiagen extraction method produced higher DNA yields compared to Nucleospin kit (data not shown). However, all extraction samples were negative for *F. hepatica* using the RAPD PCR.

When using the standard PCR as developed by Martinez-Perez et al. (2012) positive results were obtained from the Qiagen kit but negative results from the Nucleospin kit. Therefore, by using the Qiagen Kit, we increased the DNA yield and successfully amplify the target DNA. Moreover, by using the QIAamp DNA Mini Stool Kit, it is possible to eliminate PCR inhibitors. Previous studies have shown that inhibitors can partly be removed from human stool samples by using QIAamp tissue methods (Monteiro et al., 1997). There are also several options to eliminate the PCR inhibitors during extraction. For example, by using magnetic beads or bead beater methods to remove or inactivate PCR inhibitors from faecal samples (Smith et al., 2011; Wehausen et al., 2004). Therefore future approaches will need to carefully design sample preparation and DNA extraction to minimise the level of PCR inhibitors.

iii. In samples where PCR inhibitors persisted, diluting the template DNA (by a factor 50) prior to PCR helped remove the inhibiting effects (Monteiro et al., 1997). However, we have difficulty of achieving the right DNA dilution in our study as for some samples, a 1:10 dilution was sufficient to eliminate the inhibitors.

iv. Optimization of annealing temperature. Although previous studies used an annealing temperature of 63^{o}C (Martinez-Perez et al., 2012; Robles-Perez et al., 2013) this temperature did not produce a positive result in our laboratory. Therefore our approach in this study trialled four different annealing temperatures 54°C, 57°C, 63°C and 65°C to optimise the annealing temperature for the ITS2, standard and nested PCR (data not shown). An annealing temperature of 54°C cannot be used as this temperature resulted in non-specific amplification likely because it was too low the primer could bind imperfectly thus very faint bands were detected. Furthermore, if the annealing temperature was too high (65°C), the primers are not able to bind to the denatured template DNA (with no bands observed in this trial) (Hecker and Roux, 1996). However since changing the annealing temperature did not improve the efficiency of the PCR, the annealing temperature of 63°C was maintained, as it had been shown to work in previous experiments. Visible results were observed when

annealing temperature was lowered to 54°C, 57°C or even higher, the annealing temperature of 63°C was maintained in this experiment.

In summary we attempted to transfer the published PCR method to our laboratory and despite extensive optimisation it was not possible to reproduce a PCR test that accurately detected *F. hepatic*a infection prior to 8 and 10 weeks post infection and even at these later timepoints post infection animals positive by cELISA and FEC were negative by PCR.

CHAPTER 3

Improving the Design of the Composite FECRT to Detect Triclabendazole Resistance in Fluke Populations

3.1 INTRODUCTION

Parasitism is one of the most important constraints on farming systems in the United Kingdom and other regions of the world. These include gastrointestinal trichostrongylid nematodes and liver fluke trematodes. Their effects on health, production and welfare are mainly dependent on the intensity of infection. Drug control strategies have been used by farmers to control the parasites in the livestock and due to their extensive use, has resulted in the emergence of anthelmintic-resistant strains particularly in *F. hepatica* in sheep. Thus, reliable assays to detect anthelmintic resistance in populations of parasites are required for assessment of effective control of parasitic infections. This detection can be made with several *in vivo* and *in vitro* tests and there is a need to standardize and validate these tests, particularly for *F. hepatica* in sheep. Historically, most tests have been developed for nematode parasites of livestock, resulting in the guidelines of World Association of the Advancement for Veterinary Parasitology (WAAVP). Therefore, methods for detecting resistance in this chapter have been based on the WAAVP guidelines for detection of anthelmintic resistance in nematodes (Coles et al., 1992).

The *in vivo* method that is most widely used in the field is the faecal egg count reduction test (FECRT). FECRT is the test to estimate anthelmintic efficacy using faecal egg counts (FECs). The test compares the number of parasite eggs recovered in faeces taken before and after treatment or by comparing counts in faecal samples of treated and untreated sheep, the latter group acts as a control group (Coles et al., 1992). Sufficient adult parasites must be present in order to produce enough eggs for diagnostic test purposes. The FECRT is also favoured as it can be used with all anthelmintic classes. The interval between treatment and post treatment sampling should be shorter than the pre-patent period of the parasites for drugs with no residual activity and the interval between treatment and post treatment sampling

depends on the type of anthelmintic being evaluated. For example, an interval of 3-7 days is recommended for levamisole, 8-10 days for benzimidazoles and 14-17 days for macrocyclic lactones (Coles et al., 2006). Differences in post treatment sampling intervals depend on drug pharmacokinetics, excretion, persistence and their efficacy in removing different parasite life stages. Misinterpretation of anthelmintic efficacy whether false negative or false positive results could occur if resampling is done outside of these ranges. For example, a study by Grimshaw et al. (1996) falsely indicated the presence of levamisole resistance and gave misleading results in the FECRT. This study showed that % FECRT on samples taken 11 and 20 dpt with levamisole was 80% and 78%, respectively; however this is due to the maturation of immature stages that produce egg in that time period and hence underestimated the efficacy of the drug. Therefore, 3–7 days is recommended as this does not allow time for maturation and patency of immature infection at the time of resampling, but excludes the period of temporary egg suppression in resistant strains; usually up to 3 days post treatment (Coles et al., 2006), which may lead to a false "susceptible" result.

Anthelmintic resistance in nematode populations affecting sheep, is defined as a reduction in FEC of <95% (arithmetic mean) with a minimum pre-treatment count of 150 egg per gram (epg) for individual FEC according to the WAAVP Guidelines (Coles et al., 1992). In small ruminants, resistance is defined as being present if the reduction in FEC (arithmetic mean) is less than 95% and the lower 95% confidence level is less than 90% (Coles et al., 1992). The WAAVP guideline determines that if only one of the two criteria is met, anthelmintic resistance is suspected. However, such limits have not been defined for *F. hepatica* and these thresholds may not be applicable for *F. hepatica* populations or flukicide classes, hence the FECRT has not been fully standardised for this worm. Coles et al. (2001) suggested that suspected resistance in trematodes in the field needs confirming by an experimental study; e.g. an isolate collected from the field is used to experimentally infect animals, which are dosed and then slaughtered and parasites enumerated (Wood et al., 1995). For liver fluke, the only reliable test for triclabendazole (TCBZ) resistance in *F. hepatica* in the field is a 'dose and slaughter controlled study', as described by Coles et al. (2001) and Gordon et al. (2012a). Suspected TCBZ resistance can be diagnosed by using FEC and coproantigen reduction tests and these have been validated using the

WAAVP approved ‘dose and slaughter controlled study’ (Gordon et al., 2012a). These authors showed that FEC and the coproantigen ELISA (cELISA) were useful in detecting resistance. Killing animals is not possible for routine diagnosis of resistance, thus the FECRT is one of the few available tests for diagnosing resistance in liver fluke in live animals. Other diagnostic tests for detection of TCBZ resistance of *F. hepatica* include PCR (Robles-Perez et al., 2013), but we found in experimental infected sheep (chapter 2) that PCR’s evaluated did not detect infection. Therefore, in the present study, we have used the FECRT and, as there are no agreed guidelines that define presence of resistance to TCBZ, we have defined suspected resistance if the percentage reduction in composite FECs is less than 90% based on a previous study (Daniel et al., 2012). In field studies, drug efficacies from 0 to 60% have been reported when resistant *F. hepatica* was present (Gordon et al., 2012b). Furthermore, drug efficacies from 0 to 21% have been reported when sheep were experimentally infected with TCBZ-resistant *F. hepatica* isolates (Flanagan et al., 2011a).

3.1.1 Distribution of Faecal Fluke Egg Counts within populations of sheep

The first surveys of the distribution of liver fluke infection in sheep took place in the 1950s in The Netherlands (Honer and Vink, 1963a). Condemned lambs' livers from the slaughter house allowed researchers an insight into the variation in fluke burden in sheep. The study modelled parasite distribution as following a negative binomial pattern. A subsequent survey, by the same group of researchers (Honer and Vink, 1963b) found that the pattern of infection in cattle was similar to that described for in sheep in the previous study. The studies also showed that the annual patterns of liver fluke infection in sheep and cattle are different; sheep and lambs are exposed to continuous infection and may have a different physiological reaction to the infection whereas in cattle, exposure is interrupted by different times of housing (Honer and Vink, 1963b). Daily variation of *F. hepatica* egg counts in individual cattle were described in housed cattle in the winters of 1963 to 1964 and 1964 to 1965 (Honer, 1965). The authors found that marked fluctuations of FECs occurred on five different farms, suggesting temporal variation within individual animals and inter-farm variations may partly explain these differences. The quantity of faeces

produced by individual animals on each farm is under the influence of management activities; e.g. feeding, milking and other husbandry issues (Honer, 1965). Other available evidence describing the distribution of *Fasciola* eggs in cattle faeces was conducted by Duwel et al. (1990). The bulls were infected with a varying number of metacercariae and the study was carried out for six months. Daily range of variation and distribution of fluke eggs were determined three times a day (morning, noon and afternoon). The authors have shown that the distribution of fluke eggs was inconsistent within one day and the excretion of the eggs varied widely at each timepoint between animals and in each infection group (Duwel and Reisenleiter, 1990).

The pattern of nematode egg output in sheep has been described. Previous work has shown that nematode FEC in populations of sheep follow a negative binomial distribution (NBD) (Barger, 1985; Stear et al., 2006; Stear et al., 1995). A majority of individual hosts carry low numbers of parasites whereas a small number of hosts tend to have higher FEC (Gregory and Woolhouse, 1993). Other factors which will affect the overdispersion are the intensity of infection; heavily infected flocks tend to show less overdispersion (Daniel et al., 2012). An estimate of the NBD is defined by two parameters; the mean and *k*, an inverse index of overdispersion. Studies in fluke infected animals have shown high values of *k* at high mean FECs, suggesting a lower degree of overdispersion (Daniel et al., 2012). For example, if sheep are sampled in late autumn, this is when animals have been exposed to infection; a composite FECRT would give reliable results even though fewer samples are used whereas it is challenging to conduct this test during summer as fluke burdens maybe lower. It has been suggested that time and size of last infection could affect the observed degree of overdispersion in gastrointestinal nematodes (Morgan et al., 2005), but it is not known how this may affect distribution of fluke eggs.

A fundamental question has been to determine what affects the distribution of parasites within and between hosts. Age, sex, grazing history etc. are likely to affect FEC aggregation. For example, males tend to be more heavily infected than females, the reason for which may be differences in immune system. Also, FECs tend to increase with age, then may plateau in older animals, possibly again as a result of the deterioration of immune function, although egg counts may decline again, thus

reducing the degree of parasite aggregation (Hayward et al., 2009). Other factors for variation of parasite load among sheep populations may include genetic differences in susceptibility to infection, condition of the host, behaviour, seasonality and geographical area.

The success of TCBZ treatment can be evaluated by measuring the reduction in fluke FEC following treatment. This is usually based on the detection of *F. hepatica* eggs in individual sheep in experimental infections or under natural infection in the field (Flanagan et al., 2011a; Flanagan et al., 2011b; Hanna et al., 2015). Individual FEC can be uneconomical, labour intensive and time consuming. Hence there is a move to use composite faecal egg counts to reduce costs for farmers (Daniel et al., 2012). However egg counts vary over time within animals and between animals and these factors should be taken into account when developing both a FECRT and a composite FECRT. Overdispersion of individual FEC within a population has been described in nematodes, but less is known about overdispersion of *F. hepatica* FEC within populations of sheep naturally exposed to infection. Furthermore, there are several limitations for FECRT including animal-related and farm-related variability in FEC data. For example, differences in grazing management between farms will impact parasite infection intensities in sheep. When sheep are grazed in marshy areas or farms have fields with poor drainage, this may result in heavy infection of fluke in sheep, whereas other farms may have very light infection pressure, thus causing large differences in the distribution of pre-treatment FECs between farms. Spatial differences due to geographical location of the farms as well as temporal differences, including year to year variation in rainfall and the season in which sampling is conducted can also play important role in the variability in FECs. Furthermore, the non-uniform distribution of eggs in faeces from individual sheep, combined with variation in daily egg output may all impact on FEC. Other factors include the inability to measure drug efficacy in the pre-patent phase of infection leading to false negative counts and false positive FECs might occur due to the release of fluke eggs stored in the host's gall bladder, even though the flukes have been successfully eliminated following drug treatment.

Recently, for the practical purposes, a composite FECRT (cFECRT) has been developed and this test was validated for use in the field for detecting TCBZ

resistance in sheep (Daniel et al., 2012). With the cFECRT, pooled faeces from two groups consisting of 10 sheep each are tested; 5g of faeces are used from each sheep to create a composite sample of 50g and the total egg count for each composite is recorded. A particularly challenging issue when developing cFECRT is how overdispersion of fluke egg counts can affect the accuracy of this method and the sensitivity of a composite test as opposed to conducting counts on each individual sheep. There may be a larger degree of overdispersion of fluke eggs in composite samples associated with a large variance in egg counts between the 10 sheep which make up the composite. But it has been shown that composite samples from 2 groups of 10 individual sheep per sample are adequate to detect the reduced efficacy of TCBZ in the field (Daniel et al., 2012).

Other factors can limit the accuracy of cFECRT in the laboratory. First, it is difficult to thoroughly mix the composite samples and eggs may be trapped in higher fibre densities. If more fibre is present due to the larger quantities of faeces used, the more difficult it is to count the eggs in the Petri dish under the microscope. Secondly, if a large number of fluke eggs are present, it is hard to do an accurate count compared to counting smaller numbers of well-distributed eggs. However, regardless of these issues, when equal amounts of faeces from 10 sheep are mixed together compared to a single individual sample, the assumption is that the mean egg density in the composite faecal sample equals the sum of the egg density in individual sheep. Daniel et al. (2012) showed that the number of sheep required for the cFECRT was 20. If the total composite pre-treatment count is 100 or greater, a second set of samples from the same animals is collected 21 days after treatment. However, in practise, gathering the same 20 sheep for resampling is often difficult, therefore the first aim in this chapter was to determine if the same sheep had to be sampled both pre- and post treatment. By using data from two sheep farms, we determined if the same 20 sheep required sampling pre- and post treatment, or if a random sample from the population could be sampled on the two different occasions.

3.1.2 Identifying an optimal time-point for resampling

The detection of *F. hepatica* infection is based on the observation of fluke eggs in faeces. For the diagnosis of drug efficacy, the cFECRT has been evaluated, which measures composite counts at day 0 pre-treatment and 3 weeks post treatment with TCBZ. The reason for using the timepoint, 21 days post treatment was based on studies in the literature which showed fluke eggs can be retained in the gall bladder after successful treatment with flukicidal drugs (Flanagan et al., 2011a). Therefore the 3-week interval was selected to ensure that residual eggs had been flushed out of the gall bladder. Other studies have shown good results for a FECRT with a 7 day period between pre- and post-drench (Brockwell et al., 2013; Gordon et al., 2012b). For practical purposes, asking farmers to wait 3 weeks for the results of the FECRT leads to some non-compliance and also welfare issues if a resistant population of fluke are present, so providing data on drug efficacy in a timely manner is important. Hence the second aim of this study was to evaluate an earlier time point (7 days) for collection of post treatment samples. In addition, the cELISA which proved to be more sensitive in detecting early infections in sheep in Chapter 2 was evaluated with field samples alongside the FECRT.

3.2 MATERIALS AND METHODS

3.2.1 Study 1

3.2.1.1 FEC distributions on two farms

Two farms (farm 1 and farm 2) were recruited to the study in order to investigate the distribution of FEC within a population of sheep. Faecal samples were collected from all 44 sheep from farm 1 and all 105 sheep from farm 2 and individual faecal counts conducted using the sedimentation method (2.2.2). Farm 1 was sampled in June 2012 whereas for farm 2 faecal samples were collected in October 2012.

3.2.2 Study 2

3.2.2.1 Comparisons of two time points post-treatment for resampling

A total of five farms (farm A, B, C, D and E) from Wales participated in this study which was collaboration between the University of Liverpool (Department of Veterinary Parasitology) and Farming Connect, Wales. Pre- and post treatment samples from five farms were collected by the farmer or a representative from Farming Connect, Wales. On each farm, 20 sheep were identified at random, divided into two groups of 10; Group 1 and Group 2, and each group colour-coded. Individual faecal samples from Group 1 and Group 2 were then mixed together for composite counts. Faecal samples were collected on day 0 pre-treatment and then sheep were treated with TCBZ at 10 mg/kg at a dose set to the heaviest sheep within the group. Faecal samples were collected from all sheep from Group 1 and Group 2 at day 7 and day 21 post treatment using the same procedure. Samples were sent to the University of Liverpool for testing individual and composite FECs and cELISA.

3.2.3 Parasitology Techniques

3.2.3.1 Individual Fluke Egg Counts (FECs)

FECs were performed using the sedimentation technique as described in Section 2.2.2 (chapter 2) and in Appendix 2.1 (Anon, 2007).

3.2.3.2 Composite Fluke Egg Counts (cFECs)

Five grams of each faecal sample was pooled with the other samples from same group to make a total of 50 g to form the composite sample, which was then mixed thoroughly with water in a 500 ml beaker and sequentially passed through 710 μm, 150 μm and 38 μm sieves. The material retained by the 38 μm sieve was then transferred to a large square petri dish and a drop of methylene blue was added. The total numbers of eggs were counted under a dissecting microscope and eggs per 50 gram (ep50g) were calculated.

3.2.3.3 Coproantigen ELISA

The cELISA (BIO K 201, Bio-X Diagnostics, Jemelle, Belgium) was conducted according to the manufacturer's recommendations on individual faecal samples as described in previous chapter (Section 2.2.2 in chapter 2 and Appendix 2.3). For composite faecal samples, 0.5 g of faeces from each of the 10 sheep per group were used to make the 5 g composite, and then mixed up with 20 ml of the dilution buffer in a 50 ml tube. Each sample was homogenized for 10 s using the vortex and the tubes were then centrifuged at 1,000 x g for 10 min. Following centrifugation, the supernatant was collected and stored in 1.5 ml Eppendorf tubes at -20°C until needed. A full Standard Operating Procedure (SOP) for this procedure is given in Appendix 2.3.

Coproantigen values were expressed as the percentage positivity according to the formula; % = (Mean OD of the sample / Mean OD of positive control) x 100. The cut-off of the percentage positive (PP) value was 6.65% as supplied by the BIO K 201 kit manufacturer.

Statistical analysis

The percentage FEC reduction was calculated based on the WAAVP recommendation for nematodes. For the composite counts, the %FECR was calculated as (1[-post-treatment count/pre-treatment count]) x 100. Faecal egg counts were compared using the Kruskal-Wallis test (SPSS statistical software, release 20.0; SPSS Inc., USA) with results applied at the 5% level of significance. With the SPSS program, the Spearman rank correlation coefficient *R* was computed for linear correlation analysis. A *P* value of .05 or less was considered to indicate a statistically significant difference by using R. Basic descriptive statistics (mean, standard deviations and median) were computed for each variable and table and graphs of the data were produced using Microsoft Excel (Microsoft Office 2010).

Bootstrapping analysis was conducted by Ms Christina Gill, the mean FEC calculated 10,000 times by using a function in R (version 2.10.0 2010, R). The mean estimate and 95% confidence intervals were then calculated and the entire process was iterated 10,000 times.

3.3 RESULTS

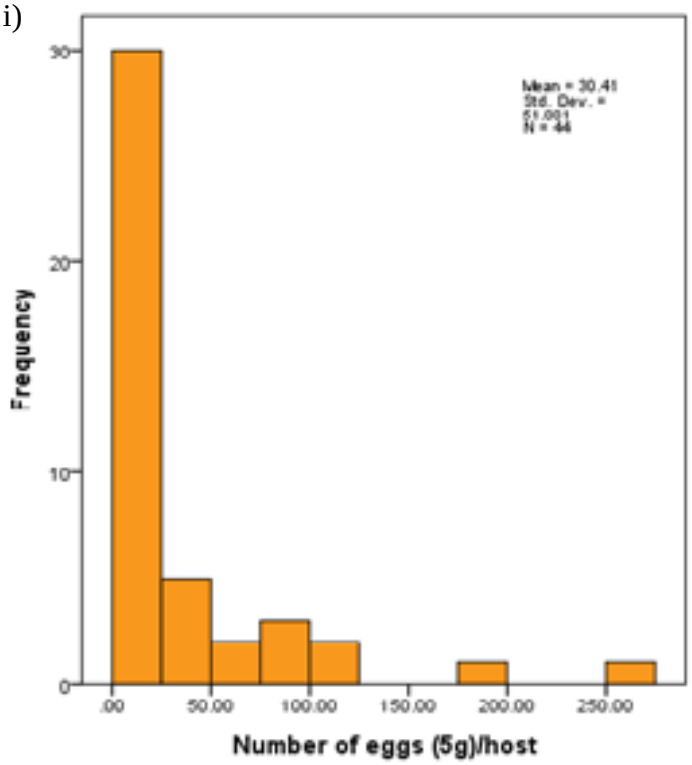
3.3.1 Does the distribution of egg counts within populations of sheep from two different farms follow a negative binomial distribution?

Table 3.3.1 gives the mean, median and range of *Fasciola* FEC values on farm 1 and farm 2. Eggs from *Paramphistomum* spp. were detected in samples obtained from both farms but were not counted in this study. The mean FEC was higher on farm 1 compared to farm 2 (Table 3.3.1). The distribution of egg counts for farm 1 and farm 2 is illustrated in Figure 3.3.1, showing that the distribution of FECs are overdispersed, with most sheep having low counts but a small number having high counts. Figure 3.3.2 shows three common statistical distributions fitted to the FECs from farm 1. The negative binomial distribution fitted the data best. The results were similar for farm 2. By using chi-squared goodness of fit test, the NBD provided a significantly better fit than the Poisson distribution. Due to the high number of individuals with zero counts, a zero-inflated model was also tested but did not improve the fit to the data.

Table 3.3.1 Mean, median and range of egg counts per 5 g (ep5g) of faeces sampled on two different farms.

Farm	No. of sheep	Mean	Median	Range
1	44	30.41	9	0 – 259
2	105	5.90	0	0 – 115

i)



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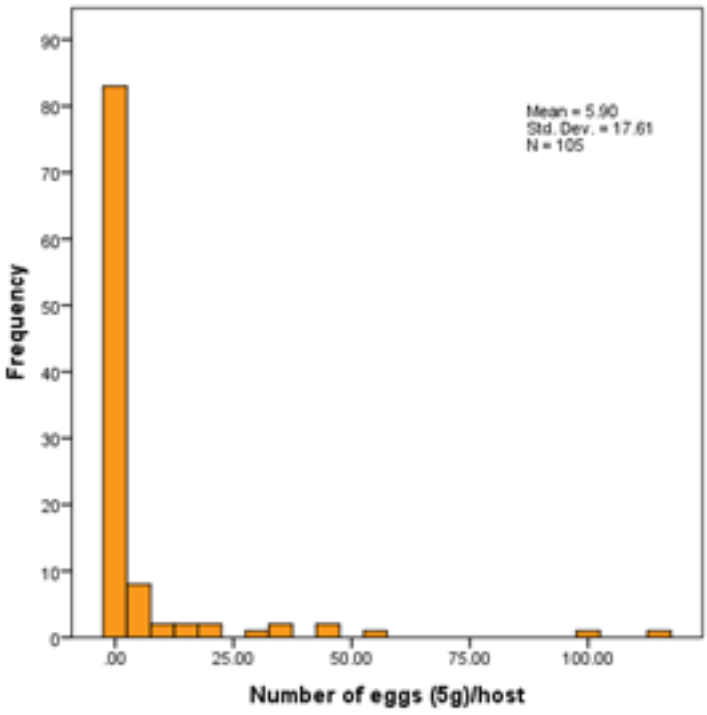


Figure 3.3.1 Observed faecal egg counts frequency distributions for two different farms i) Farm 1 and ii) Farm 2.

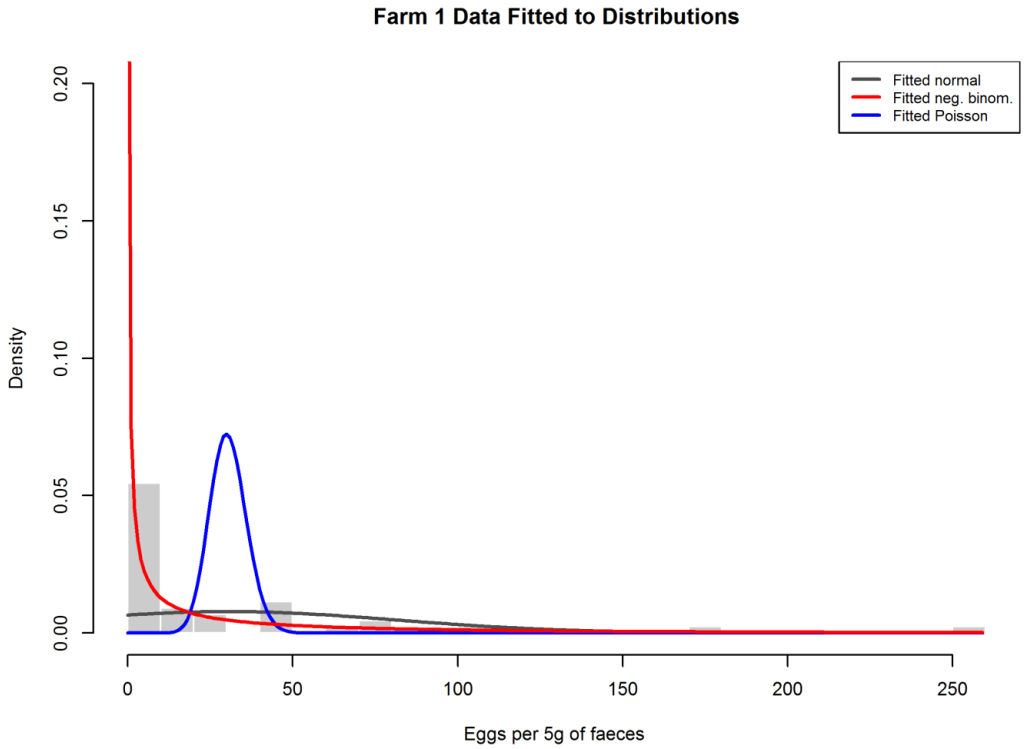


Figure 3.3.2 The FEC data for farm 1 was analysed to identify the model which best fitted the distribution, either a normal distribution (black line), a negative binomial distribution (red line) or a Poisson distribution (blue line). The results showed that the negative binomial distribution fitted the data best.

3.3.2 For the FECRT do we need to sample the same 20 sheep pre-treatment and post-treatment?

We used a bootstrap analysis to simulate sampling 20 sheep from our two populations (farm 1 and farm 2) 10,000 times. Analysis for farm 2, with 105 sheep, showed that the calculated mean FEC after 10,000 re-iterations was within the range of the true mean ± 5% in 5.3% of cases and in 10.5% of cases for farm 1 with 44 sheep. On both farms the spread of the mean FECs for different random samples was large, with 95% being between 11.7-55.6 and 0.6-15.25 eggs per 5g for farm 1 and 2, respectively. These results suggest that to detect a 90% reduction in faecal egg counts, the same 20 sheep should be sampled pre- and post treatment (see appendix 3.1 for analysis summary).

3.3.3 Can the post-treatment samples be collected at day 7 or day 21?

Five farms submitted samples for the FECRT. Samples from two groups of the same 10 sheep were taken at the time of treatment (day 0), day 7 and 21 post treatment. Composite FECs were carried out for each group at each timepoint. The composite samples were also tested by cELISA. In addition, individual counts for each of the 20 sheep were conducted and individual samples were also tested in the cELISA. Four of the farms had pre-treatment composite counts of less than 100 eggs and were excluded from this analysis. The cut-off of 100 eggs in composite count is required due to potential underestimation of pre-drench faecal egg count (Daniel et al., 2012).

Table 3.3.2 summarises the data for the composite FECs taken pre-treatment, 7 and 21 days post treatment. The total composite FEC on day 7 post treatment was 115 eggs compared to 69 on day 21 post treatment. The percentage reduction in FEC on day 7 was 21% compared to 52% on day 21. These results suggest that sampling on day 7 would over estimate the level of resistance on this farm. Table 3.3.3 summarises the composite cELISA PP values and composite counts recovered at day 0 (pre-treatment) and day 7 and 21 post treatment for groups 1 and 2. In group 1, there was an increase of composite FEC from 48 eggs to 110 eggs on day 0 and day 7, respectively and decreased to 37 eggs on day 21. The results for composite FEC for group 2, give a lower number of eggs recovered from day 7 (5 eggs) and rose to 32 eggs on day 21; on day 0, 97 eggs were detected. These results were illustrated in Figure 3.3.3 together with the composite cELISA at each of the three timepoints. Composite coproantigen results were positive at each sampling point. Overall, the samples collected 7 days after treatment with TCBZ, showed a large increase in PP values in both groups, thus indicating that sampling at day 7 potentially overestimated the resistance on this farm. For day 21, both tests showed a reduction, indicating that sampling on day 21 was a better time point to assess treatment efficacy. This was determined further by calculating the correlation between the composite FEC and cELISA (Figure 3.3.4). Weak correlation was found ($R = 0.0365$) indicating there was no correlation between composite FEC and cELISA, thus suggesting that cFECRT is more effective at detecting resistance to TCBZ compared to the composite cELISA.

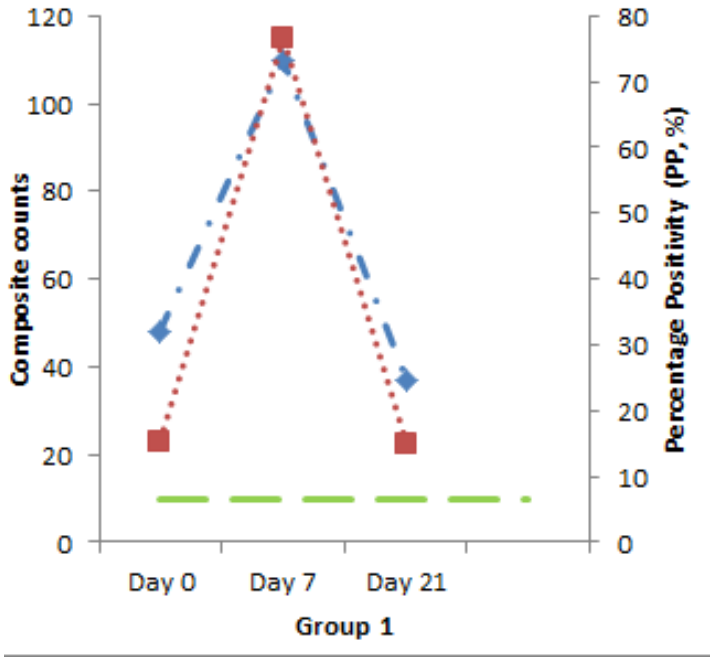
Table 3.3.2 The composite faecal egg counts, carried out using 2 composite samples from 10 individual sheep (n = 2 x 10) on different sampling timepoints from farm A.

Sampling	Total composite FEC	% FECR
Day 0	145	-
Day 7	115	21%
Day 21	69	52%

Table 3.3.3 The composite faecal egg counts and coproantigen ELISA (PP), carried out from 2 composite samples from 10 individual sheep (n = 2 x 10) on different sampling timepoints from farm A.

Sampling	Composite FEC	Composite PP	Composite FEC	Composite PP
	Group 1	Group 1	Group 2	Group 2
Day 0	48	15.3	97	61.92
Day 7	110	76.42	5	96.59
Day 21	37	14.88	32	8.51

i)



ii)

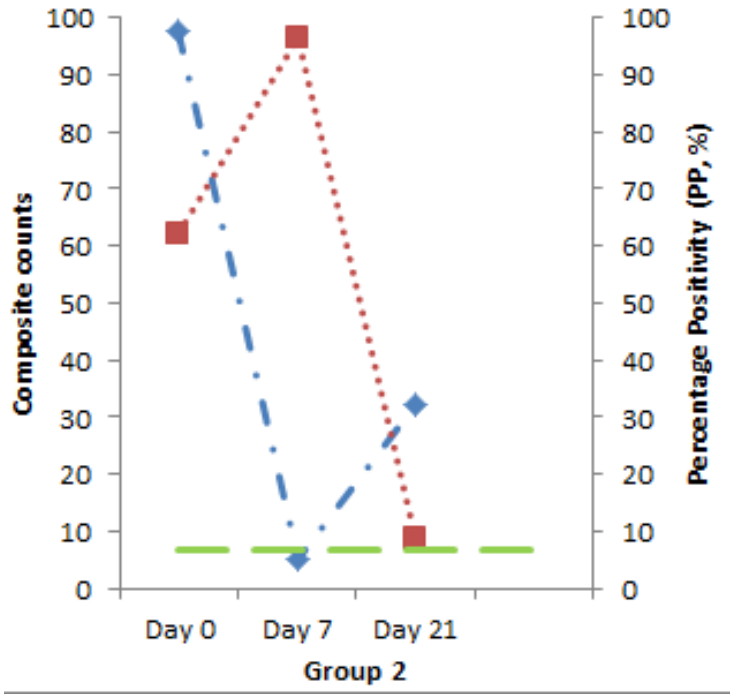


Figure 3.3.3 Composite counts (eggs/50 g) (blue dashed-dots line) and coproantigen ELISA (PP, %) level (red dots line) from farm A for i) Group 1 and ii) Group 2. The coproantigen ELISA positive cut-off value is 6.55% (green dashed line).

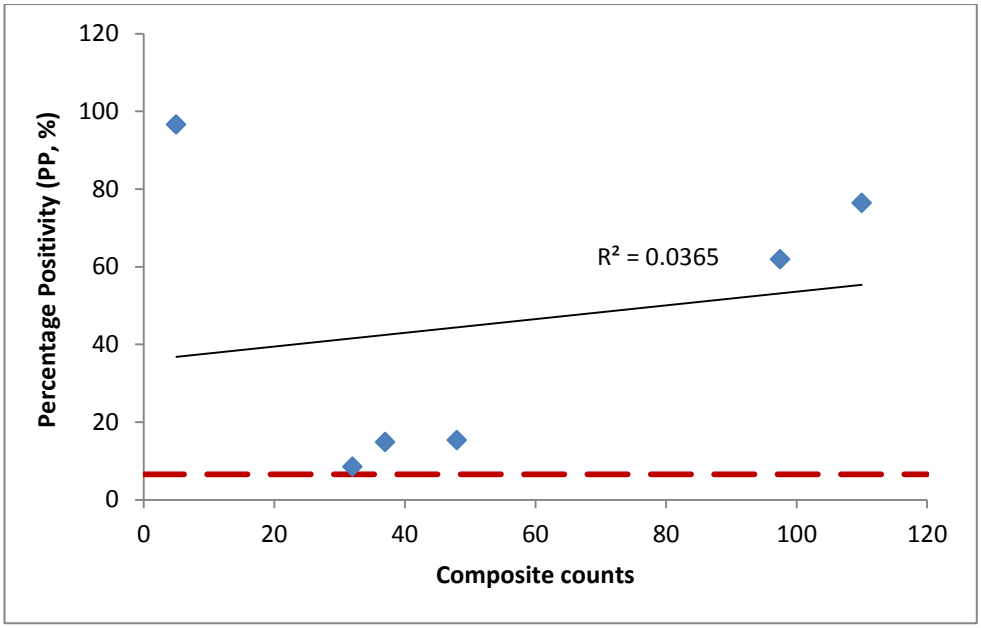


Figure 3.3.4 Correlation between composite counts (eggs/50 g) for two groups of 10 sheep sampled on day 0, 7 and 21. The positive cut-off value is 6.55% (red dashed line).

3.3.4 Is there a correlation between individual FEC and Coproantigen results?

Figure 3.3.5 summaries FEC from 20 individual sheep from all five farms. For farm A the individual FECs remained positive after treatment on 7 and day 21. The total numbers of eggs found was 1311 on day 7 post treatment compared to pre-treatment samples was 2359 eggs and treated sheep continued to shed fluke eggs 21 days following treatment, at which point 869 eggs were detected. Low or zero fluke egg counts were recorded on the other four farms and they were excluded from the study. However, on farm C, eggs were recovered on day 21 post treatment, despite the zero pre-drench FEC values. The composite FEC also detected eggs (128 eggs) at day 21 but was zero count on day 0 and 7 (data not shown).

There was no significant difference by using the Kruskal-Wallis test ($p > 0.348$) in total egg counts of 20 individual sheep FECs between day 0 pre-treatment and day 7 and day 21 post treatment on farm A (Figure 3.3.6). Furthermore, a visual inspection of their histogram and normal Q-Q plots (data not shown) showed that the distribution of FEC was not normally distributed either on day 7 or 21 post treatment, with a skewness of 3.319 (SE = 0.512) and a kurtosis of 12.231 (SE =

0.992) for day 7 post treatment and a skewness of 1.856 (SE = 0.512) and a kurtosis of 3.713 (SE = 0.992) for day 21 post treatment.

The mean epg results of both groups on farm A at all sampling points (mean ± standard deviation) and median (min-max) are shown in Table 3.3.4. Faecal egg counts and cELISA were carried out on 20 individual sheep at each timepoint on farm A, the results are shown in Table 3.3.5. Number of eggs recovered from each individual sheep varied widely. The results show one animal with negative FEC on day 0, and three and two sheep were negative on day 7 and 21 post treatment respectively. For the cELISA, of these 20 animals, 9 sheep were positive for coproantigen on day 0 and number of positive animals increased to 13 on day 7. Only 8 animals had positive cELISA results by day 21. One sheep whose cELISA was positive had a FEC of 8.6 epg, however one sheep with 10 epg had a negative coproantigen result.

The correlations of the FECs and cELISA (PP) values for individual animals over the 3-time point sampling period are shown in Figure 3.3.6. The correlation between the two tests varied between days of sampling, with R^2 values of 0.0468 ($p = 0.360$), 0.5492 ($p > 0.0001$) and 0.0923 ($p = 0.1926$), respectively, for day 0, 7 and 21. These results show that there is a highly significant relationship between FEC and cELISA on day 7 post treatment but not at the other two timepoints.

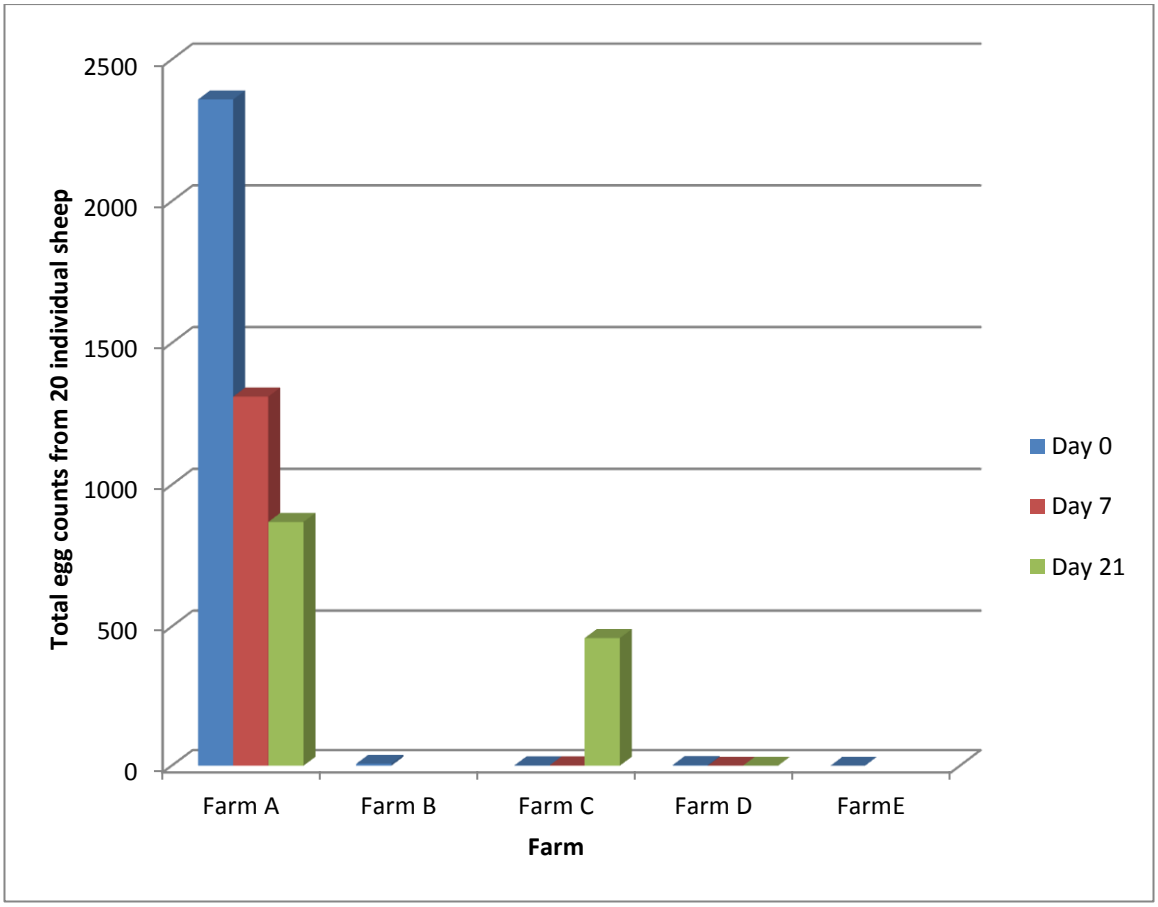


Figure 3.3.5 Results of total fluke egg counts (sum of ep5g for each individual sheep) for 20 sheep tested on day 0 pre-treatment, day 7 and day 21 post treatment for the five farms.

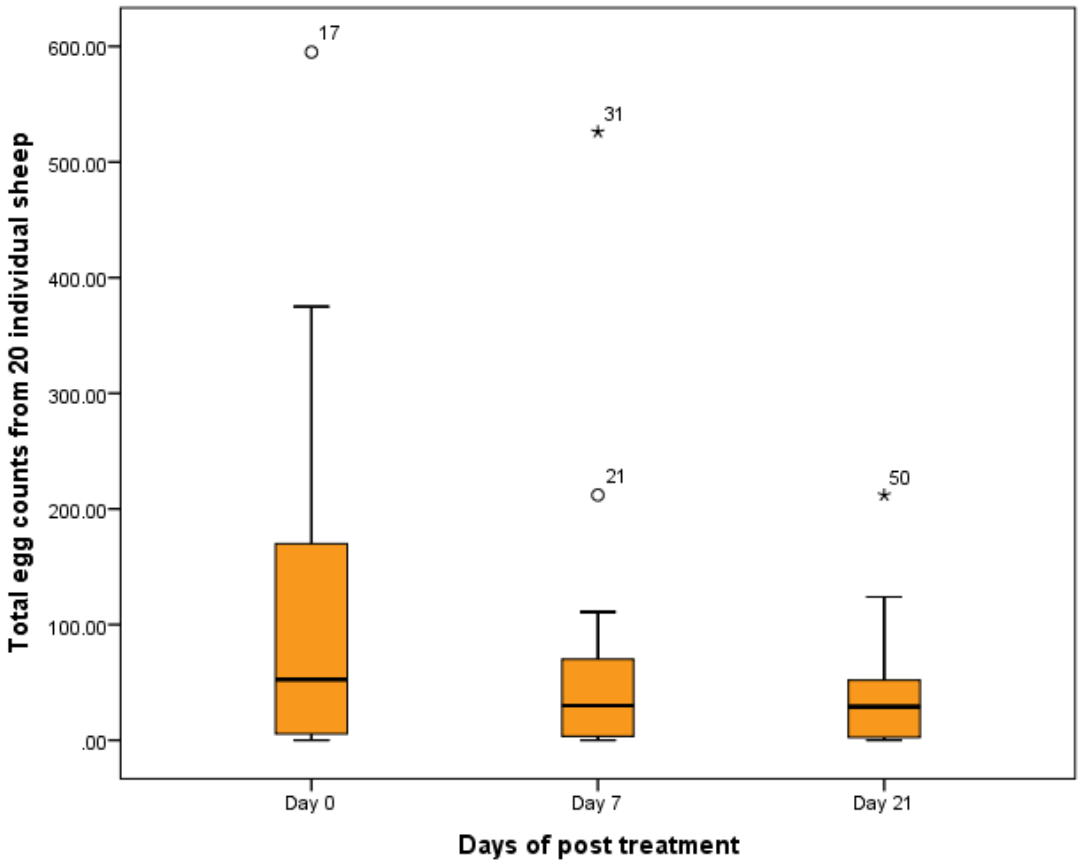


Figure 3.3.6 Fluke egg counts for 20 sheep from farm A sampled on 0, 7 and 21 days post treatment.

Whiskers indicate minimum and maximum values, boxes indicate 25th and 75th percentile values and the dash indicates the median values. Note that the outliers for 'out' values (small circle) and 'far out' (marked with a star).

Table 3.3.4 Mean fluke egg counts (epg) and PP values on day 0, 7 and 21 for sheep from farm A, treated with triclabendazole on day 0. Values shown represent the mean, median and standard deviation.

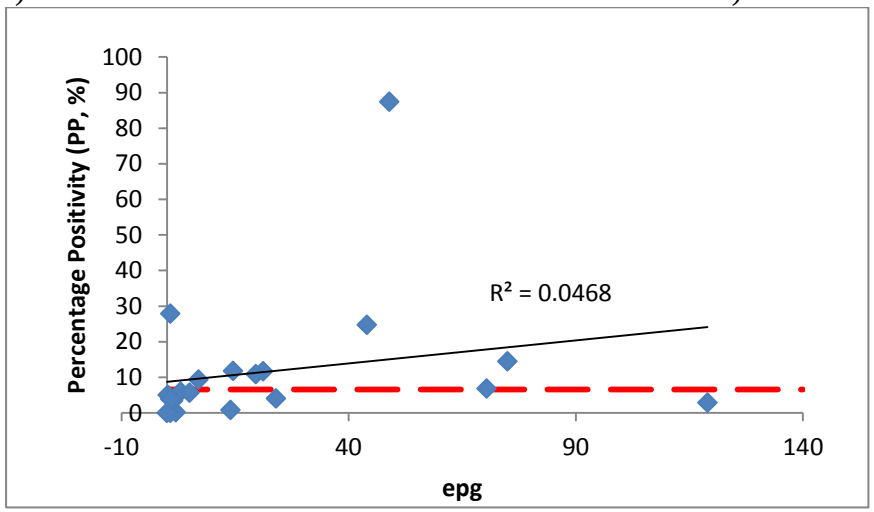
Day of sampling	Epg (Mean ± SD)	PP (Mean ± SD)	epg Median (min-max)	PP Median (min-max)
Day 0	23.6 ±32.3	11.8 ± 19.3	10.5 (0.2-119)	5.8 (0-87.4)
Day 7	13.1 ± 24.0	23.3±27.6	6.0 (0-105.2)	11.1 (0-88.0)
Day 21	10.5 ± 12.6	10.9 ±16.0	6.3 (0-42.4)	3.2 (0.33-65.9)

Table 3.3.5 Individual counts, both on FEC (epg) and coproantigen ELISA (PP, %) were taken from 20 sheep at day 0 pre-treatment, day 7 and 21 post treatment. The positive cut-off value for coproantigen ELISA is 6.55%.

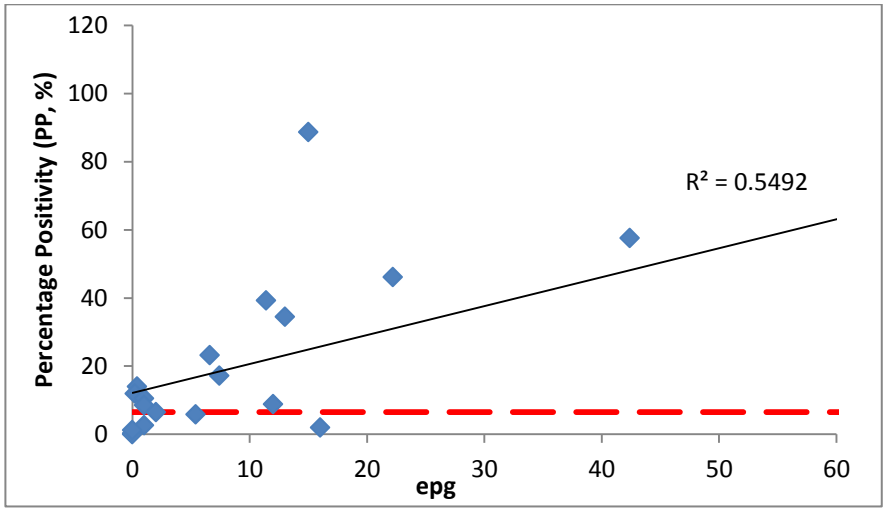
Sheep	Day 0		Day 7		Day 21	
	FEC	PP	FEC	PP	FEC	PP
1	2	0.16	42.4	**57.56**	0	1.07
2	3	5.93	1.0	2.61	3.4	2.36
3	0.8	**27.84**	22.2	**46.13**	10.0	2.95
4	7	**9.35**	13.0	**34.47**	8.2	2.04
5	70.4	**6.77**	0.4	**13.96**	24.8	**19.99**
6	1.4	3.38	0	0.23	24.4	**18.15**
7	0.2	4.97	0	0	0.2	**21.75**
8	0.8	0	6.6	**23.19**	1	**21.37**
9	14	0.77	0.2	**11.87**	0	0.84
10	0.8	3.85	11.4	**39.27**	42.4	**33.13**
11	49	**87.36**	105.2	**88.00**	18.0	**8.44**
12	0	0	1.0	**10.48**	4.4	1.58
13	5	5.62	5.4	5.77	2.3	0.33
14	21.2	**11.61**	1.0	**8.51**	0.4	1.60
15	14.6	**11.73**	16.0	1.96	36.0	**6.79**
16	44.0	**24.66**	7.4	**17.21**	8.6	**65.89**
17	119.0	2.79	15.0	**88.69**	10.0	2.36
18	24.0	3.98	12.0	**8.81**	0.2	0.96
19	75.0	**14.45**	0	1.23	15.8	3.53
20	19.6	**10.87**	2	6.46	0.6	3.82

NB: **bold values** represent positive coproantigen results; individual sheep were not eartagged, so sample 1, day 0 does not correspond to the same sheep as sample 1, day 7 or day 21.

i)



ii)



iii)

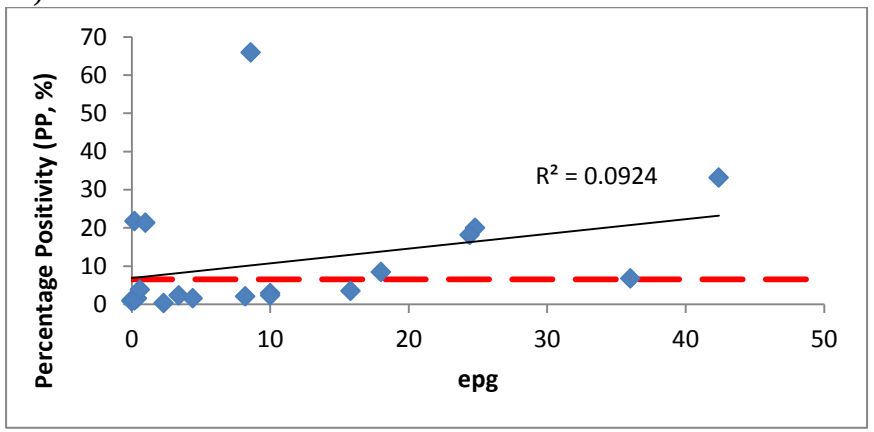


Figure 3.3.7 Faecal egg counts (epg) and coproantigen ELISA (PP, %) levels for i) Day 0 pre-treatment ii) Day 7 and iii) Day 21 day post treatment. The positive cut-off value is 6.55% (red dashed line).

3.4 DISCUSSION

3.4.1 Study 1

The variability of egg distribution patterns of *F. hepatica* in sheep has not previously been fully addressed. Due to this lack of information, results in this study provide a baseline of information regarding patterns of egg distribution in naturally infected sheep and highlight the degree of variability in fluke egg output between individual sheep and between farms.

The analysis of the FEC from a total of 149 sheep on two farms, with different levels of exposure showed that a high number of zero counts were detected and the pattern of egg output followed a negative binomial distribution. However, the results on these farms were limited to FEC data, the location of the farm and the season when the samples were collected. No data were collected on animal signalment such as age, date of birth, sex and breed, all of which have been shown to be significantly associated with egg count variation in nematodes (Stear et al., 1995). Those variables were influenced by the time of sampling and size of last infection (Morgan et al., 2005). This finding is in agreement with our study. In our study, farm 1 was sampled in June (summer) whereas for farm 2, samples were collected in October (autumn). Different stages of the parasite would have been present in the sheep at these times of year. In the summer, it is likely that adult parasites are present, either a residual burden acquired the previous autumn, or derived from metacercariae picked up from the pasture in spring. In contrast, in October, the majority of the fluke burden would most likely be juvenile flukes, derived from metacercariae released as a result of the summer infection of snails. These parasites would not be sufficiently mature to be shedding eggs. The FEC was higher for farm 1, sampled in June. However other factors can also influence egg ouput such as differences in exposure on sheep farms in different regions, depending on the farm environment and management practices (Vercruysse and Claerebout, 2001). Studies of *F. hepatica* have shown that the excretion of fluke eggs varies at different timepoints and their distribution is inconsistent at the individual level and within animals over time (Duwel and Reisenleiter, 1990). Similarly, FECs following natural nematode infection are very variable both within and between animals (Stear et al., 2006). Our

results confirmed that fluke egg output is best described by a negative binomial distribution but we did not obtain any further data on variation in egg output within individual animals over time.

The general principles of infection patterns were applied in the present study with the understanding that parasite count data is often overdispersed and this can be defined by a NBD (Stear et al., 2006; Stear et al., 1995). Helminth infections are often characterised by being overdispersed with a large proportion of the population having low egg count (e.g. high number of zero counts as seen in our data). A study by Barger et al. (1985) revealed that, in nematodes, a NBD provided a good description of the distribution of four trichostrongylid species; *Haemonchus, Ostertagia, Trichostrongylus* and *Nematodirus.* In contrast, not all FECs in sheep population or parasite distributions amongst hosts showed a good fit to the NBD (Bliss and Fisher, 1953; Stear et al., 1995). Studies conducted in natural nematode infected sheep have reported that the dominant species is *T. circumcincta*, and these data show that due to the presence of other species (*Cooperia* spp., *Trichostrongylus axei* and *vitrinus*) a poor fit of the NBD was observed. In this regard, it is complicated because these other species can all contribute to the egg count and their eggs are morphologically indistinguishable and the biotic potential differs between different nematode species. In the present study, we also detected eggs of *Paramphistomum* spp. on both farms, suggesting that this species can also be involved in egg count in sheep, however we specifically excluded paramphistome eggs in our counts and our data followed a good fit of NBD.

The concept of bootstrapping was introduced by Efron (1979). Using this approach is useful to estimate the population distribution using information based on re-sampling from a known sample. Bootstrapping has been used extensively with equine FECRT data (Denwood et al., 2010; Kaplan, 2002) and were also applied to fluke FECRT data on sheep farms to evaluate the method used in the current study (Daniel et al., 2012). In the present study, our results convincingly showed by using bootstrapping methodology, resampling the same 20 sheep gave us the most reliable method of estimating the FEC reduction rather than sampling another randomly identified 20 sheep on the second sampling visit. This is supported by the recommendation provided by the WAAVP and also from other recent studies (Hanna

et al., 2015). In the cFECRT method, in which 20 sheep are sampled before and after administration of drug, this number of sheep was validated taking into consideration the variation in FEC due to differences in FEC across individual sheep, time of sampling etc (Daniel et al 2012). Other parasites and other systems also show heterogeneities in parasite loads and exhibit a highly aggregated distribution and this leads to some individuals that harbour low egg counts and interestingly, one host can excessively contribute to high egg count (Anderson and May, 1991). This was also apparent from our data, when individual counts were done, in several groups, one sheep of the 20 sampled, had excessively high counts compared to the other sheep (Table 3.3.5). The second factor affecting the sampling method used is the number of sheep in the flock. In a small flock, if they are resampled at random, it is more likely that sheep that were sampled on the first occasion will be resampled. In contrast in a large flock, it is likely that different sheep will be sampled. Increasing the number of samples taken is another option, however, the analysis conducted by Daniel et al. (2012) showed that increasing the sample size up to 60 sheep, did not improve the power of the method.

We have used a threshold of a reduction of less than 90% to indicate evidence of resistance. However it remains unclear whether this indicates true reduced efficacy for fluke infections in sheep. Different cut-off values have been used; for nematodes < 95% reduction is used based on WAAVP guidelines and other studies in horses have used < 90% reduction to detect BZ resistance (Kaplan and Nielsen, 2010). Using a reduction threshold of < 90% to determine efficacy is an arbitrary value and until we know the exact variation in FECs as well as the genetics of resistance and the flow of genes in a population of fluke, it is difficult to define a definitive cut-off. Experimental infections with *F. hepatica* susceptible isolates have shown FEC reductions of between 95 and 100% by 14 to 28 days dpt, supporting our choice of 90% as a conservative estimate of susceptibility (Flanagan et al., 2001a).

In conclusion, this study highlights that FECs vary in naturally infected sheep within a population and between different populations and sampled at different times. In order to avoid making erroneous conclusions regarding the final interpretation of drug efficacy in the field, we propose to examine the same 20 sheep for pre- and post

treatment egg counts per farm but suggest that more information is required before a reduction of < 90% can be used definitively to indicate true resistance.

3.4.2 Study 2

This study sought to determine if the post treatment sample could be taken on day 7 pt rather than on day 21 pt, which is the current recommended time interval. The 21 day period between treatment and resampling is based on the suggestion that eggs can be trapped in the gall bladder even after the death of the parasites (Fairweather, 2011b; Flanagan et al., 2011a; Gordon et al., 2012b; Mitchell et al., 1998). Data from the experimentally infected sheep described in Chapter 2, showed that the FEC had declined by 98% by 7 days post treatment. Hence we compared day 7 and day 21 pt in a field situation.

Of the five farms which participated in this study, only one had sufficiently high pre-treatment counts to complete the study. Our findings showed that on both day 7 and day 21 pt the %FECR was < 90% indicating that resistance was present on this farm. However the %FECR was 21% on day 7 and 52% on day 21, suggesting that by waiting until day 21 pt is likely to reduce the risk of over estimating resistance. This interval will allow ample time for the disintegration of susceptible adult fluke following treatment and allow for the clearance of any fluke eggs potentially stored in the gall bladder. In experimental studies in which a FECRT was used, a reduction in the efficacy of TCBZ was calculated at 14 dpt although this period of sampling was not tested in the present study (Flanagan et al., 2011a). In comparison, other studies have used a 21 days period following treatment for the diagnosis of drug efficacy in sheep (Daniel et al., 2012; Hanna et al., 2015). From our experimental trial (chapter 2), FECs and cELISA were still positive on day 10 pt using a susceptible isolate. In that study 4/6 treated sheep still had a positive egg count but the %FECR was 98% on day 7 pt. However the results from farm A in this study suggest that waiting 21 days may be more accurate, especially if resistant parasites are present. Variation in the shedding of fluke eggs after treatment with TCBZ has been reported in the literature (Flanagan et al., 2011b) which discusses the issues of the late immature fluke migratory stages, which mature during the 3 week period between treatment and resampling and can potentially lead to an increase in the FEC if the fluke are resistant to the drug used. In contrast, experimental study in cattle

have shown good results for FECRT at 7 dpt; all six treated animals had returned to FEC negative (Brockwell et al., 2013) and in naturally infected sheep, treatment success was recorded within one week of treatment with TCBZ (Gordon et al., 2012b). Re-sampling at day 14 pt also showed good results which suggest that it is a suitable interval between pre and post treatment sampling to calculate the drug efficacy. Studies have shown that in naturally infected sheep, treated with closantel, oxyclozanide and nitroxynil, FEC was reduced by 100% by 14 dpt but not in the case of TCBZ-treated sheep (Mooney et al., 2009), in the study performed by Flanagan et al. (2011a) a 95% FEC reduction at day 14 pt were reported for TCBZ-susceptible *F. hepatica* infected sheep. Based on our study, a 3 week interval between treatment and sampling is needed to detect loss of TCBZ efficacy in the field.

The FECRT currently used in the field to detect evidence of drug failure is based on composite FECs (Daniel et al., 2012). However data from the experimental infection in chapter 2 suggested that the cELISA was more sensitive at detecting early infections. Hence we evaluated the coproantigen test for its ability to detect infection and resistance in the field. The results for farm A showed that the composite cELISA increased on day 7 pt and then declined, but remained above the positive threshold on day 21 pt. There was no correlation between the composite cELISA and the CFEC. Nevertheless the cELISA supported the conclusion that resistance was present on this farm, but the % reduction in PP value was not calculated. Other studies have used the criteria of the PP value declining to negative by day 14 pt as assurance that treatment was successful (Flanagan et al., 2011b; Gordon et al., 2012b).

The cELISA was evaluated in the present study using individual samples. Results from farm A showed that positive coproantigen (PP values) were detected from 13/20 sheep at 7 dpt compared to 9/20 sheep on day 0 (Table 3.3.5). By day 21, 8/20 sheep remained positive by cELISA. Fluke eggs were detected in these animals. False positive and false negative results of FEC and cELISA are discussed in the previous chapter. In the present study, the discrepancy between FEC and cELISA is difficult to clarify, due to an absence of information about the actual fluke burden in these animals which could only be ascertained at post-mortem. Similarly others

have shown that in the field, animals with low FEC, had negative cELISA values (Hanna et al., 2015). However, results 21 dpt showed that many more animals give positive FEC than cELISA. Studies by Flanagan et al. (2011b) used 14 dpt as the resampling time-point and the authors reported that all sheep that were infected with TCBZ-susceptible *F. hepatica* and following TCBZ-treated sheep had zero coproantigen levels by 14 pt. In practice, the coproantigen detection method will be useless if the test fails to meet the required level of sensitivity when applied on a large scale in a field-based study.

The coproantigen values increased at day 7 pt in this study before declining on day 21 pt. Following drug treatment, for a sensitive population, adult fluke have been shown to die within 48 hours (Hanna et al., 2010), however all the fragments of the parasites are unlikely to be completely cleared before 7 dpt, hence the coproantigen levels detected at day 7 could be due to dead flukes that are releasing the *F. hepatica* coproantigen. This highlights the importance of a pre- and post-drench time window in order to remove the dead fluke. To avoid this problem, even though three weeks is a long time, the results from the present study validate resampling at 21 dpt. Moreover, the data from this field study taken together with the data from the experimental trial described in chapter 2, suggest that the cELISA requires further validation before it could be used to replace the cFECRT in the field.

In conclusion, this study shows the sensitivity of cFECRT after drug treatment, comparing two different resampling time-points in naturally individual infected sheep. Day 21 was identified as the optimum time point to collect the post-treatment sample to avoid the danger of over estimating resistance. The commercial BIO K 201 ELISA is a sensitive test, however in the present study, inconsistent results comparing the cELISA and FECs were obtained. Evaluating the cFECRT in this chapter, has led to the conclusion that it is the optimum test to use in a wider field study to determine the prevalence of TCBZ resistance in the UK.

CHAPTER 4

The Prevalence of TCBZ Resistance in Sheep Farms in the UK Using the Composite Faecal Egg Count Reduction Test

4.1 INTRODUCTION

Liver fluke infection caused by the trematode parasite *Fasciola hepatica* affects sheep and cattle in many parts of the world. It is a common cause of morbidity and mortality in sheep and can cause acute, sub-acute or chronic disease. In sheep, acute infection with liver fluke has a negative impact on their welfare, productivity and results in significant financial losses, costing the sheep farming industry millions of pounds every year. Chronic fluke infection in sheep results in poor condition, reduced wool quality, submandibular oedema, apparent pneumonia (panting due to pain) and infertility (low scanning rates) (Sargison and Scott, 2011b).

In the past, liver fluke disease (fasciolosis) in the UK has been a problem mainly during the late autumn and the late winter. However, climate change is affecting the life cycle of *F. hepatica* and the distribution of the intermediate snail host, which has resulted in changes to the seasonal pattern of disease in the UK over the last few years (Charlier et al., 2011; Van Dijk et al., 2010). Between 1995 and 2013, the incidence of fasciolosis rose in England and Wales as reported by The Animal and Plant Health Agency (APHA) (Anon, 2013). Apart from changes in temperature, rainfall patterns and rising water levels caused by global warming , the spread of fasciolosis is also greatly influenced by the introduction of infected sheep to previously negative farms and regions (Mitchell, 2002), changes in farming practices associated with climate change and environmental regulations (Kenyon et al., 2009; Pritchard et al., 2005).

The pro-benzimidazole anthelmintic, triclabendazole (TCBZ) is the drug of choice for acute fasciolosis in sheep due to its high efficacy against immature stages of *F. hepatica* (Fairweather and Boray, 1999a). In sheep, clinical disease due to early immature liver fluke is a major problem and only TCBZ drug can kill this stage of

liver fluke from two days post infection. Studies have found that at a dose rate of 10 mg/kg, the efficacy was 93 to 98% against this age of fluke (Boray et al., 1983). The authors also reported that at the dose rate of 5 mg/kg, the TCBZ had 92 to 98% efficacy against liver flukes aged four to eight weeks, respectively and 100% efficacy was achieved against 12 week old flukes. A list of commercially available anthelmintics on the market for treatment of liver fluke infections is shown in Table 1.2 and 1.3. In the absence of vaccines for *F. hepatica*, these anthelmintics have historically been used in the control of fasciolosis. However, there is evidence that TCBZ is losing its effectiveness due to the reliance on repeated TCBZ treatment to prevent acute disease. It is now 30 years since the discovery and licencing of triclabendazole for prophylactic and chemotherapeutic use (Boray et al., 1983). As a result TCBZ resistance in *F. hepatica* populations in both sheep and cattle has emerged. A major concern now has been raised over the extent of resistance to TCBZ in fluke populations. The impact of not detecting the presence of TCBZ resistance on sheep farms can result in serious economic losses as well as affecting animal welfare (Sargison and Scott, 2011b).

The first case of resistance was documented in naturally infected sheep in Australia (Overend and Bowen, 1995) and more recently resistance was reported in Great Britain (Daniel et al., 2010), Ireland (Mooney et al., 2009), Scotland (Gordon et al., 2012b; Kenyon et al., 2009; Sargison and Scott, 2011b), Wales (Thomas et al., 2000) and a number of other European countries (Alvarez-Sanchez et al., 2006; Brennan et al., 2007; Gaasenbeek et al., 2001). The summary of resistance cases in the UK and worldwide are listed in Table 1.4 and 1.5. Therefore, there are great concerns that if the resistance level to TCBZ in fluke populations has increased, sheep farmers may have limited alternatives to choose from. The resistance level will always be different on each farm and as such there is no single right answer to fluke control for everyone.

There is pressing a need to identify factors that significantly influence drug resistance on farms. Over reliance and repeated use of TCBZ is one of the factors that may result in drug resistance. However, there is a school of thought which states that anthelmintic resistance is over-diagnosed; if this is true, rather than perpetuating misinformation, this may actually lead to a heightened awareness of the issue and a

subsequent change to more sustainable drug use and parasite control management strategies, before overt resistance develops (Sargison and Scott, 2011a). Farm management also plays an important part in the development of drug resistance. Therefore, it is necessary to identify the risk factors associated with presence of resistance on farms in order to investigate any links with the development of anthelmintic resistance. Fluke management practices such as pasture management, the drenching programme and choice of anthelmintics for the flock is very important for sustainable parasite control. These factors also relate to climate change in the UK and this influences *F. hepatica* life cycle and survival of intermediate host snails (Charlier et al., 2011; Sargison, 2012).

Aim of study

The aim of this chapter was to investigate prevalence of resistance to TCBZ in fluke populations infecting sheep in the UK. Two studies were conducted: 1) sheep farms in the county of Cumbria in the North West of England, and 2) sheep farms located in three areas of Britain: South West England, Wales and North East England. The composite faecal egg count reduction test (cFECRT) was used to evaluate the TCBZ efficacy on these sheep farms. In study 2, questionnaires were sent out to the farmers to investigate anthelmintic utilization practices and on-farm sheep management to identify risk factors for drug resistance.

4.2 MATERIALS AND METHODS

4.2.1 Study 1

This study was conducted in collaboration with a farmer's co-operative group, the Cumbria Farmers Network (CFN). The CFN was set up in October 2005 and has over 500 members in Cumbria (http://www.thefarmernetwork.co.uk/about-us/). The farms that participated in this study were selected by the CFN such that selected farms were evenly distributed throughout Cumbria and were representative of all local farm types (hill or lowland) and soil types in the region. Twenty farms were

initially included in the study, but only sixteen farms subsequently submitted samples. Of the sixteen farms, three had pre-treatment FEC below 100 eggs per gram (epg) and were therefore not included in the study. Of the 13 remaining farms, samples were collected pre-treatment (day 0) and post treatment (on day 21). The field work was conducted from August to November 2013.

4.2.2 Study 2

On submission of a proposal and a confidentiality agreement and their subsequent approval, RADAR (Rapid Analysis and Detection of Animal-related Risks) provided details of 750 sheep holdings from the Agriculture census data in from three geographical areas of Britain, South West England, Wales and North East England. The details provided included name, address and flock size and included flocks with more than 200 adult breeding sheep. A sampling strategy, stratified according to county and number of sheep farms according to the database, showed that 126 farms (sample size based on a prevalence of 20%, with 7% precision and 95% confidence) was required. This estimate was based on two previous studies which showed that the prevalence of fluke infection in dairy herds in Britain was 76% (McCann et al., 2010b) and prevalence of TCBZ resistance on sheep farms was 28% (Daniel et al., 2012). In addition 12 study farms from the APHA's SCOPS project also participated in the study. The field work was conducted from December 2014 until April 2015.

Ethical approval for both these studies was obtained through the University of Liverpool's ethical review process (VREC82).

The address list of sheep farmers, obtained from RADAR, was used in order to make primary contact and issue an invitation to join this study. Two hundred and fifty farms in the three areas were selected randomly from the list. Each farmer was sent a pack containing an invitation letter to participate in the study (see Appendix 4.1), a consent form (see Appendix 4.2) and participant information sheet (see Appendix 4.3). Respondents were given the opportunity to read the participant information sheet that explain the purpose of the study and their participation in this survey was voluntary and anonymous. It was also explained that there were no risk and harm to

completing a survey. Respondents were requested to send back the consent form and sign the form as the confirmation that the farmer would like to participate in the study.

It was also requested that samples should not be collected from sheep that had been treated with an anthelmintic for fluke within the past 3 months. Once a farmer sent back the consent form agreeing to participate in the study, their details were given to APHA, York. Staff from APHA then contacted and visited the farm to collect the faecal samples.

4.2.2.1 Questionnaire

The questionnaire was divided into six sections and consisted of 51 questions over nine pages. See Appendix 4.4 for a copy of the questionnaire. The questionnaire was comprised of six sections:

i) The first section included the general information such as names, addresses and had to provide the background details of their respective farms.

ii) The second section was entitled 'About your sheep management'. This part was designed to understand participants' management practices on various elements of farm and flock. There were twenty questions comprised on both close-ended and open-ended questions for participants to describe all the aspect in their farm management that are undertaken to prevent the introduction of liver fluke infection.

iii) The third section focused on liver fluke status of the farm (two close-ended questions and one open-ended question).

iv) The fourth section covered fluke control in regard to the use of anthelmintic product and drenching practices. This part was designed to identify deworming history of the farm as well as investigating how the farmer handled their newly purchased sheep in particular deworming, before being turned out to the pasture.

v) The fifth section of the questionnaire asked about sheep fluke FEC. Participants were asked to describe their fluke control programme by response to: a) the frequency of using FEC, b) conducting any drug resistance test on the sheep, c) if yes, provided results of the test.

vi) In the last section of the questionnaire, questions enquired about fluke control advice that was received by the participants.

To increase the reliability and validity of the questions, the questionnaire was reviewed by three supervisors. They were asked to review the survey design and contents. Further work of developing the questionnaire evolved around the idea of one question for one minute and making the question as clear, short and not time consuming to the farmer.

4.2.2.2 Data collection

Questionnaires were distributed on the first visit to the farm. Staff from APHA left the questionnaire with the farmer and collected it on the second visit 21 days post treatment. Data collection from the completed questionnaire survey were entered into an Excel database (Microsoft 2010) and for each individual farmer record, a numeric farm ID was created. Results of FECs were entered into the same spread sheets. The originals of the completed questionnaires were kept confidentially at the Department of Veterinary Parasitology Office at University of Liverpool.

4.2.3 Parasitology techniques

4.2.3.1 Composite Faecal Egg Count Reduction Test (cFECRT)

The cFECRT was used to establish drug efficacy. Floor faecal samples were collected from 2 groups of 10 sheep on day 0 and immediately afterwards animals were treated with TCBZ (Fasinex) using the recommended sheep dose. Dosage was set for the weight of the heaviest animal in the group; on some farms, sheep were divided into groups according to weight to ensure the correct dose was used. The dosing gun and Fasinex was provided by the project to ensure correct dosing

procedure and was supervised by APHA staff (Sargison and Scott, 2011a). The faecal samples were sent to the University of Liverpool for processing. In the laboratory, the faeces were mixed up with plenty of water to make a faecal slurry. The faecal slurry was then washed through a sieve stack with sequentially smaller aperture sizes mesh (710, 150 and 38 μm). Extra care was taken to prevent overflow through the series of sieves which can result when a large amount of sediment is retained on either of the bottom two meshes. Under running tap water, the faecal slurry was washed until the water running through the sieves was clean. The retained particulate matter containing eggs and debris on the 38 μm sieve was then rinsed into the 500 ml beaker. A simple sedimentation technique was performed. Fifty ml of the sediment was transfer to a petri dish and one drop of methylene blue was added. Eggs were counted under the dissecting microscope using 4 x magnifications. Twenty one days after the first sampling, faecal samples were collected from the same two groups of sheep.

Statistical analysis

The reduction in FECs was estimated on samples obtained before and after treatment with TCBZ by using the following equation:

$$\text{Reduction (\%)} = 100 - \left[\frac{\text{post treatment counts}}{\text{pre treatment counts}} \times 100 \right]$$

Resistance, or TCBZ treatment failure, is declared when the percentage of reduction is less than 90%. A reduction of FEC was calculated using the Microsoft Excel 2010.

Questionnaire survey results were analysed using descriptive statistics (Minitab 17.0 statistical software).

4.3 RESULTS

4.3.1 Prevalence of Resistance to Triclabendazole in Cumbria, North West England.

Twenty farms were identified to take part in the study, four farms did not send samples for analysis and an additional three farms were excluded from the study because their pre-treatment FEC were below 100 epg.

For the remaining 13 farms, the percentage reductions in cFECRT ranged from 0-79% and are presented in Table 4.3.1. Resistance or drug failure, as defined as less than 90% reduction in FEC, was present on all farms with farms G to M, showing a 0% reduction in egg output at 21 dpt. The geographical location of each farm is shown in Figure 4.3.1.

Table 4.3.1 Summary of pre-treatment, post-treatment and reduction (%) in composite faecal egg counts (in 50 gram of faecal samples) in sheep treated with 10mg/kg (Fasinex®; Novartis) for 13 farms.

Farm ID	Total eggs Pre- treatment	Total eggs Post treatment	%Reduction
A	2991	640	79
B	5316	1764	67
C	4492	2380	47
D	4062	2394	41
E	6380	4793	25
F	676	661	2
G	218	497	0
H	391	419	0
I	243	406	0
J	1545	3315	0
K	4376	7947	0
L	6954	13137	0
M	21664	65000+	0

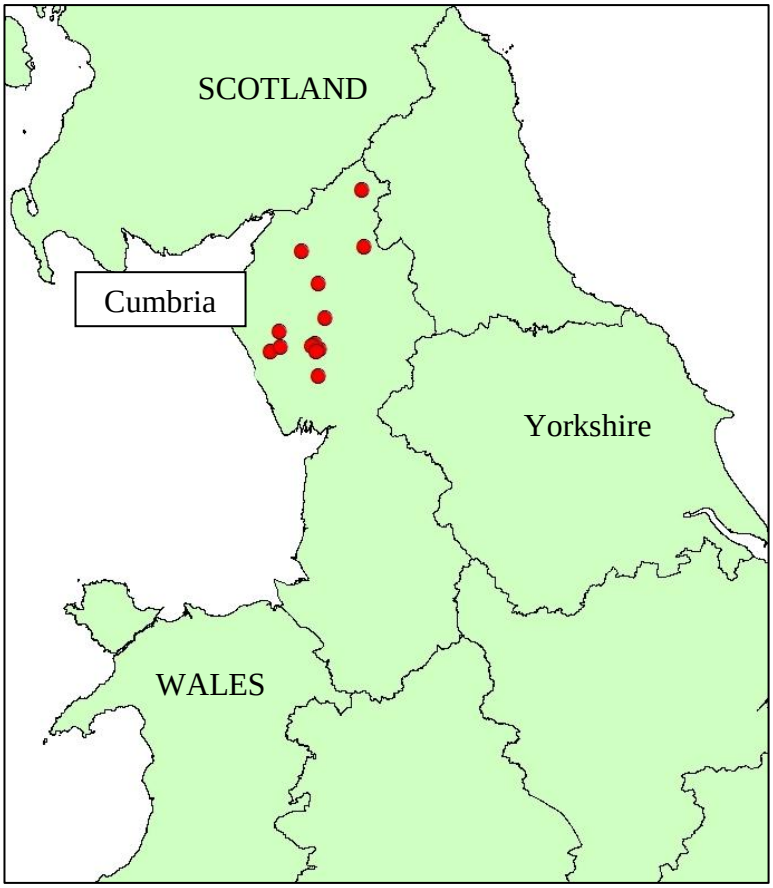


Figure 4.3.1 Map of the North of England showing the location of *F. hepatica* resistance study farms in Cumbria (Red: 13 sheep farms).

4.3.2 Prevalence of Resistance to Triclabendazole in England and Wales.

A total of 250 farms were contacted from three different geographical regions areas of Britain; South West England (100 participant letters), Wales (50 participant letters) and North East England (100 participant letters) to ask if they would participate in the study. Thirty four farmers replied (13.6%) of which 30 agreed to participate in the study (South West England: 16, Wales: 5 and North East England: 13), giving a total response rate of 12%.

Pre-treatment samples were collected from 30 farms and a questionnaire survey conducted to obtain information on historical drug use and other risk factors. In addition, 12 farms participating in a SCOPS project were also sampled. Of the 42 farms, the composite counts were too low to conduct the FECRT on 35 farms. Hence seven farms, 16.7% (95% CI 8-31%) of the sampled farms were used in the drug efficacy study. Of these 7 farms, two were SCOPS farms, one was from Wales

and the remainder were all in North East England. All 7 farms received treatment and second samples were submitted at 21 days post treatment. The pre- and post treatment egg counts and the percentage of reduction for all seven farms are presented in Table 4.3.2. TCBZ resistance was considered as present when the FEC reduction was less than 90%. Figure 4.3.2 shows the location of study farms.

4.3.3 Questionnaire survey responses

All 7 farms returned questionnaires on the second sampling visit (day 21 pt) having met our requirements of pre-treatment FEC above 100 epg. These farms were in lowland areas (1 farm), upland areas (2 farms), hill sheep farms (2 farms) and 2 farms in the upland marginal/hill areas of England (Table 4.3.3). One of the conditions of entry into the trial was that the sheep had not been treated with an anthelmintic for at least 12 weeks before faecal collection. This requirement was important to ensure that the sheep were infected with sufficient liver fluke parasites for the purpose of this study.

The total number of ewes ranged from 350 to 900 on the 7 farms. Among the sampled farms, 57% had cattle on the farm, 14% had cattle and goats whereas another two farms had only sheep (Table 4.3.3). Analysis showed that there was no significant difference (T-test $p = 0.82$) between resistance status and total number of ewes. Due to the small number of farms further analysis on differences between resistance status and farm characteristics was not performed.

Results of management practices for all 7 farms are presented in Appendix 4.5. The survey indicates that sheep were either moved to the current pasture within the last month (farm 1 and 4) or in the last 1 to 3 months (farm 3, 5, 6 and 7). Cattle were reared with sheep on 5 farms and with horses on farm 1. The majority of farmers (6 out of 7) sent their sheep away for winter grazing, and all farms produced their own forage; for example hay, silage and straw. All farmers used several types of fertilizer including commercial fertiliser, manure, lime, slurry and slag. The questionnaire asked respondents to rate the drainage of the grazing field by observation and the results showed that 2 out of 7 rated the drainage as poor, although all farmers except

for farm 1 reported problems with boggy fields. Most farmers except farm 6 and 7 had tried to improve the drainage on their farms. The water sources present on each farm can be seen in appendix 4.5.

The questionnaire survey indicated that Flukiver (closantel) was the most commonly used drugs to treat fluke. Fasinex and Endofluke (both triclabendazole) were also used (Table 4.3.4). Most of the respondents indicated that when calculating the dose of anthelmintic, the weight of the sheep was important. The reasons provided for choosing these products included veterinarian's advice, recommendation by the shop and, less frequently, based on information in magazines or television. The factors that influenced the farmers to use anthelmintics for treating liver fluke included because they were following a fluke programme advised by the vet or animal health officer. It appeared that farmers had their own specific time of treatment as they responded that 'I always fluke at the same times of year'; e.g. after lambing period, September/October/November or December/January, when animals show symptoms such as poor body condition and after scanning. Of these 7 farms, 2 of the respondent farmers reported that the frequency of treatment with anthelmintics was twice per year and 2 other farms applied treatment four times annually. Quarantine drenching to treat new stock against liver fluke, was not applied on these studied farms.

Six farms (85.7%) stated that FECs were never used and one farm used this test to monitor liver fluke infection (Table 4.3.5). The results also showed that no investigations had been conducted on drug resistance previously (based on FECRT and coproantigen diagnostic test) on six farms (85.7%), however the other farm had used FECRT.

Table 4.3.2 Summary of pre-treatment, post treatment and reduction (%) in composite faecal egg counts (in 50 gram of faecal samples) in sheep treated with 10 mg/kg (Fasinex®; Novartis) for 7 farms.

Farm ID	Total eggs Pre- treatment	Total eggs Post treatment	%Reduction
1 (Wales)	2518	13	99
2 (North East)	258	0	100
3 (North East)	1000	107	89*
4 (North East)	2284	1	100
5 (North East)	12155	8947	26*
6 (North East)	210	32	85*
7 (North East)	2552	2008	21*

*indicates farms where resistance is suspected.

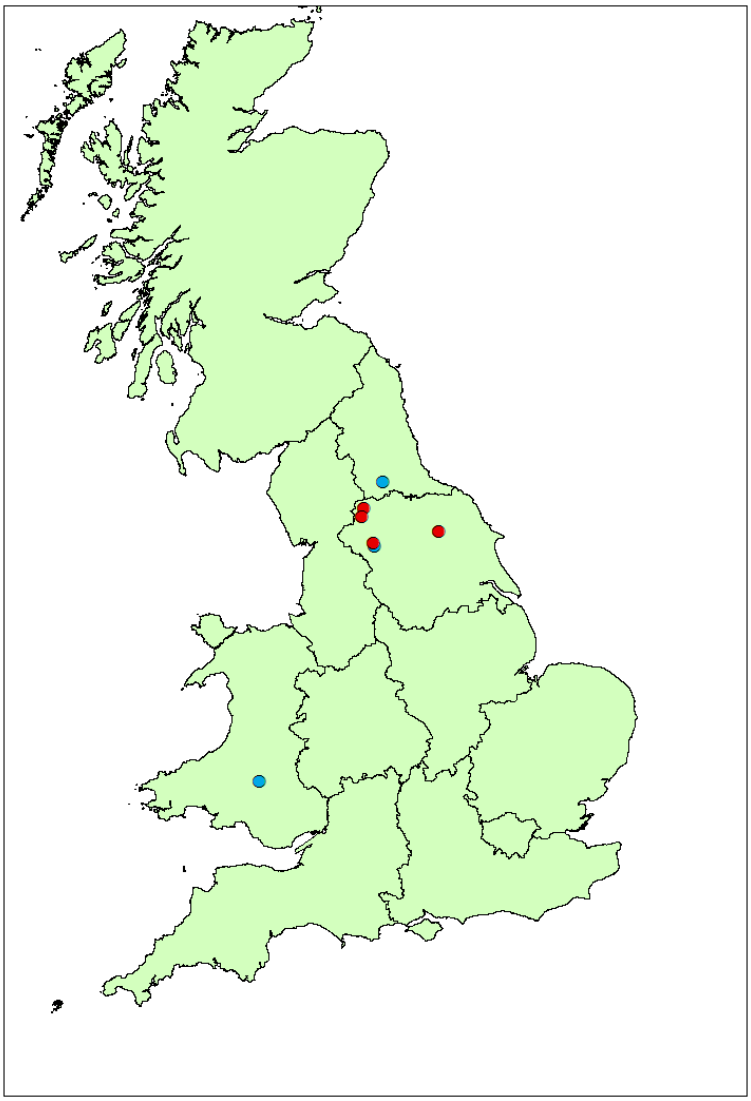


Figure 4.3.2 Map of the Great Britain showing the location of *F. hepatica* resistance study farms (Red: Resistant farm; Blue: Susceptible farm).

Table 4.3.3 Responses of farmers to questionnaire survey on farm descriptors (n=7).

Variable	Level of variable	Resistant (n)	Susceptible (n)
Type of farm	1 = Lowland	1	0
	2 = Upland	1	1
	3 = Hill	1	1
	4 = Upland + hill	1	1
Farm size (acres)	1 = 0-250	1	2
	2 = 251-500	2	1
	3 = 501-750	1	0
sheep numbers (range)	1 = 301-600	2	2
	2 = 601-1000	2	1
Cattle	1 = Yes	3	1
	2 = No	1	1
	3 = Cattle + goats	0	1
Sampling times	1 = January	1	0
	2 = February	2	3
	3 = March	1	0

Table 4.3.4 Responses of farmers to questionnaire survey on sheep fluke control on anthelmintic usage practice on each farm.

Variable	Level of variable	Count/farm	Responses
When last treated	0 = Missing responses	0 = 1/7	14.3%
	1 = By October 2014	1 = 1/7	14.3%
	2 = By November 2014	2 = 1/7	14.3%
	3 = By December 2014	3 = 3/7	42.8%
	4 = By January 2014	4 = 1/7	14.3%
Product used when last treated	1 = Endofluke	1 = 2/7	28.6%
	2 = Flukiver	2 = 3/7	42.8%
	3 =Triclafas	3 = 1/7	14.3%
	4 = Flukanide	4 = 1/7	14.3%
Commonly used drench	0 = Missing responses	0 = 1/7	14.3%
	1 = Flukiver + Fasinex	1 = 1/7	14.3%
	2 = Flukiver + Supaverm + Endofluke	2 = 1/7	14.3%
	3 = Flukiver + Fasinex + Combined cattle/sheep	3 = 1/7	14.3%
	4 = Endofluke + Fasimec Duo	4 = 1/7	14.3%
	5 = Flukiver + Fasinex + Albensure + Endofluke + combined cattle/sheep	5 = 1/7	14.3%
	6 = Flukiver + Fasinex + Trodax + Alverm + Tribex + Supaverm + Rycoben + + combined cattle/sheep	6 = 1/7	14.3%
Treatment frequency (per year)	0 = Missing responses	0 = 1/7	14.3%
	1 = 1	1 = 1/7	14.3%
	2 = 2	2 = 2/7	28.6%
	3 = 3	3 = 1/7	14.3%

	4 = 4	4 = 2/7	28.6%
Quarantine drench	0 = Missing responses	0 = 1/7	14.3%
	1 = Yes	1 = 0/7	0%
	2 = No	2 = 6/7	85.7%

Table 4.3.5 Responses of farmers to questionnaire survey on sheep fluke control on faecal egg count (FEC) on each farm.

Variable	Level of variable	Count/farm	Responses
FEC used to monitor fluke	1 = Yes	1 = 1/7	14.3%
	2 = No	2 = 6/7	85.7%
Previous investigation of drench resistance study	1 = FECRT	1 = 1/7	14.3%
	2 = No	2 = 6/7	85.7%
	3 = Coproantigen Test	3 = 0/7	0%

4.4 DISCUSSION

4.4.1 Study 1

The key aim of this study was to generate some pilot data on the prevalence of TCBZ resistance on sheep farms, as there is only limited information available for England and Wales. Our data confirms the presence of TCBZ resistance on sheep farms in Cumbria, which was detected on each of the 13 farms included in the study, 8 of which failed to show any reduction in egg counts, demonstrating a high level of resistance to this anthelmintic. This finding supports the results of drug efficacy trials in sheep conducted by Daniel et al. (2010), who confirmed TCBZ resistance on 6/13 farms in South West Wales and one farm in South West Scotland. These results are in agreement with anecdotal evidence from farmers and the APHA suggesting that TCBZ resistance is present on sheep farms in the UK.

Treatment of susceptible isolates of liver fluke with TCBZ in experimentally and naturally infected sheep can result in reductions in FECs of between 96.5% and 100% (Rapic et al., 1984; Turner et al., 1984; Wolff et al., 1983). These findings are supported by field trials which demonstrated that the drug was fully effective with 100% reduction in egg output after treatment (Maes et al., 1990; Stansfield et al., 1987). This is in contrast to the situation seen in the field when TCBZ resistance in sheep was detected years later- the first case was reported in Australia in 1995 (Overend and Bowen, 1995) and was followed by further reports of drug failure in several other countries (Gordon et al., 2012b; Mooney et al., 2009; Sargison and Scott, 2011b). Recently, cases of TCBZ-resistant *F. hepatica* were recorded in South West Wales and South Wales Scotland, with egg count reductions of <95% (Daniel et al., 2010), suggesting that TCBZ resistance may be wide spread in Great Britain. This agrees with our results since TCBZ resistance was also detected on all 13 sheep farms tested in Cumbria. However, study by Daniel et al. (2012) failed to find TCBZ resistance in England, where ten farms were sampled, including one in the Cumbria area. In the present study, the most important finding is the high prevalence of TCBZ resistance, with a degree of resistance evident on all farms. On seven of these, there was an increase in egg numbers after treatment indicating that TCBZ was

ineffective at controlling neither adults nor immature *F. hepatica* was removed. As for other farms, the drug was not fully effective against all stages of flukes.

The cFECRT was used in the present study as a diagnostic tool and this test has been validated in a previous study by Daniel et al. (2012). The cFECRT estimates TBCZ efficacy by comparing FECs from animals before and 21 dpt by using two groups of 10 animals each. It has been suggested that <95% reduction in FEC is indicative of resistance (Daniel et al., 2010; Flanagan et al., 2011a) and this figure is also suggested by the WAAVP guidelines for the determination of drug resistance in nematodes (Coles et al., 1992). In this study, we chose to use a less stringent cut-off of <90% reduction in cFECRT as the threshold for TCBZ resistance.

In estimating TCBZ efficacy in *F. hepatica*, a threshold of 100 epg was identified as a pre-requisite for the test to be conducted (Daniel et al., 2012). In WAAVP guidelines, they recommend that for nematode infection in sheep, only animals with pre-treatment egg count > 150 epg, using the modified McMaster method, should be included in the study (Coles et al., 2006). Using the McMaster method, a multiplication factor is applied, so 1 egg counted is equivalent to 50 epg. In the TCBZ FECRT used here, actual eggs are counted to give the final count. For the CFN study, three farms had counts below the 100 epg threshold and were excluded from the study. In comparison for the second study, only 7/42 farms had counts of above 100 epg.

The biggest challenge in controlling fasciolosis is that TCBZ is the only drug on the market that has a high efficacy against young immature flukes (Fairweather and Boray, 1999b). Excessive use of TCBZ has led to an increase in the incidence of anthelmintic resistance and results from this study highlight the severity of the problem we are facing now. It is not surprising that the widespread use of this drug appears to have selected for TCBZ resistant fluke populations on the farms sampled in this study. There are a number of factors that are likely to have contributed to this occurring.

4.4.2 Study 2

Of major concern, TCBZ resistance has now been reported in the UK (Gordon et al., 2012a; Hanna et al., 2015; Mooney et al., 2009; Sargison and Scott, 2011b) and other cases confirmed worldwide (Moll et al., 2000; Ortiz et al., 2013). The Department of Veterinary Parasitology at University of Liverpool has monitored the anthelmintic resistance status of *F. hepatica* on sheep farms for many years by comparing pre- and post- TCBZ treatment FECs and is revealing the growing development of resistance of *F. hepatica* to TBCZ across the UK. Most flukicidal drugs on the market (e.g. closantel, nitroxynil) are effective in treating chronic fasciolosis, however TCBZ remains the drug of choice due to its activity towards immature flukes (which cause acute fasciolosis) in sheep. Any evidence of TCBZ resistance in liver fluke populations on farm would make TCBZ treatment inadvisable and would remove one of the key anthelmintics available to control acute fasciolosis. Therefore, estimation of anthelmintic efficacy using cFECRT was conducted in this study to document the anthelmintic resistance status of sheep farming in three different regions of Britain. These regions were selected as representing sheep rearing areas of the country, but where the prevalence of infection is likely to vary due to the prevailing climatic conditions. Thus Wales has a high rainfall and is known to have a high prevalence of *F. hepatica* infection, South West England has a moderate prevalence of infection and North East England has a drier climate and a lower prevalence (McCann et al., 2010a).

Results of the present study showed that compliance in participating in the study was relatively low (12%) and 35/42 farms sampled had fluke egg counts below the 100 egg threshold. This was much lower compared to Study 1 (Cumbria sheep farms). This may be due to the warmer, drier summer of 2014 (http://www.nadis.org.uk/), leading to a lower fluke challenge (Skuce et al., 2014) or because awareness of fluke is high as a result of the massive losses associated with fasciolosis following the wet year of 2012 (SCOPS, 2012).

The evaluation of CFECRT showed that TCBZ was ineffective against *F. hepatica* infection on four out of seven farms that had FEC of 100 or above. Two farms (farm 5 and 7) had %FECR of 26% and 21% respectively whereas the other two farms had %FECR of 89% and 85%, suggesting that the population of parasites showed partial

resistance. These findings suggest that even in an area where the fluke challenge is comparatively low, resistance is still a threat.

In the present study, results of the questions on use of drenches confirmed that six out of seven farms used TCBZ to treat liver fluke in sheep. The use of closantel (Flukiver) and nitroxynil (Trodax) are other treatment options for sheep. Closantel and nitroxynil have 91 to 99% efficacy against the adult flukes and were fully effective against TCBZ-resistant flukes with the high fluke burden in sheep flocks in Northern Ireland (Hanna et al., 2015). Another study also showed that these drugs have 100% efficacy against *F. hepatica* in a naturally infected hill sheep flock in the west of Ireland (Mooney et al., 2009). Our survey also confirmed that the farmers have used more than one different type of drug on each studied farm. Typically each farm uses specific brands of anthelmintic drugs (see Table 4.3.4). Whilst TCBZ should be reserved for the treatment of immature fluke in sheep, it is likely that farmers used various brands of drug without realizing it is the same active compound (TCBZ). Over reliance or repeated TCBZ treatment may occur due to this confusion; farmer may change one brand to another, thinking that they are alternating the active product. This means that the emergence of drug resistance in *F. hepatica* in sheep may occur due to farmers' lack of knowledge about the active ingredient in different products. Our study has confirmed that 4 out of 7 farms studied showed resistance to TCBZ treatment; however, several studies have reported that lack of efficacy of the drug is not necessarily associated with the actual resistance. It is appears that the problem may be related to, for example, underdosing or dependent on the efficacy of the various brands of TCBZ drug available (Fairweather, 2011a; Sargison, 2012; Sargison and Scott, 2011a; van Dijk et al., 2015). It is highly recommended that these drugs should be used strategically to combat infection, by knowing the status of the farm; e.g stage of infection present and resistance status. TCBZ is used for its high efficacy against both immature and mature flukes, however if over used for long period of time there is a strong potential for the development of resistance to the drug within the fluke population on a farm. Our survey also showed that veterinarians are an important source of information for farmers regarding fluke control strategies and are usually involved in the decision of the product use.

Time after last drench is very important in determining when to collect faecal samples. In our study, we requested farmers should only send sheep faecal samples that were taken at least 12 weeks after the last drench. However, two farms confirmed that the pre-treatment faecal samples were collected at 5 and 9 weeks after the sheep were last treated for farm 3 and 5, respectively. Egg production capacity of *F. hepatica* is very high and an individual fluke may produce 5,000 to 20,000 eggs per day (Happich and Boray, 1969b). The pre-patent period for *F. hepatica* is about 8 to 12 weeks (Andrews, 1999). These parasites need this period of time to be at full egg laying capacity and by this time young fluke will have matured. In a previous study, groups of Wistar rats were infected with 20 metacercariae by stomach tube (Valero et al., 2006). The authors demonstrated that the pre-patent period depended on the infection level and the pre-patent period decreased when the burden of flukes increased. In contrast, experimental studies on sheep infected with 200 metacercariae have shown that the pre-patent period was 63 days and by increasing number of metacercariae (infected with 2000 metacercariae) the pre-patent period was prolonged, reported at 13 to 15 weeks after ingestion (Boray, 1969). Therefore, 12 weeks after treatment is the time to get a more meaningful interpretation of parasite burden from FECs. Selection bias can be minimize by ensuring this period of time of last drenched for the parasites to mature and produce eggs. Other factors include time of year, climate and grazing strategy. Interestingly for farms 3 and 5, despite the treatment 4 and 9 weeks previously, both had an egg count above the threshold and both had evidence of resistance in the FECRT.

Identification of risk factors for *F. hepatica* infection may help in developing control strategies in sheep and these can be assessed through the questionnaire survey. However, major problem that we faced is the limited sample size. The sample size was too small because we only received back seven completed questionnaires from those farms that had the second sample collected. Questionnaires were not obtained from farms who had FEC below the 100 egg threshold. Our aim at the outset was to obtain data from 126 sheep farms around the UK, but we did not achieve this and therefore it is difficult to detect statistical differences between the interaction between TCBZ resistance status and various variable responses. However, with the seven replies were adequate to give an overview of fluke farm practices.

This survey showed that 5 out of 7 farms grazed cattle on a pasture, before and after sheep were grazed. Such a practice could potentially facilitate poorer liver fluke control since the same parasite can infect both sheep and cattle. Whilst for nematode infections it is good to rotate with other susceptible livestock such as cattle and horses, this is not the case for fluke. Sharing grazing with sheep at the same is potentially hazardous as cross infection is likely to happen and is associated with a risk of transferring resistant strains of *F. hepatica*. Other studies have also identified co-grazing sheep is a risk factor for infection in cattle and vice versa and wild life has been implicated in acting as a reservoir of infection (Bennema et al., 2009; Charlier et al., 2011).

Results from the survey indicate that 85.7% (6/7) of the respondents let the sheep to go to other farms for winter grazing. This approach has been practised by farmers for many years and allows sheep to graze lowland pasture protected from extreme winter weather. Of these six responses, three of the farms demonstrated resistance to TCBZ. Infective stages of *F. hepatica*, metacercariae, remain viable on the pasture for several months and can survive over winter (Boray and Enigk, 1964). However, milder conditions may increase the risk of *F. hepatica* metacercariae surviving on pasture, meaning that sheep which are transferred to lowland pastures during winter may be at higher risk of picking up infection. Also, sheep which graze on other farms may be exposed to a more diverse population of *F. hepatica* than those sheep which remain on a single farm, and may potentially bring these parasites, some of which may be TCBZ resistant, back to their own farm. In a study in Northern Ireland, all flocks with high levels of fluke burden were found from lowland areas, whereas all the farms in upland areas had lower burden (Hanna et al., 2015).

Our survey showed that only one farm used FEC to monitor fluke on their property and this farm had previously had a FECRT done, but evidence suggested that the fluke population on this farm was susceptible (farm 2).

Other factors should be taken into account when controlling *F. hepatica* infections on sheep farms including optimal use of anthelmintics. It has been suggested that development of anthelmintic resistance can be slowed, if the frequency of anthelmintic treatment in sheep is minimized (Taylor and Hunt, 1989). Our results have shown that sheep on farm 1 were drenched once per year, farm 3 and 6 twice

per year, farm 4 three times per year and farm 5 and 7 four times in a year. Based on the resistance status of these farms, farm 5 and 7 drenched the flock more frequently compared to other farms and showed the highest level of resistance. However, anthelmintic resistance can also be selected at lower treatment frequencies if the same drug is used for over many years. To control infection and delay the development of resistance, choosing the right drug product and the times of year to treat treatment should depend on the disease pressure on the farm especially if a flock continues to graze on heavy contamination pasture. Strategic treatment times may also be given according to the weather conditions and the advent of milder, wetter weather. TCBZ can be used for autumn treatment if there is no evidence of resistance whereas for farms with evidence of TCBZ resistance, farmers are advised to use closantel and nitroxynil to treat the flock. This strategic control programme based on fluke forecast - closantel is used during the high risk autumn treatment whereas nitroxynil or albendazole is used when predominantly adult flukes are present.

Underdosing due to body weight estimation is a critical issue that needs to be addressed in farm livestock (Sargison, 2012; van Dijk et al., 2015). Lack of proper body weight estimation during drenching may lead to under dosing and hence emergence of anthelmintic resistance. Results from the questionnaires have shown that nearly 2/3rd of the farmers used scales to estimate body weight. Results from this survey indicate that underdosing of sheep may not occur and that farmers are well educated about weighing before drenching. Furthermore, farmers also need to monitor the dosing gun as faulty equipment can cause incorrect dosage given to the sheep. Underdosing may also arise following incorrect storage of the drugs; when drug are not stored properly, this may impact the efficacy of the drug, thus should not be used in sheep (Sargison and Scott, 2011a). These factors were not an issue in our study as we provided the drug and the dosing gun and the dose was given under the supervision of APHA staff. However, the survey only considers a single time-point snap-shot of practices; correctly calibrated dosing guns and properly stored drugs were provided and dosing was supervised, no observation of dosing or examination of equipment previously used for anthelmintic treatments was available and so the accuracy of these drug administration's cannot be commented on.

Therefore, it must be kept in mind that underdosing could easily have occurred in previous years and would therefore have selected for resistance.

The present study investigated farm practice after the introduction of new stock which may affect the spread of anthelmintic resistance. Giving a quarantine drench to all new stock could prevent the introduction of resistance *F. hepatica* isolates onto a sheep farm (Sargison, 2012). Farmers are given advice that they should purchase sheep from the flocks of known liver fluke status when restocking. All of the respondent farmers said they did buy in new stock for their properties which included ewes and tups. However, none of the respondents performed quarantine drenching to these newly purchased sheep within 48 hours of arrival on the farm. This indicated that these farms were at risk of importing sheep carrying resistant populations of fluke. There are several recommendations available to farmers to reduce the risk of introducing in resistance strains of liver fluke to the farm: the paddock used as quarantine should be rested for a year; e.g. not be grazed until pastures have been cut for silage or hay or the land ploughed. It has been suggested that sheep introduced onto a farm should be treated using a sequentially administered combination of a benzimidazole and a salicylanilide derivative drug (Sargison, 2012). Given the acknowledgement of development of TCBZ resistance around the UK, this is likely to be effective way preventing TCBZ resistance to build up. Quarantine drenching is an important aspect of an effective parasite management plan and failure to practice this may increase the emergence of resistance on the sheep properties.

Results from our survey showed that all the respondents produced their own forage. There was a variety type of forage including hay, silage and straw. However, there is uncertainty about the survival of the metacercariae of *F. hepatica*, on forage, particularly silage or hay. Work by Boray et al. (1964), suggested that metacercariae will survive on hay with the relative humidity of >90% when stored at a low temperature. Another study, however, found that metacercariae survived for two to three months when placed in hay at low temperature and low relative humidity (Enigk and Hildebrandt, 1964). Other studies in the UK also demonstrated that approximately 50% of metacercariae encysting on herbage in September will survive in winter conditions (Ollerenshaw, 1967). However, it is not clear if metacercariae

will survive in hay under normal farm conditions in the UK. Therefore, feeding the animals with hay or silage during winter is probably not a potential source of liver fluke infection. In contrast, freshly cut grass from the high risk-flukey pasture is not recommended.

Fertilisers were commonly used on fields grazed by sheep (commercial fertilizer, manure, slurry, lime and slag), which can have an effect on soil nutrients, as well the vegetation. This could provide better habitats for the snail, providing the water plants and mud required by the snail that are rich in organic matter, due to application of the fertilisers and calcium which is needed for shell development. Work from Rondelaud et al. (2004) have demonstrated that lower calcium ion concentration (723 mg/l) present in waters induces slower growth of infected snails, and limits shell height to 8 mm. The authors also concluded that lower calcium ion content would create less favourable conditions for the development of redia within the snails. This means that calcium in snail habitat is needed and is a very important factor for the development of the liver fluke life cycle. In addition, our survey indicates that 6 out of 7 farms (except for farm 7) were under the Environmental schemes; therefore have strict guidelines for fertiliser policies. The schemes also encourage the creation of wetlands, important for migratory birds and invertebrates, but which can increase the risk of *F. hepatica* (Pritchard et al., 2005). This also includes the introduction of *G. truncatula* to the ecosystem (van Leeuwen, 2012).

It is of great importance that the flock is moved to the lower risk pasture in order to not to exposure the sheep to *F. hepatica* infections. This survey showed that 85.7% (6/7) of the farms had a boggy field at some part of the year on their properties. This means that using a temporary fencing around the high flukey-risk areas is highly recommended. Our results also showed that all the farms bordered wet areas including ponds, rivers, streams and marshy areas. These conditions will also favour the life cycle of the liver fluke through propagation of intermediate snail host, *G. truncatula* (Charlier et al., 2011). By controlling the snail habitat (wet areas) and reducing numbers of snails through effective drainage could reduce fluke risk on the farm but is not possible for those farms in Environmental schemes (Charlier et al., 2011; Sargison, 2012). Studies have shown that snails located at the extremity of drainage furrows in meadows had a higher prevalence of *F. hepatica* infections

compared to snails colonizing lower parts of the drainage ditches or river bank (Rondelaud and Dreyfuss, 1996). Farms 5 and 7 rated their properties as having a poor drainage where the sheep were currently grazing. The survey results indicated that methods used to improve the drainage were harrowing, rolling, sub-soiling, aeration and maintenance the existing drains. It is likely that the life cycle of *F. hepatica* can be disrupted if drainage is improved. However, on farms 6 and 7 no drainage improvement methods were applied. Installation of water troughs also play an important factor to reduce the exposure of infections of sheep on the farm (Charlier et al., 2011). The main purpose of this approach is to avoid wet areas building up round the watering places. Majority of the farmers had several main water sources on their farms including troughs, piped, stream and spring. It is the best for the flock to graze fluke free areas as snail intermediate hosts can breed around water contaminated by infected sheep faeces. Clearly, water resources, particularly irrigation systems, can contribute to the introduction and spread of liver fluke infections.

Last but not least is regarding the surveys in this study. It is believed that giving feedback and useful information to the participants will encourage them to participate further in future projects and other research related activities. Data for each farm was fed back to the farmers as soon as the egg counts had been completed and an advice sheet sent with the results.

In conclusion, the present study has provided evidence of TCBZ resistance in *F. hepatica* in sheep in the UK. The cFECRT is useful diagnostic tool in detecting resistance. Although, this is small scale study, and insufficient data was collected to ascertain the prevalence of triclabendazole in different regions of Britain, the survey findings highlighted the fluke control measures and fluke management adopted by farmers at the present time. The worrying sign of TCBZ resistance is now more severe than is commonly recognized. In future, farmers should put a hand together with the veterinarians, meetings and training to gain more knowledge on various aspects of stock management and control strategies for the flock. A better understanding of the best use of anthelmintics can effectively slow down the spread of anthelmintic resistance.

CHAPTER 5
General Discussion

The chapter summarises the research objectives and the significant findings and conclusions of the study. Recommendations related to this study and future research regarding the issue are also addressed.

Liver fluke infection is typically highly prevalent in sheep in the UK and recently there has been a significant rise in both acute and chronic fasciolosis cases. It is a serious production-limiting disease in sheep and cattle due to mortality, reduction in milk and meat production, secondary bacterial infection, high-cost of anthelmintic treatment and condemnation of livers at slaughter (Garcia et al., 2008; McKenna et al., 2002; Sargison and Scott, 2011b; Schmidt and Roberts, 2005). Previously, East Anglia and South-East Scotland were regarded as low-risk areas for fasciolosis, however due to changing climatic conditions, specifically warmer and wetter springs and summers and milder winters, there has been an increased fluke prevalence in livestock throughout the UK (Kenyon et al., 2009; Pritchard et al., 2005; Van Dijk et al., 2010). Over the past 10 to 15 years a number of cases of liver fluke infection were reported (see Figure 1.2) with evidence of geographical distribution (from west to the east) and different temperature and rainfall patterns (Fox et al., 2011). Unusually wet summers between 2007 and 2012 have led to the 2012/2013 liver fluke season with high fluke burdens on pasture which resulted in outbreaks of acute fasciolosis (SCOPS, 2012). The fasciolosis risk depends on the distribution and numbers of the intermediate mud snail hosts (*Galba truncatula*). These snails survive well in shallow water, ditches, boggy field, marshy environment and banks of slowly-moving streams but not in the standing water (Frömming, 1956). In addition, larger bodies of semi-permanent water can provide suitable habitats for other lymnaeid snails, e.g. *Radix peregra*, *Lymnaea stagnalis* and *Galba glabra* that can also act as intermediate hosts for liver fluke (Relf et al., 2009). In years of high-fluke risk with the changing weather patterns favourable for snail development and establishment of snail habitats the number of snails on pasture in the UK has the potential to rapidly increase; increasing the risk of fasciolosis.

The epidemiology of fasciolosis continues to evolve in the UK in response to the changes in the patterns of anthelmintic usage and the development of anthelmintic resistance (Kenyon et al., 2009; Pritchard et al., 2005; Sargison, 2012). One of the most effective fluckicide drugs in controlling fasciolosis is TCBZ, which belongs to the benzimidazole family; TCBZ has high efficacy against immature (from as early as 2 dpi) and mature adult fluke (Fairweather and Boray, 1999a). However, reports of TCBZ resistance in fluke populations have been reported in a number of countries due to over-reliance on this drug (Alvarez-Sanchez et al., 2006; Daniel et al., 2012; Gordon et al., 2012a; Moll et al., 2000; Mooney et al., 2009; Ortiz et al., 2013; Overend and Bowen, 1995). Furthermore, infections of liver fluke in humans are a significant problem in both tropical and subtropical regions, even including developed countries (Mas-Coma et al., 2009). Recently, a case of TCBZ-resistant *F. hepatica* in humans was reported in The Netherlands (Winkelhagen et al., 2012). Despite several treatments with TCBZ, the drug showed no efficacy in the patient, thus highlighting a serious zoonotic threat posed by liver fluke infection, particularly with resistant parasites (Winkelhagen et al., 2012). Other cases in humans also have been reported in Peru, Bolivia, Egypt, Iran, Puerto Rico and Portugal (Mas-Coma et al., 1999a).

Given these circumstances, it is important to monitor changes in the prevalence of this disease and to study the important issue of TCBZ resistance. There are numerous anecdotal and a smaller number of confirmed reports of poor TCBZ efficacy in both sheep and cattle. This PhD study has therefore focused on an evaluation of diagnostic tests that are available to detect early infection of *F. hepatica* in sheep and to identify the most appropriate diagnostic test for detecting TCBZ efficacy. In the second part of the study, we explored ways of making the composite FECRT (cFECRT) method for detecting TCBZ resistance in the field, more user friendly. The final aim of our study was to determine the current status of TCBZ resistance in *F. hepatica* on sheep farms in Britain using the cFECRT.

5.1 Sensitivity of coproantigen ELISA compared with FEC and PCR assay

At present, methods for detecting infection with liver fluke and monitoring the efficacy of anthelmintic drugs, at the farm-level comprise FEC/FECRT, coproantigen ELISA and PCR. The cELISA in particular, developed by Mezo et al. (2004) may be very promising as this test can detect pre-patent infections and has been shown to be highly specific and sensitive for the diagnosis of acute and chronic *F. hepatica* infections in sheep.

In Chapter 2, we compared these three diagnostic tests, ITS2 PCR, cELISA and FEC for their ability to detect *F. hepatica* infection in experimentally infected sheep and to determine drug efficacy in a controlled efficacy test. Comparison of the performance of the cELISA, FEC and PCR following TCBZ treatment of sheep experimentally infected with a TCBZ susceptible isolate of *F. hepatica* provided a great opportunity to identify their relative merits. The results from the present study have confirmed findings by others, that cELISA (BIO K 201, BIO-X Diagnostics, Belgium) is more sensitive than FEC at early detection of liver fluke infection in sheep. Coproantigens were first detected at 5 weeks of infection, and all sheep were positive by 8 wpi. In contrast, eggs were first detected in faeces at 7 weeks of infection, two weeks later than *Fasciola* coproantigens. Previous studies have found similar results in experimentally infected sheep (Flanagan et al., 2011a; Flanagan et al., 2011b; Mezo et al., 2004). In terms of detecting infection the cELISA is the most sensitive and is capable of detecting immature fluke around 5-6 weeks of age (see Figure 2.3.1). Although a number of PCR protocols claim a greater sensitivity (Martinez-Perez et al., 2012; Robles-Perez et al., 2013) it was not possible to reproduce these published results. It is difficult to provide an explanation for this failure as even faecal samples late in infection, where eggs were clearly visible as a source of fluke DNA, did not produce a positive PCR reaction. Similar observations have been made by other research groups (Skuce et al., personal communication) and it suggests that if the PCR is not capable of detecting fluke DNA in an experimental system it is of little value as a diagnostic in a field setting. The FEC performed largely as expected in the experimental trial and highlighted its value as a diagnostic for patent fluke infection.

These experimental infections highlight that the cELISA may be a better indicator of TCBZ failure. However, in natural infections, the cELISA does not always perform quite as well. This was seen in Chapter 3 in field study with naturally infected sheep. This study showed the sensitivity the commercial BIO K 201 ELISA after drug treatment is a sensitive test, however inconsistent results comparing the cELISA and FECs were obtained. Similarly under field conditions, Kajugu et al. (2015) showed that animals that had low but positive FEC results (1-7 epg) were not always positive for cELISA; only 10 of 36 animals were positive.

In contrast, other studies demonstrated that many more animals gave positive cELISA results than FECs (Hanna et al., 2015). It could be that the cELISA may be able to detect the presence of immature flukes in the bile ducts, or that the cELISA continues to detect coproantigen released from disintegrating fluke (Flanagan et al., 2011a; Flanagan et al., 2011b). The results in chapter 3 clearly showed a big discrepancy between FEC and cELISA compared to chapter 2. It would be interesting to have liver fluke burden of naturally infected sheep, to better understand the sensitivity of cELISA and support the findings of the present study. One of factors that might explain the difference between experimental studies and natural infection is that experimentally infected sheep are given a single high dose of metacercariae; for example, in our study 200-215 metacercariae were used to infect the sheep. In comparison, sheep that are exposed to continuous low levels of infection in the field may give a different diagnostic value, both on cELISA and the FEC. Another reason may be that experimental sheep were housed indoors throughout the study and were therefore not exposed to infection with other parasites e.g paramphistomes whereas naturally infected sheep are at pasture and are exposed to numerous potential parasitic infections. The FEC is highly specific as distinction can be made between different species of egg. However the cELISA (MM3 assay) is also reported as highly sensitive (100%; Mezo et al., 2004) and specific for *F. hepatica* infections: studies have confirmed that there was no cross-reactivity to paramphistomum, coccidian and/or gastrointestinal nematodes (strongyle-type and *Nematodirus* spp.) under field conditions (Gordon et al., 2013; Kajugu et al., 2015). This suggests it may be more of a sensitivity issue. What is evident is that the coproantigen detection method will be useless if the test fails to meet the required level of sensitivity in field-based studies and much further evaluation is required.

The cELISA was not tested on a large scale here and it would be beneficial to compare the coproantigen detection methodology to the cFECRT in a large scale field-based study.

After TCBZ treatment we observed a significant reduction in egg counts even when liver fluke survived TCBZ treatment. Live fluke (ranged from 1-13) were recovered from five out of six sheep at 10 dpt. The presence of flukes which had survived the treatment suggests variability within this population, which was a recent field isolate. The efficacy of TCBZ against this isolate of *F. hepatica* was 97% with $p<0.001$ with reference to the untreated group. It has been claimed that cELISA BIO K201 has a very high sensitivity that can detect as few as one adult fluke (Mezo et al., 2004), this result was not supported in the present study. One fluke was recovered from 2 sheep but only one sheep positive by cELISA and the other was sheep not. Both sheep were positive by FEC. These observations may suggest that capture of coproantigen by this diagnostic test may give false negative results. Similarly, Gordon et al. (2012b) found false negative coproantigen results with naturally infected sheep in Scotland, 5 sheep with 2-6 fluke burdens. One possible reason is that the presence of coproantigen level in faeces may be irregular, e.g in sheep harbouring one fluke burden and this is not necessary coinciding with FEC positivity. This scenario just like FECs, variation in daily egg output of sheep.

Several issues have been raised concerning fluke eggs which may remain trapped in the gall bladder of the sheep after adult *F. hepatica* is eliminated after treatment (Fairweather, 2011b) or the disintegration of dead flukes which continue to produce coproantigen and release it to the faeces (Hanna et al., 2010). However our study and that of others (Flanagan et al., 2011b; Hanna et al., 2015; Hutchinson et al., 2009) have all shown that, despite the presence of low numbers of eggs, that the %FECR for a susceptible isolate is >95%, although for field studies, several authors suggest that complimentary methods, both FEC and cELISA should be used (Hanna et al., 2015; Kajugu et al., 2015). Finally it would have been interesting to note what difference it would have made if the experimental study in chapter 2 had been performed at >14 days post TCBZ treatment, as the effect of retained eggs or coproantigen should have been reduced at this timepoint.

5.2 Improving the design of cFECRT and evaluating the interval between treatment and post treatment sampling

Undoubtedly improving the delivery of timely and efficient diagnostics for TCBZ resistance to farmers would result in greater success in treatment of infected animals using effective drugs. Hence in chapter 3, we conducted a study to improve the design of the cFECRT to question the need to re-sample the same sheep and retaining a 21 day post treatment interval. Firstly, we determined whether it was necessary to sample the same 20 sheep pre- and post treatment, or if two random groups of 20 sheep could be sampled for the two counts. The bootstrap analysis showed that the same 20 sheep had to be sampled pre-treatment and at 21 dpt because of the variation in egg counts within a population of sheep. Secondly, the cELISA and FEC were compared on individual samples and composite samples from 20 sheep on five farms. Faecal samples were collected prior to treatment, 7 and 21 dpt to determine the optimum time of collecting the post treatment sample. A comparison revealed that the individual cELISA values were more informative than the composite cELISA values for the detection of infected sheep (see Table 3.3.3 and 3.3.5). The cFECRT test provides more information about the extent of resistance within a population by giving a %FECR; the cELISA, both individual results and composite result, could only be considered positive or negative. This study also showed that the cFECRT was more accurate if the second sample is collected at 21 dpt, and can help avoid false positive results compared to FEC reduction at day 7 pt. This is due to the issue of fluke eggs that trapped in the gall bladder after successful drug treatment and removal of fluke from bile ducts (Flanagan et al., 2011a; Flanagan et al., 2011b; Sargison, 2012).

Only one of the five farms that participated in this study had FECs that were high enough to conduct the analysis. Also the results suggested that the fluke on this farm were resistant. This limited the amount of analysis that could be done, a bigger study on more farms would strengthen the conclusions from this study, that the cFECRT is a useful field test to detect drug failure.

5.3 TCBZ resistance prevalence studies

Evaluating the cFECRT in this chapter, has led to the conclusion that it is the optimum test to use in a wider field study to determine the prevalence of TCBZ resistance in the UK. Given that we do not have a clear picture of TCBZ resistant in England and Wales Chapter 4, describes a study to establish the prevalence of TCBZ resistance in sheep in England and Wales. This was conducted with a view to identifying factors influencing the development of TCBZ resistance. Preliminary studies in England and Wales showed that TCBZ resistance was evident on 7 out of 25 farms analysed (Daniel et al., 2012), however those farms were not randomly selected for that study. Therefore we set out to conduct a study to ascertain the prevalence of TCBZ resistance in sheep in England and Wales, and the study was performed in 2 phases. In the first phase, all 13 farms, in the county of Cumbria showed evidence of drug failure. In addition, on 8 of these 13 farms, there was no reduction in egg output post treatment, thus indicating a high level of drug failure on those farms. Even given this small number of farms, the findings are disturbing and particularly concerning as *F. hepatica* infection pressure is high. Despite several flock health plans to reduce the spread of anthelmintic resistance in sheep, these efforts do not seem to be very effective. Therefore, it is very important for the farmers to follow advice from veterinarians including avoiding frequent usage of TCBZ.

In the second phase, a survey of TCBZ resistance was conducted in three regions of Britain; North East England, South West England and South Wales. A questionnaire was included to identify risk factors associated with drug failure. This study had a poor response rate; although 250 farms were contacted, only 30 farms took part in the study. Of those 30 farms, together with 12 from the APHA's SCOPS study, only 7 farms had pre-treatment counts of 100 epg or higher. Evidence of TCBZ failure was observed in 4 of those 7 farms, all from North East England. There were too few results from the questionnaire to identify risk factors for resistance. We can conclude from these results that TCBZ resistance is common on sheep farms in some parts of the UK. This study identified hotspots of resistance in Cumbria and North East England, and other studies showed extensive resistance in Wales. However further investigation is needed to establish a national prevalence.

In an effort to maintain a healthy sheep flock, together with controlling economic losses due to fasciolosis (Sargison and Scott, 2011b) farm management strategies might help farmers to have a highly effective long-term control programme for liver fluke infection in sheep and in the emergence of anthelmintic resistance. These include the frequency of drenching and the choice of product.

Whilst TCBZ should be reserved for the treatment of acute infection in sheep, farmers are advised to avoid using the same class of anthelmintic drugs every year so that the longevity of the compounds will be prolonged. Another important factor which is considered to increase the selection for anthelmintic resistance is the administration of correct drug dose (Sargison, 2012). In this survey, nearly 2/3rd of the farmers used scales for estimation of body weight, thus suggesting that underdosing due to inaccurate judgement of sheep bodyweight did not occur. However, farmers also need to monitor the dosing gun as faulty equipment can cause incorrect dosage given to the sheep. Underdosing may also arise if drugs are not stored properly or if they go out of date. There are extensive industry guidelines from organisations such as SCOPS (sustainable control of parasites of sheep) which are there to advise farmers on how to best use anthelmintics (Sargison and Scott, 2011a).

Other recommendations include quarantine treatments of newly introduced sheep using a sequentially administrated combination of a benzimidazole and a salicylanilide anthelmintic drug to reduce the spread of resistant *F. hepatica* into new areas or farms (Sargison, 2012). Farm management can have a big effect on level of infection. For example by improving drainage systems, or fencing off wet and boggy areas that contain snail habitats and grazing sheep on low-risk pastures at high risk times of year, can all reduce risk of infection (Pritchard et al., 2005) whilst reducing reliance on drugs to control infection.

In summary, although cFECRT is the most practical and validated test to diagnosis liver fluke infection better methods for reliable detection at an early stage of infection in sheep are urgently needed. The *F. hepatica* cELISA, according to our results, is useful for detecting immature fluke infections but more work is required to establish how useful this test is for diagnosing TCBZ resistance in the field. In order to developed more sensitive cELISA, first, we need to revive the cut-off value for

positivity percentage that recommended by the manufacturer. We noted a high rate of false negative results in cELISA on field study and could be explained by the facts that high PP (%) of cut-off value (6.65% and 9.32%) resulted in insensitivity of this test. For example, field study in beef and dairy cattle by Brockwell et al. (2014) was able to improve the sensitivity of cELISA by using lower cut-off value, 1.3%, and thus they removed the false negative results. Therefore, further testing is required to establish lower custom cut-off PP (%) to improve sensitivity. Secondly, the manufacturer's protocol for faecal processing prior to testing can be altered to optimise the availability of the faecal antigen and further improve sensitivity. In the protocol, faecal sample was diluted in the dilution buffer, centrifuged for 10 min and the supernatant were stored at -20 °C until used. Recent work has shown that they leave the solution for overnight so that the faecal antigen will be more dissolved and increase the level of coproantigen for further use (Novobilsky A, personal communication). The ITS2 PCR used in the present study may hold significant promise as *Fasciola* DNA can be detected from as early as 2 wpi in experimentally study (Robles-Pérez et al., 2013). However, improve developed method needs to be further optimised as this assay failed to detect liver fluke infection in our laboratory. It is important to be forethoughtful, what is the source of the *Fasciola* DNA, whether it will ever be possible to detect DNA related to infection status, particularly in field naturally infected sheep and how does the DNA signal respond to TCBZ treatment, therefore, suggestions are made for further validation and investigation of the potential molecular based method in our laboratory. Furthermore, the prospect for loop mediated isothermal amplication (LAMP) testing to detect fasciolosis and TCBZ resistance in the laboratory are discussed.

Liver fluke is becoming increasingly common in sheep and poses a very real threat to livestock production of sheep in the UK and worldwide. Other major issues include resistance to TCBZ. Since anthelmintic resistance is inheritable and irreversible once fully fixed within a population, it is important to detect TCBZ resistance at early stage in the field so that we can maintain the efficacy of currently available drug as long as possible. At the moment there does not appear to be resistance to the other drugs that are used to treat *F. hepatica*, namely closantel and nitroxynil. However by reducing the use of TCBZ, greater pressure will be applied to these other drugs. There is a lack of effective diagnostic tools to detect infection and

the efficacy of TCBZ for the treatment and tests to measure efficacy of closantel and nitroxynil are urgently needed. Finally a large scale of TCBZ prevalence study needs to be conducted that would help to confirm our findings and provide a solid information to indicate options for sustainable liver fluke control in sheep livestock industry in the UK.

STANDARD OPERATING PROCEDURE (SOP) FOR CHAPTER 2

APPENDIX 2.1

Modified sedimentation technique for counting of eggs

Equipment/chemical

Bowl (500ml capacity)
Coarse sieve (38µm, 150µm and 710µm)
Counter (Clay Adams)
Weighing balance (accuracy ±0.1g)
Glove
Microscope
Petri dish (90ml)
Tap water
Wooden tongue depressor

Procedure

1. Bowl was place on the balance and adjusts the weight to zero.
2. Five grams of faeces were weighed.
3. The faeces were mixed manually with tap water in a bowl. Allowed pelleted faecal samples to soak and soften before the next step.
4. The faeces were mixed well and ensure that no spilled for liquid faecal samples as the eggs can settle out.
5. The sieves were stacked according to the size of aperture which is the sieve with the smallest aperture should be at the bottom and the largest at the top.
6. The faecal slurry were poured thorough the sieves under the running tapped water. Wash it through with plenty of water until water runs clear from the bottom sieve.
7. The top sieve was removed and repeats again Step 6 for the two remaining sieves.
8. The 150µm sieve was removed and gently washed through the sediment on the surface of the 38µm sieve. The sediments in the sieve were then backwash into a 500ml beaker.
9. Topped up with clean tap water in the beaker and allowed to settle for 4 minutes.
10. The supernatant were discarded leaving approximately 100ml at the bottom of the beaker and topped up again with water for another 4 minutes.
11. Step 10 was repeated until the water was clean.

12. The clear sediments were poured off down to 50ml or less if possible without losing the contents and transferred it to a large square petri dish.
13. The petri dish was placed on microscope and all eggs were counted when seen within the ruled or squared areas that was sit the dish on top.
14. If the eggs were not need for further testing, added a couple of drops of methylene blue as this will help the eggs to stand out (yellowish colour) under the microscope.
15. One egg represent 1 eggs per gram.

As for composite FEC, 5g of individual sample was pooled with the other samples from same group to make a total of 50g to form the composite sample. All the steps above were applied to give eggs per 50g.

APPENDIX 2.2

Fluke recovery

Equipment/chemical

String
Scalpels
Forceps
Scissors
Faecal pots
Tray
RPMI media; warmed to 37 ^{o}C
PBS; warmed to 37 ^{o}C
Petri dishes
Gloves

Procedure

1. Before removal of the liver, the duodenum was ligated with string either side of the point of entry of the bile duct, this to prevent any loss of flukes. Ensure that when removed from the carcass, the gall bladder remains attached and intact. Sample of faeces were taken from the spiral colon and stored in the refrigerator (Day 10 post-treatment).
2. The gall bladder was dissected away from the liver using forceps and scalpel blade. The whole gall bladder and contents were stored into the faecal pot for later examination.
3. The main bile duct was cut using sharp scissor to recover the adult flukes. Firm but gentle pressure was applied along the route of the ducts in order to bring the flukes out. The flukes were collected into a Petri dish with PBS warmed to 37^{o}C.
4. The portion of duodenum closest to the liver was squeezed in case any fluke had travelled down the common bile duct.
5. All adult flukes were rinsed in warm PBS and then incubated in 1ml of RPMI media at 37 ^{o} C for 2 hours in order for the parasite to purge any eggs and stomach contents.
6. The liver was sliced in approximately 1cm thick portions and then transferred into tray of warmed PBS (or saline) to 37^{o}C. Each slice was squeezed to reveal any hidden flukes. The liver content was incubated at 37^{o}C for 2 hours to allow any immature fluke to emerge.

7. After 2 hours, the content was removed and discarded, then washed through a 38 µm mesh sieve to remove excess blood. The liver parenchyma was examined for immature fluke. Any flukes recovered were transferred into a container of PBS at 37 ^{o}C (or saline).
8. The inner surface of the gall bladder was washed to detect any remaining eggs (likely in heavy burdens some adult fluke may be found in the gall bladder or even the duodenum). The bile was washed through a 38 µm with plenty of water; eggs that retained in the sieve were then transferred into a faecal pot and stored in the fridge in clean tap water.
9. In the laboratory:

- The adult flukes from the petri dish were removed and counted. For partial flukes recovered, the numbers of posterior and anterior ends were counted as well to determine the liver burden. All the flukes were stored frozen in the Eppendorf (1 per adult) for further use.
- Eggs remaining in the wells were transferred into Eppendorf and washed with tap water followed by centrifuging for 30 s. The supernatant was discarded and replaced with clean water. This step was repeated until the water is clear and there are no debris left in with the eggs. The solution was stored in the fridge for later processing.

APPENDIX 2.3

Coproantigen: Bio-X *Fasciola hepatica* antigenic ELISA Kit (BIO K 201)

Composition of the Kit

Two 96 well Bio-X K201 microplates
50x avidine (500µl)
1x chrome solution (2ml)
50x conjugate (500µl)
5x dilution buffer (50ml)
2 vials of positive reference antigen
1x stopping solution (15ml)
1x substrate solution (30ml)
20x washing solution (100ml)

Equipment

Adhesive for microplates
Beakers (50ml)
Distilled water
Dispenser tips
Gloves
Graduated automatic (mono- and multichannel) pipettes
Graduated cylinders
Lid
Microplate reader
Microplate shaker
Microplate washer
Plastic tubes
Tube rack
Reagent reservoir for multichannel pipettes

Procedure

The reagents were removed from the fridge and must be kept at 21°C before use (at least half an hour before use). For microplates, removed it from the fridge and then followed by its packaging.

(a) Faecal material

1. The faecal samples were diluted in the dilution buffer. 0.5 grams of faecal material were mixed up with 2ml of the dilution buffer.
2. All samples were vortexed for 10 s prior to centrifugation.
3. The tube was centrifuged at 1000g for 10 minutes to concentrate the solutions.

4. The supernatants were collected and stored in 1.5 ml Eppendorf tubes at -20°C until testing.

(b) Bio-X Diagnostics microplates preparation

1. The diluted faecal samples of 110µl were dispended into the wells as followed: sample 1 in wells A1 and B1, sample 2 in wells C1 and D1 and the same goes with sample 3 and sample 4.
2. The plates were incubated at 21ºC or were placed on a plate agitator at room temperature for 2 hours.
3. The plates then were rinsed with the washing solution, prepared as instructed in 'Composition of the Kit' as follows:
    a. The contents in the microplates were poured off sharply over a sink until it gets empty.
    b. The plates were then dried out by tapping the plate upside down with clean absorbent paper towels to ensure no liquid in the wells left.
    c. The plates were filled once more with the washing solution using a spray bottle and empty the wells again by flipping the plate over a sink.
    d. Step 'c' was repeated at least two more times, and proceeds to the next process. Avoid the formation of bubbles in the wells to ensure it was washed properly by the solution.
4. 100µl of diluted anti- *Fasciola hepatica* conjugate was added to each well.
5. The plates were covered with the lid/cling film and incubated at 21ºC for 1 hour.
6. The lid/cling film was removed and then the plates were again washed at least three times with the washing solution as described in Step 3.
7. 100µl of diluted peroxidise-linked conjugate solution was added to each well.
8. The plates were covered with the lid/cling film and incubated at 21ºC or for 1 hour.
9. The lid/cling film was removed and then the plates were again washed for three times with the washing solution as described in Step 3.
10. 100µl of the chromogen solution was added to each well.
11. The plates were then incubated at 21ºC and away from light for 10 minute.
12. 50µl of stop solution was added to each well.

13. The net optical density of each well was calculated using a plate reader and a 450nm filter.
14. Coproantigen values are expressed as the percentage positivity (PP) according to the formula; % = (Mean OD of the sample / Mean OD of positive control) x 100. The cut-off value for percentage positive (PP) provided by manufacturer's guidelines.

(c) Kit antigens preparation

(i) anti-Fasciola hepatica conjugate solution

20µl of the biotin-linked anti-*Fasciola hepatica* conjugate was added with 0.98 ml of the reagent dilution buffer (per strip).

(ii) avidine-peroxidise conjugate solution

20µl of avidine-peroxide conjugate was added with 980µl of the reagent dilution buffer (per strip).

(iii) chromogen solution

500µl of chromogen was added with 9.5ml of the substrate solution (approximately for 1 plate preparation). The solution must be absolutely colourless when used. Any change of colour or when its turned into blue colour in the pipette, the solution is already contaminated.

(iv) positive reference

0.5ml of distilled or demineralised water was added to reconstitute the antigen.

(v) stopping solution

1 M phosphoric acid stop solution.

(vi) substrate solution

Hydrogen peroxide substrate solution must be kept at 4°C.

(vii) washing solution

The concentrated washing solution was diluted 20 fold in distilled water.

APPENDIX 2.4

QIAamp DNA Stool mini kit – Faecal DNA extraction protocol

Composition of the Kit

QIAamp Mini Spin Columns
Collection Tubes (2 ml)
InhibitEX® Tablets
Buffer ASL (140 ml)
Buffer AL (33 ml)
Buffer AW1 (19 ml)
Buffer AW2 (13 ml)
Buffer AE (15 ml)

1. The faecal samples were diluted in the lysis buffer. A total of 0.5 grams of faecal material were mixed up with 2.8 ml of buffer ASL in a 15 ml tube.
2. All samples were vortexed for 1 min prior to centrifugation until sample properly homogenized, then heat the sample in the water bath for 5 min at 95^{o}C**.**
3. The sample was centrifuged at full speed @2500g for 8 min to concentrate the solutions.
4. A 1.5 ml volume of the supernatants were collected and transferred into 15 ml tube with an InhibitEX tablet. Vortex until the tablet is completely suspended, then leaves the samples for 3 min at room temperature and then centrifuges at full speed @2500g for 6 min.
5. A total of 400 µl was transferred into a new 1.5 ml tube and centrifuge at @2500g for 3 min.
6. A total of 200 µl supernatant into the 1.5 ml tube and 15 µl of proteinase K were added and then added 200µl Buffer AL and vortex for 15 s. The sample must be mixed very well.
7. The samples were incubated in water bath at 70^{o}C for 10 min.
8. 200 µl of ABS ethanol was added and mixed by vortex followed by centrifuging for 1 min at 16 000 x g.
9. Entire sample was transferred onto the QIAmp spin column (plus the 2 ml collection tube) without moistening the rim, label the lid and then centrifuged for 1 min at 12 850 x g.

10. 500µl of buffer AW1 was added to the column and centrifuged at 12 850 x g for 1 min. The spin column was discarded from the tube and placed it in another collection tube.
11. 500µl of buffer AW2 was added to the spin column and centrifuged at 12 850 x g for 3 min. This step was repeated and the spin column was transferred into a new collection tube and centrifuged at 12 850 x g for 1 min to eliminate traces of AW2.
12. The column was transferred into a new 1.5 ml tube and 60µl of buffer AE was added directly onto the QIAamp® membrane, incubated at room temperature for 5 min, and centrifuged at 12 850 x g for 1 min. The eluted DNA was store at -20°C until analysis.

PCR

PCR reaction was based on a 25 µl volume containing:

	PCR mix	Volume (µl)
1	DNA template (1:50 diluted)	4
2	H_20	6
3	Primer ITS2 F 10 µm	1.25
4	Primer ITS2 R 10 µm	1.25
5	2 x Biomix Red*	12.5

**Biomix™ Red (Bioline Reagents Limited, London, UK)*

1. Master mix was mixed up for 14 tubes (12 samples and positive and negative samples).
2. 21 µl of the master mix was added to each tube.
3. 4 µl of DNA templates was added into the tubes.
4. The thermocycyler was set as followed:

PCR cycling conditions:

95°C for 2 min
95°C for 30 sec
63 °C for 30 sec
72°C for 45 sec
} 40 cycles
72°C for 10 min

5. The amplification products were analysed by electrophoresis.

DNA Gel Electrophoresis

This is used to separate macromolecules based upon their size. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through an agarose matrix. Agarose gel electrophoresis can be used for the separation of DNA fragments ranging from 50 base pair to several megabases using specialized apparatus. The distance between DNA bands of a given length is determined by the percent agarose in the gel. The PCR mix requires a dye front to observe movement through the gel and to ensure that the DNA doesn't run off the ends. However, the Biomix red contains a dye in the master mix so it is not necessary to add any dye.

1. Make up agarose gel at 1.5%- 1.5g agarose was added to 100ml TAE and heat in the microwave (take care as the agarose will boil over).

   The large frames should take 100ml agarose, whilst 50 ml of agarose should be sufficient for the small casting frames.
2. Once the agarose is melted and cooled (enough to be handled) 10 ul of Sybr Safe was added (for a 100 ml, or 5µl for 50 ml) and mixed by swirling the agarose.
3. Once mix, poured into the gel tank and leave to set (check that there are no leaks).
4. 5 µl of 100 bp ladder was added into the first lane of the gel.
5. 5 µl of PCR product was added into each well.
6. Run gel for 30 minutes at 100V- check to see if it has run through.
7. Gel was placed into G:Box Syngene to visualise bands.

APPENDIX 2.5

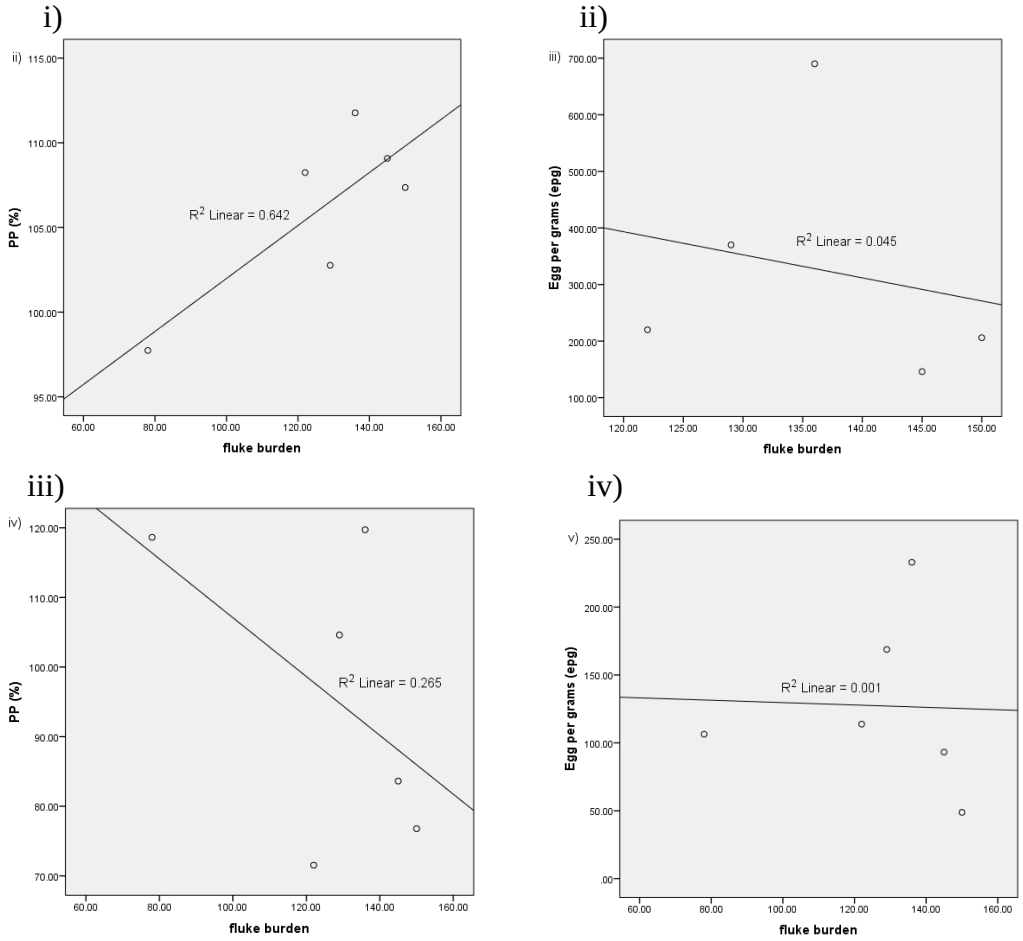


Figure 2.5. i) The correlation between fluke burden and PP (%) on 14 wpi; ii) The correlation between fluke burden and FEC on 14 wpi; iii) The correlation between fluke burden and PP (%) on 10 dpt; iv) The correlation between fluke burden and FEC on 10 dpt.

STANDARD OPERATING PROCEDURE (SOP) FOR CHAPTER 3

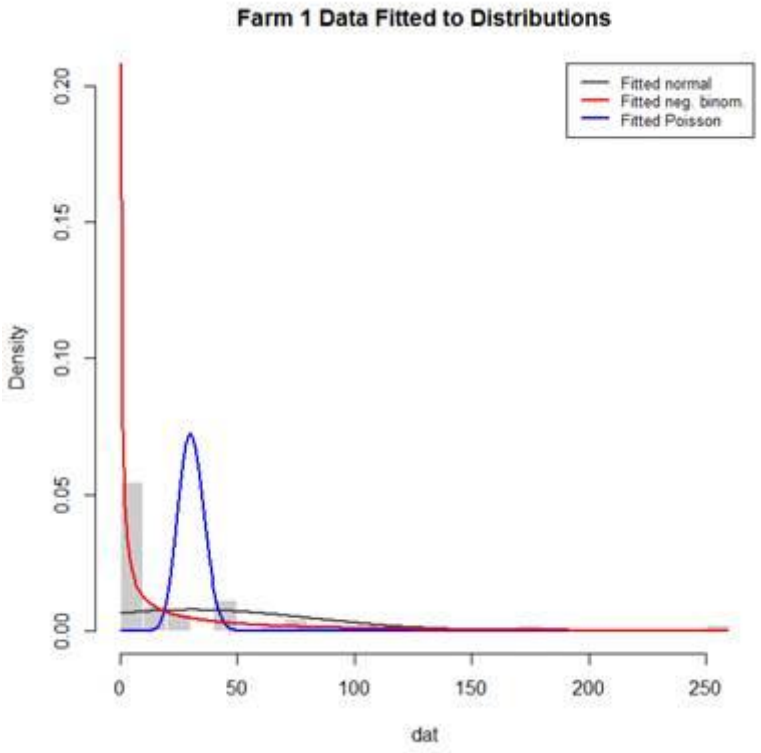
APPENDIX 3.1

Letter from Ms Christina Gill

Finding the best model for your data

The negative binomial distribution is often used to describe count data (for example FEC). Originally the NBD was designed to work out the probability of an event occurring. The NBD is not suitable for all count data, so you have to either show that works for your data, or otherwise assume it works based on the results of previous studies. Another distribution sometimes used to model count data is the Poisson distribution. However, parasite count data is often overdispersed (this means that the spread of your data is greater than the mean) and this can be modelled with the NBD (unlike the Poisson distribution, where the mean is assumed to equal the variance). Other distributions you may have heard of are the normal distribution and the binomial distribution.

If you look at the histogram from your data from Farm 1 below (graph for Farm 2 is similar), it looks like a negative binomial distribution is the best fit for your data:

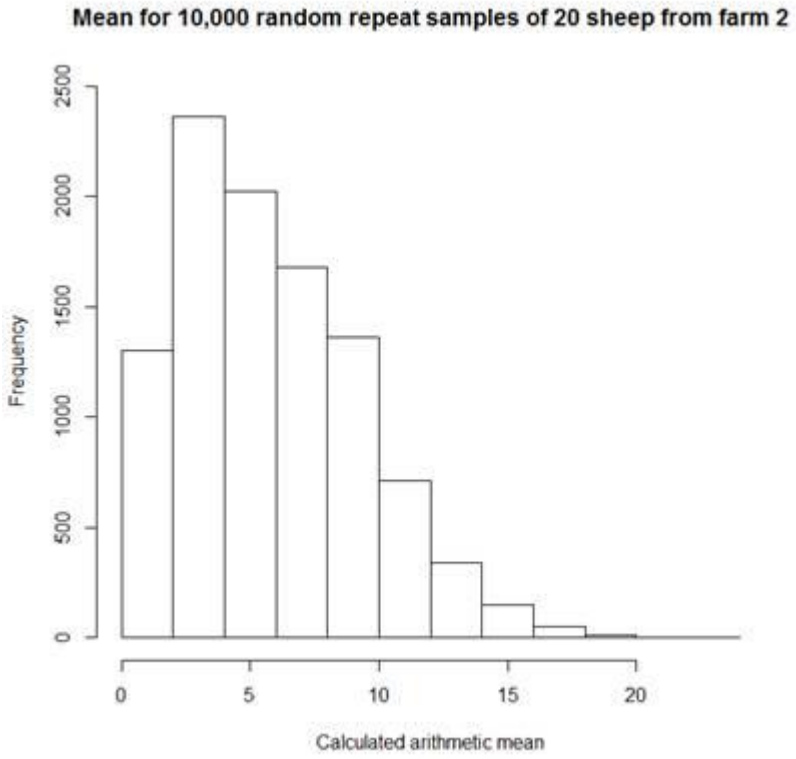


I tested the fit of the data in R using a chi-squared goodness of fit test. This confirmed that the NBD fits your data, while the Poisson distribution does not, as expected. Sometimes, this kind of count data is even better described by what is called a zero-inflated negative binomial distribution. This is essentially the same as the NBD, except that there are an increased number of individuals with zero counts. You would expect this to occur in cases where the chances of having a zero count are controlled by a separate process to the one controlling the rest of the distribution (there is some more information about this here: http://en.wikipedia.org/wiki/Zero-inflated_model). I tested this on your data, but using a zero-inflated negative

binomial distribution did not improve the fit for your data (I evaluated this by doing a test that compares whether a model fits the data better than another called the "likelihood ratio test"). So your data is best described by a negative binomial distribution. This is useful to know if you want to do any testing on hypothetical populations of sheep, for example if you need to know what happens when your flock size is doubled.

Do you need to resample the same 20 sheep?
Whether or not you need to resample the same 20 sheep, or can sample any 20 sheep will depend on a number of factors. The first factor is individual variation (the variance/spread of your data). The higher the spread of your data, the less likely it will be that the results from two different sheep will be comparable. This will likely vary by farm. The second factor is the number of sheep in the flock. If you have a small flock, you are more likely to pick at least some sheep that you have sampled the first time. In a large flock, you are likely to sample different sheep. Finally, the degree of correlation between pre-treatment and post treatment counts is important also. If the correlation is high, then testing the same sheep is better, relatively speaking, than sampling different sheep (you can check this on your pre and post-treatment data using Spearman's or Pearson's correlation).

For the farm where you have samples from 105 sheep, you can estimate the accuracy of taking samples from 20 sheep: The mean FEC for all sheep on this farm is 5.90 eggs per 5g of faeces. If we randomly sample 20 of these sheep and calculate the mean FEC (per 5g) and do this 10,000 times here is a histogram of the results:



You can see that the mean values are also following the NBD (this is due to the small sample size of 20). You can also see that there is a wide spread of means.

Actually, the calculated mean from 20 samples only falls within the range of the 'true' mean +/- 5% in 5.3% of cases (farm 2). Doing the same thing for farm 1, the result is slightly better at 10.5%. This is because the variance is lower on this farm and the total number of sheep is lower also. You can work out a 95% confidence interval for this mean, but since the results are not normally distributed, this can be a little tricky. It is approximately 11.7-55.6 and 0.6-15.25 eggs per 5g for Farm 1 and Farm 2, respectively).

If we compare a random sample of 20 sheep from Farm 2 to another random sample of 20 sheep from Farm 2 (and repeat this 10,000 times), then we find a reduction of 95% or more in FEC 0.5% of the time (so on a farm where treatment has no effect on FEC at all, we would get a false negative for resistance 0.5% of the time). On the other hand, if we assumed that at the next sampling each individual sheep had exactly a 95% reduction in FEC then we would detect that in only 49.4% of cases if we sample different sheep at each visit (so we would get a false positive for resistance more than 50% of the time). However, your calculated FEC reduction is not just a binary variable - the actual percentage FEC reduction value gives you additional valuable information. For research purposes I think you need this to be fairly accurate, otherwise your results will be poorly comparable to other studies. Therefore we would resample the same 20 sheep (if your purpose was to diagnose resistance then the loss of accuracy may be less important).

One thing I think you still need to do is check the degree of correlation between pre- and post-treatment values for the 21 or so sheep where you have this data. If they correlate poorly, you may want to think about increasing your sample size.

STANDARD OPERATING PROCEDURE (SOP) FOR CHAPTER 4

APPENDIX 4.1

UNIVERSITY OF LIVERPOOL

Veterinary Parasitology
Institute of Infection and Global Health/School of Veterinary Science
University of Liverpool
Liverpool Science Park IC2
146 Brownlow Hill
Liverpool
L3 5RF

Dear

We would like to invite you to participate in a study being run by the University of Liverpool. The purpose of the study is to determine how widespread resistance to fluke treatment is in sheep across England and Wales. Specifically we are investigating resistance to the drug triclabendazole (TCBZ), which is found in products such as Fasinex, Combinex, Tribex and Cydectin Triclamox.

If you wish to take part in the study, the first step is to complete the consent form attached to this letter and return it to us in the stamped addressed envelope. Please include your mobile number and email address on the consent form.

When we have received your consent form, staff from either the AHVLA or the University of Liverpool will ring you to arrange to visit your farm to collect dung samples from and treat 20 individual sheep. We will bring a pack of Fasinex to treat these sheep and we will also give you a short questionnaire to complete giving us some information on whether you have a history of fluke on your farm, the treatment history and management of your flock. We will visit your farm again to take a second set of samples from the same 20 sheep, 21 days later. We will collect the questionnaire from you on this second visit.

The samples will be tested for liver fluke eggs. Once the analysis is complete and we have received the questionnaire, **we will send you the details of all the counts from your farm together with advice on those results**.

Details of what we are asking you to do are given overleaf. There is also a Participant Information Sheet giving some more information.
For further information call Juriah Kamaludeen on 07588 816 818 or Professor Diana Williams on 07968 075462, email juriahk@liv.ac.uk or write to Juriah Kamaludeen at the address above.

Thanks very much for your co-operation.

Yours sincerely

Juriah Kamaludeen, Jane Hodgkinson, Gina Pinchbeck and Diana Williams

APPENDIX 4.2

UNIVERSITY OF LIVERPOOL

CONSENT FORM

Title of Research Project: To establish the national prevalence of triclabendazole (Fasinex) resistance in fluke populations affecting sheep.

Researcher(s): Juriah Kamaludeen, Dr Gina Pinchbeck, Dr Jane Hodgkinson and Professor Diana Williams

Participant Name:

Farm Address:

Contact telephone number:

Email address:

Please tick box

1. I confirm that I have read and have understood the participant information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my rights being affected. ☐
3. I understand that, under the Data Protection Act, I can at any time ask for access to the information I provide and I can also request the destruction of that information if I wish ☐
4. I agree to take part in the above study. ☐

Participant Name	Date	Signature
Researcher	Date	Signature

[Version Number]
[Date]
[Principal Investigator Initials]

1 for subject; 1 for researcher

1

APPENDIX 4.3

The University of Liverpool
Veterinary Parasitology Group, Liverpool Science Park IC2, Brownlow Hill, Liverpool, L3 5RF

Participant Information Sheet

The purpose of this study is to investigate the occurrence and identify the factors that affect resistance to the commonly used drug triclabendazole, found in Fasinex, in liver fluke infected sheep in England and Wales.

We would like you to participate in this study but before you decide whether to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information and feel free to ask us if you would like more information or if there is anything that you do not understand. We would like to stress that you do not have to accept this invitation and should only agree to take part if you want to.

Thank you for reading this.

Frequently Asked Questions

1. What is the purpose of the study?

   This study will look at the level of liver fluke infection in your flock and investigate the effectiveness of triclabendazole (Fasinex) treatment.

   We would also like to obtain information about farm management practices that might influence the occurrence of drug resistance.

2. Why have I been chosen to take part?

   Your name has been selected at random from a list of sheep farmers in England and Wales by the AHVLA.

3. Do I have to take part?

   Participation is voluntary and you are free to withdraw from the study at anytime without explanation and without any disadvantage.

4. What will happen if I take part?

   Enclosed are details of what we will do should you decide to participate.

5. Are there any risks in taking part?

   There are no risks involved.

6. Are there any benefits in taking part?

   Yes. In return for participating in this study, you will receive test results showing the level of fluke infection in your herd. You will also find out if the drug is still effective on your farm. We are happy to discuss management and control of fluke on your farm with you and your vet.

   In the long term, this study will advantage all farmers as we will find out more about how common drug resistance is in England and Wales and we will be able to advise on how to reduce the risk of resistance occurring in the future.

7. What if I am unhappy or if there is a problem?

   If you are unhappy, or if there is a problem, please feel free to let us know by contacting Juriah Kamaludeen on 07588 816 818 or Professor Diana Williams on 07968 075462 and we will try to help. If you remain unhappy or have a complaint which you feel you cannot come to us with then you should contact the Research Governance Officer on 0151 794 8290 (ethics@liv.ac.uk). When contacting the Research Governance Officer, please provide details of the name or description of the study (so that it can be identified), the researchers involved, and the details of the complaint you wish to make.

8. Will my details be kept confidential?

   All your personal details and questionnaire answers will be stored securely and confidentially until the end of the study (Dec 31st 2015) and then destroyed. Any publications using the data obtained from this study will ensure that individual participating farms cannot be identified.

   All completed questionnaires will be kept in paper-format in a locked cabinet within the Department of Infection Biology, Institute of Infection and Global Health, University of Liverpool. Keys for this cabinet will be kept in a separate locked room.

   All digital data will be stored in a secure University database (M:Drive) and identifying details will be stored in a separate database to the questionnaire answers.

   Only the researcher and the three supervisors will have access to the data.

   All digital data will be password protected.

   The files containing the data will be stored on a shared network file only accessible by Dr Jane Hodgkinson, Prof D Williams, Dr Gina Pinchbeck and Juriah Kamaludeen. No files will be stored on computer hard drives to prevent loss of data due to computers being stolen or left unattended.

Digital data will be given a reference number pertaining to that individual farm, and only the key, linking reference number and farmer details, will be able to connect farm data to an individual address. This reference number list will be stored securely and a hard paper copy will be stored with the completed questionnaires.

Only Dr Jane Hodgkinson (primary supervisor), Prof D Williams (second supervisor), Dr Gina Pinchbeck (third supervisor) and Juriah Kamaludeen (PhD student) will have access to the reference number list.

9. What will happen to the results of the study?

Results generated from each farm, will be sent to that farm as soon as possible after the laboratory test is finished. The results from the whole study will be published as a scientific paper and summaries sent to the farming press. A final report will be sent to all participating farmers. We will ensure that no identifying information about the farms participating in the study is included in any of these publications.

10. What will happen if I want to stop taking part?

Participants can withdraw at anytime, without explanation. However, the results of the liver fluke tests may not be made available to them.

11. Who can I contact if I have further questions?

Please contact:
Juriah Kamaludeen or Diana Williams
Veterinary Parasitology Group
Department of Infection Biology
Institute of Infection and Global Health University of Liverpool,
Liverpool Science Park IC2,
146 Brownlow Hill,
Liverpool.
L3 5RF

Office: 0151 795 0234/0220

APPENDIX 4.4

UNIVERSITY OF LIVERPOOL

Liver fluke and drench resistance in England and Wales

We are conducting an investigation into resistance to the commonly used product: triclabendazole (found in Fasinex, Combinex, Tribex, Cydectin Triclamox) in liver fluke on sheep farms in England and Wales. Through this study we hope to identify the factors that lead to resistance, so that we can advise farmers on how to reduce the risk of resistance spreading in future.

We will use a Composite Faecal Egg Count on faecal samples from your sheep to find out whether they are infected with liver fluke. If positive, we will repeat the sampling following treatment and assess the level of resistance on your farm. We are also interested in the management practices and fluke treatments you use in your sheep.

- **All responses will be kept entirely confidential**
- **The information that you provide will be only be used by researchers at the University of Liverpool and will be kept as an anonymised record**
- **Once completed, please post this questionnaire back in the pre-paid envelope**

Thank you for completing this survey

How to complete the questionnaire

- This questionnaire is about FLUKE in your sheep. Please read the instructions for each question carefully.
- Please write in **BLOCK CAPITALS** as clearly as you can.
- Please use a **Tick** '√' in the box provided as shown: ☑ Yes ☐ No
- Please write in the space provided or follow instructions that explain what to do. There are no right or wrong answers.

  For example: **LIVERPOOL**
- If you make a mistake or change your answer please completely fill in the incorrect box and put a cross in the correct box, as shown:

  ■ Yes ☑ No

SECTION 1: About your farm

1. Today's Date:

2. Please provide your name:

3. Your address:

4. Postcode:

5. Email address / Telephone:

6. What is the size of your flock? ☐☐☐☐ Sheep

7. Do you have other livestock on your farm other than sheep? Please give numbers in the boxes.

☐ None
☐ Dairy
☐ Beef sucklers
☐ Beef stores
☐ Beef finishers
☐ Goats
☐ Other (*Please specify*)

8. Are there any other animals kept on the farm? ☐ Yes ☐ No

8a. If Yes, what are they?

☐ Horse ☐ Dog ☐ Other (*please specify*)

9. How many years have you kept sheep on your farm? ☐☐ Years

10. What is the **total** size of your farm? ☐☐☐☐☐ acres/hectares (*delete as applicable*)

11. Is your farm organic? ☐ Yes ☐ No

12. Is your farm: ☐ Upland ☐ Lowland ☐ Hill

13. Does your farm have Less Favoured Areas (LFA) status? ☐ Yes ☐ No

14. Do you have any environmental schemes? ☐ Yes ☐ No

14a. If Yes, please provide basic details:

SECTION 2: About your sheep management

15. When were your sheep moved onto their current pasture? (If you keep sheep in more than one group, please answer for the primary flock e.g. breeding ewes)

☐ Within the last month
☐ 1 to 3 months ago
☐ 4 to 6 months ago
☐ Other (*Please specify*)

16. How many times a year do you move your sheep to different pasture?

17. Are the fields used for grazing sheep used for any other stock?

☐ Yes before the sheep
☐ Yes after the sheep
☐ No

17a. If Yes, what are they?

☐ Dairy cattle
☐ Beef cattle
☐ Other (*Please specify*)

18. Do you let your sheep go to other farms for winter grazing?

☐ Yes ☐ No If No, go to question **19**

18a. How many years have you sent sheep elsewhere for winter grazing?

18b. Which sheep do you send for winter grazing?

19. When do your sheep lamb?

Start date: End:

20. Do you house your sheep for lambing?

☐ Yes ☐ No

20a. If Yes, when are the sheep housed?

Date housed: Date turned out:

21. How many ewes do you have on your farm?

22. Do you breed all your own replacements?

☐ Yes ☐ No

23. Do you buy in new stock?

☐ Ewes ☐ Tups ☐ Other (Please specify)

24. Do you graze sheep on silage or hay aftermath?

☐ Yes ☐ No

25. Do you produce your own forage? ☐ Yes ☐ No

25a. If yes, what type of forage that you produce? **Please TICK ALL which apply**

☐ Hay
☐ Silage
☐ Straw
☐ Other (*Please specify*)

26. Do you fertilise fields grazed by sheep on your farm? **Please TICK ALL which apply**

☐ Commercial fertiliser
☐ Slag
☐ Calcified seaweed
☐ Manure
☐ Slurry
☐ Pot ash
☐ Lime
☐ Treated human sewage
☐ No
☐ Other (*Please specify*)

27. What is the main water source for your sheep? **Please TICK ALL which apply**

☐ Trough
☐ Bowser
☐ Piped
☐ Spring
☐ Stream
☐ Pond
☐ Other (*Please specify*)

27a. If a piped water source, where does it come from?

☐ Bore hole
☐ Mains water
☐ From a stream/river
☐ Don't know
☐ Other (*Please specify*)

28. How would you rate the drainage of the field your sheep are currently grazing?

☐ Very good ☐ Good ☐ Average ☐ Poor

29. At any time of the year, do you get 'boggy fields' on your farm? ☐ Yes ☐ No

30. Do the fields used for sheep have, or border any of these?

- ☐ Ponds
- ☐ Rivers
- ☐ Stream
- ☐ Marshy areas (e.g. marked by the presence of reeds)

31. How would you describe your gateways?

- ☐ Muddy all year
- ☐ Dry
- ☐ Muddy in the winter
- ☐ Hard-core used

32. Do you use creep feeders? ☐ Yes ☐ No

32a. If Yes, how often the feeders are moved?

33. Do you use any method to improve drainage? **Please TICK ALL which apply**

- ☐ Harrowing
- ☐ Aeration
- ☐ Rolling
- ☐ Sub-soiling
- ☐ None
- ☐ Other (*Please specify*)

34. Do you regularly clean out your ditches on your farm?

☐ Yes ☐ No ☐ Not applicable

34a. If Yes, please provide basic details:

SECTION 3: About sheep health

35. Have you ever had liver fluke infection on your farm?

☐ Yes ☐ No ☐ Unknown

35a. If Yes, how would you rate the level of liver fluke infection on your farm?

☐ High ☐ Low

☐ Medium ☐ Don't know

36. Have you had any abattoir returns listing fluke? If yes, please provide further details.

SECTION 4: About your sheep fluke control

37. Do you drench your sheep against liver fluke?

☐ Yes ☐ No ☐ Sometimes If No, go to question **44**

If yes, answer Question 38-43

38. When were your sheep last treated for fluke and what product did you use?

dd / mm / yr

☐☐☐☐☐☐

Product:

39. How many times per year do you treat your sheep for fluke? ☐☐

40. How do you calculate the dose of drench to give to your sheep?

41. What type of drenches do you currently or usually use to treat fluke?
Please TICK ALL which apply

- ☐ Albensure
- ☐ Allverm
- ☐ Flukiver
- ☐ Ovispec
- ☐ Rycoben
- ☐ Fasinex
- ☐ Trodax
- ☐ Albex
- ☐ Combinex cattle/sheep
- ☐ Ivomec Plus Injection for cattle
- ☐ Mebadown Super
- ☐ Supaverm
- ☐ Tribex
- ☐ Other (*Please specify*)

42. How do you decide which product to use?

- ☐ Following advice from a vet
- ☐ Recommended by the shop
- ☐ Based on information in magazines/television
- ☐ Recommended by neighbour
- ☐ Other (*Please specify*)

43. How do you decide when to treat fluke in your sheep?

- ☐ Following a fluke programme advised by the Vet or animal health officer
- ☐ When my sheep look poor
- ☐ I fluke my sheep when I remember
- ☐ I always fluke at the same times of year (Please provide brief details):

44. Do you give a 'quarantine drench' to treat new stock against liver fluke?

(A 'quarantine drench' is defined as drenching newly purchased sheep within 48 hours).

☐ Yes ☐ No ☐ Unsure

45. Do you treat other stock on your farm for fluke?

Animal	Yes / No	When	What with
Cattle			
Horses			
Goat			
Other (*specify*):			

SECTION 5: About your sheep fluke faecal egg count (FEC)

46. Have you ever used faecal egg counts (FECs) to monitor fluke?

☐ Yes ☐ No

46a. If Yes, when have you used FEC monitoring?

☐ Within the last year
☐ 2 – 5 years ago
☐ 5 – 10 years ago
☐ Other (*Please specify*):

46b. How frequently do you use FEC?

☐ Every 3 months
☐ Every 6 months
☐ Never
☐ Once yearly
☐ Occasionally
☐ Other (*Please specify*):

47. Have you ever used a drug resistance test for fluke in your sheep?

☐ Faecal Egg Count Reduction Test
☐ Coproantigen Test
☐ No

If No, go to question **48**

47a. If applicable, please indicate below the results of the most recent drug resistance test for fluke done on your farm.

Date if known	Drench used	Result

SECTION 6: About your fluke control advice that you received

48. From which sources did you obtain your information for fluke control in sheep?
Please TICK ALL which apply

- ☐ Health advisers
- ☐ Feed supplier
- ☐ Seminars
- ☐ Farming Press/Media
- ☐ Veterinarian
- ☐ Neighbour
- ☐ Internet
- ☐ Other (*Please specify*):

49. Of these sources, which is the **MOST** important source of information for you?

50. Do you follow the **NADIS** fluke forecast? ☐ Yes ☐ No

51. Have you heard of SCOPS? ☐ Yes ☐ No

51a. If yes, do you follow SCOPS recommendations when treating your sheep for fluke?

Do you have any other comments regarding fluke control, this questionnaire or related matters?

'End of questionnaire'

Thank you for completing this survey. Your participation is much appreciated.
Now please return the questionnaire in the prepaid envelope provided.

Veterinary Parasitology, Department of Infection Biology,
Institute of Infection and Global Health, University of Liverpool,
Liverpool Science Park IC2, 146 Brownlow Hill, Liverpool, L3 5RF.

APPENDIX 4.5

The use of different management practises in the different 7 sheep farm.

Management Factor/Farm ID	1	2	3	4	5	6	7
Sheep move to current pasture a) 1 to 3 months b) Within the last month c) Housed at Christmas for lambing in March d) Housed the ewes mid-January	b	d	a	b	a	a	a
Used grazing with other livestock a) Yes before the sheep b) Yes after the sheep c) No	a, b	a, b	**	b	a, b	c	a, b
If yes, grazing with beef cattle a) Yes b) No	No	Yes	Yes	Yes	Yes	**	Yes
Let sheep for winter grazing a) Yes b) No	Yes	Yes	Yes	Yes	Yes	Yes	No
Type of forage that produced a) Hay b) Silage c) Straw	b	a, b	a, b	b	a, b	a, c	a, b

Fertilise fields grazed by sheep a) commercial fertilise b) manure c) slurry d) lime e) slag	a, b	b, d	a, b, d	a, b d, e	a, b d, e	a	b, d
Main water source for your sheep a) through b) piped c) stream d) spring	c, d	a, c, d	c, d	a, b, c, d	d	a, b, d	a, b, c
Rate the drainage of the grazing field	very good	average	average	average	poor	good	poor
Get 'boggy fields' on farm a) Yes b) No	No	Yes	Yes	Yes	Yes	Yes	Yes
Method for improved drainage a) Harrowing b) Rolling c) Aeration d) Sub-soiling e) Maintain existing drains f) None	a	a, d	e	a, d, e	c	f	f
Clean ditches a) Yes b) No	No	Yes	NA	Yes	NA	NA	No

c) NA

** missing data; NA (not applicable)

References

Abdel-Rahman, S.M., O'Reilly, K.L., Malone, J.B., 1998. Evaluation of a diagnostic monoclonal antibody-based capture enzyme-linked immunosorbent assay for detection of a 26- to 28-kd *Fasciola hepatica* coproantigen in cattle. Am J Vet Res 59, 533-537.

Almazan, C., Avila, G., Quiroz, H., Ibarra, F., Ochoa, P., 2001. Effect of parasite burden on the detection of *Fasciola hepatica* antigens in sera and feces of experimentally infected sheep. Vet Parasitol 97, 101-112.

Alvarez-Sanchez, M.A., Mainar-Jaime, R.C., Perez-Garcia, J., Rojo-Vazquez, F.A., 2006. Resistance of *Fasciola hepatica* to triclabendazole and albendazole in sheep in Spain. Vet Rec 159, 424-425.

Alvarez, L., Moreno, G., Moreno, L., Ceballos, L., Shaw, L., Fairweather, I., Lanusse, C., 2009. Comparative assessment of albendazole and triclabendazole ovicidal activity on *Fasciola hepatica* eggs. Vet Parasitol 164, 211-216.

Alvarez, L.I., Mottier, M.L., Lanusse, C.E., 2007. Drug transfer into target helminth parasites. Trends Parasitol 23, 97-104.

Alvarez, L.I., Solana, H.D., Mottier, M.L., Virkel, G.L., Fairweather, I., Lanusse, C.E., 2005. Altered drug influx/efflux and enhanced metabolic activity in triclabendazole-resistant liver flukes. Parasitol 131, 501-510.

Alvinerie, M., Floc'h, R., Galtier, P., 1991. Plasma protein binding of nitroxynil in several species. J Vet Pharmacol Ther 14, 170-173.

Anderson, N., Luong, T.T., Vo, N.G., Bui, K.L., Smooker, P.M., Spithill, T.W., 1999. The sensitivity and specificity of two methods for detecting *Fasciola* infections in cattle. Vet Parasitol 83, 15-24.

Anderson, R.M., May, R.M., 1991. Infectious Diseases of Humans.Oxford, U.K.: Oxford University Press. 13.

Andrews, S.J., 1999. The life cycle of *Fasciola hepatica*. In: Dalton, J.P. (Ed.), Fasciolosis, CAB International, Oxon, pp. 1-29.

Anon, 2007. AHVLA Standard Operating Procedure, OP PA026, Fluke egg demonstration by sedimentation AHVLA Standard Operating Procedure, SOP PA026, Fluke egg demonstration by sedimentation.

Anon, 2009. Evidence of fluke metacercariae overwintering on infected pastures. Vet Rec 164, 799-802.

Anon, 2009a. VLA VIDA data.

Anon, 2010. VLA Small Ruminant Disease Surveillance Report. Vol 14, No. 1. https://www.defra.gov.uk/vla/reports/docs/rep_survrep_qtlysr0101.pdf.

Anon, 2013. VIDA REPORT 2013. https://www.gov.uk/government/statistics/veterinary-investigation-diagnosis-analysis-vida-report-2013.

Barger, I.A., 1985. The statistical distribution of trichostrongylid nematodes in grazing lambs. Int J Parasitol 15, 645-649.

Bennema, S., Vercruysse, J., Claerebout, E., Schnieder, T., Strube, C., Ducheyne, E., Hendrickx, G., Charlier, J., 2009. The use of bulk-tank milk ELISAs to assess the spatial distribution of *Fasciola hepatica*, *Ostertagia ostertagi* and *Dictyocaulus viviparus* in dairy cattle in Flanders (Belgium). Vet Parasitol 165, 51-57.

Bennema, S.C., Ducheyne, E., Vercruysse, J., Claerebout, E., Hendrickx, G., Charlier, J., 2011. Relative importance of management, meteorological and environmental factors in the spatial distribution of *Fasciola hepatica* in dairy cattle in a temperate climate zone. Int J Parasitol 41, 225-233.

Bennett, J.L., Kohler, P., 1987. *Fasciola hepatica*: action in vitro of triclabendazole on immature and adult stages. Experimental Parasitology 63, 49-57.

Beretta, C., Locatelli, A., 1969. Anthelmintic and diffusion properties of 2-iodo-4-cyano-6-nitrophenol affected by blood serum or by serum albumin solutions *in vitro*. Br Vet J 125, 483-491.

Bliss, C.I., Fisher, R.A., 1953. Fitting the negative binomial distribution to biological data. Biometrics 9, 176-200.

Boray, J.C., 1963. The ecology of *Fasciola hepatica* with particular reference to its intermediate host in Australia. Proceedings of the 17th World Veterinary Congres Hanover 1, 709.

Boray, J.C., 1969. Experimental fascioliasis in Australia. Adv Parasitol 7, 95-210.

Boray, J.C., 1986. Trematode infections of domestic animals. In: *Chemotherapy of parasitic diseases*: (Edited by Campbell W.C.and Rew R.S.), Plenum Press, New York and London, pp. 401-405.

Boray, J.C., 1999. Liver Fluke Disease in Sheep and Cattle. Agfact AO.9.5.7, second ed. revised by G.W. Hutchinson, S. Love. NSW Agriculture.

Boray, J.C., Crowfoot, P.D., Strong, M.B., Allison, J.R., Schellenbaum, M., Von Orelli, M., Sarasin, G., 1983. Treatment of immature and mature *Fasciola hepatica* infections in sheep with triclabendazole. Vet Rec 113, 315-317.

Boray, J.C., Enigk, K., 1964. Laboratory studies on the survival and infectivity of *Fasciola hepatica*- and *F*. gigantica-metacercariae. Z Tropenmed Parasitol 15, 324-331.

Brennan, G.P., Fairweather, I., Trudgett, A., Hoey, E., McCoy, McConville, M., Meaney, M., Robinson, M., McFerran, N., Ryan, L., Lanusse, C., Mottier, L., Alvarez, L., Solana, H., Virkel, G., Brophy, P.M., 2007. Understanding triclabendazole resistance. Exp Mol Pathol 82, 104-109.

Brockwell, Y.M., Elliott, T.P., Anderson, G.R., Stanton, R., Spithill, T.W., Sangster, N.C., 2014. Confirmation of *Fasciola hepatica* resistant to triclabendazole in naturally infected Australian beef and dairy cattle. Int J Parasitol Drugs Drug Resist 4, 48-54.

Brockwell, Y.M., Spithill, T.W., Anderson, G.R., Grillo, V., Sangster, N.C., 2013. Comparative kinetics of serological and coproantigen ELISA and faecal egg count in cattle experimentally infected with *Fasciola hepatica* and following treatment with triclabendazole. Vet Parasitol 196, 417-426.

Canevari, J., Ceballos, L., Sanabria, R., Romero, J., Olaechea, F., Ortiz, P., Cabrera, M., Gayo, V., Fairweather, I., Lanusse, C., Alvarez, L., 2014. Testing albendazole resistance in *Fasciola hepatica*: validation of an egg hatch test with isolates from South America and the United Kingdom. J Helminthol 88, 286-292.

Carr, A.W., McCracken, R.O., Stillwell, W.H., 1993. Uncoupling of rat liver mitochondrial oxidative phosphorylation by the fasciolicide triclabendazole and its sulfoxide and sulfone metabolites. J Parasitol 79, 198-204.

Charlier, J., Bennema, S.C., Caron, Y., Counotte, M., Ducheyne, E., Hendrickx, G., Vercruysse, J., 2011. Towards assessing fine-scale indicators for the spatial transmission risk of *Fasciola hepatica* in cattle. Geospat Health 5, 239-245.

Charlier, J., De Meulemeester, L., Claerebout, E., Williams, D., Vercruysse, J., 2008. Qualitative and quantitative evaluation of coprological and serological techniques for the diagnosis of fasciolosis in cattle. Vet Parasitol 153, 44-51.

Charlier, J., Hostens, M., Jacobs, J., Van Ranst, B., Duchateau, L., Vercruysse, J., 2012. Integrating fasciolosis control in the dry cow management: the effect of closantel treatment on milk production. PLoS ONE 7, e43216.

Christensen, N.O., Nansen, P., 1976. The influence of temperature on the infectivity of *Fasciola hepatica* miracidia to *Lymnaea truncatula*. J Parasitol 62, 698-701.

Coles, G.C., Bauer, C., Borgsteede, F.H., Geerts, S., Klei, T.R., Taylor, M.A., Waller, P.J., 1992. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet Parasitol 44, 35-44.

Coles, G.C., Jackson, F., Pomroy, W.E., Prichard, R.K., von Samson-Himmelstjerna, G., Silvestre, A., Taylor, M.A., Vercruysse, J., 2006. The detection of anthelmintic resistance in nematodes of veterinary importance. Veterinary Parasitology 136, 167-185.

Coles, G.C., Rhodes, A.C., Stafford, K.A., 2000. Activity of closantel against adult triclabendazole-resistant *Fasciola hepatica*. Vet Rec 146, 504.

Coles, G.C., Stafford, K.A., 2001. Activity of oxyclozanide, nitroxynil, clorsulon and albendazole against adult triclabendazole-resistant *Fasciola hepatica*. Vet Rec 148, 723-724.

Conceicao, M.A., Durao, R.M., Costa, I.H., da Costa, J.M., 2002. Evaluation of a simple sedimentation method (modified McMaster) for diagnosis of bovine fascioliosis. Vet Parasitol 105, 337-343.

Crossland, N.O., 1976. The effect of the molluscicide N-tritylmorpholine on transmission of *Fasciola hepatica*. Vet Rec 98, 45-48.

Dalton, J.P., 1998. Fasciolosis. CAB International.

Daniel, R., van Dijk, J., Jenkins, T., Akca, A., Mearns, R., Williams, D.J., 2012. Composite faecal egg count reduction test to detect resistance to triclabendazole in *Fasciola hepatica*. Vet Rec 171, 153, 151-155.

Daniel, R., van Dijk, J., Williams, D., 2010. Faecal egg count reduction test to detect lack of efficacy of triclabendazole. Vet Rec 167, 759.

Denwood, M.J., Reid, S.W., Love, S., Nielsen, M.K., Matthews, L., McKendrick, I.J., Innocent, G.T., 2010. Comparison of three alternative methods for analysis of equine Faecal Egg Count Reduction Test data. Prev Vet Med 93, 316-323.

Dreyfuss, G., Rondelaud, D., 1994. Comparative study of cercarial shedding by *Lymnaea tomentosa* Pfeiffer infected with either *F. gigantica* Cobbold or *F. hepatica* Linne. Bulletin de la Societe Francaise de Parasitologie 12, 43-54.

Dreyfuss, G., Vignoles, P., Rondelaud, D., Vareille-Morel, C., 1999. *Fasciola hepatica*: characteristics of infection in *Lymnaea truncatula* in relation to the number of miracidia at exposure. Exp Parasitol 92, 19-23.

Dumenigo, B.E., Espino, A.M., Finlay, C.M., Mezo, M., 2000. Kinetics of antibody-based antigen detection in serum and faeces of sheep experimentally infected with *Fasciola hepatica*. Vet Parasitol 89, 153-161.

Dumenigo, B.E., Mezo, M., 1999. Monoclonal antibody sandwich immunoassay detection of coproantigen to evaluate the efficacy of treatment in natural ovine fasciolosis. Res Vet Sci 66, 165-167.

Duwel, D., Reisenleiter, R., 1990. [*Fasciola hepatica*: coprological diagnosis in comparison to the worm burden in sheep and cattle]. Angew Parasitol 31, 211-217.

Efron, B., 1979. Bootstrap methods: Another look at the jackknife. The Annals of Statistics 7, 1-26.

Elliott, T.P., Spithill, T.W., 2014. The T687G SNP in a P-glycoprotein gene of *Fasciola hepatica* is not associated with resistance to triclabendazole in two resistant Australian populations. Mol Biochem Parasitol 198, 45-47.

Endah Estuningsih, S., Widjayanti, S., Adiwinata, G., Piedrafita, D., 2004. Detection of coproantigens by sandwich ELISA in sheep experimentally infected with *Fasciola gigantica*. Trop Biomed 21, 51-56.

Enigk, K., Hildebrandt, J., 1964. Survival of metacercariae of *Fasciola hepatica* in hay.

Espino, A.M., Diaz, A., Perez, A., Finlay, C.M., 1998. Dynamics of antigenemia and coproantigens during a human *Fasciola hepatica* outbreak. J Clin Microbiol 36, 2723-2726.

Espino, A.M., Marcet, R., Finlay, C.M., 1997. *Fasciola hepatica*: detection of antigenemia and coproantigens in experimentally infected rats. Exp Parasitol 85, 117-120.

Fairweather, I., 2005. Triclabendazole: new skills to unravel an old(ish) enigma. Journal of Helminthology 79, 227-234.

Fairweather, I., 2009. Triclabendazole progress report, 2005-2009: an advancement of learning? J Helminthol 83, 139-150.

Fairweather, I., 2011a. Raising the bar on reporting 'triclabendazole resistance'. Vet Rec 168, 514-515.

Fairweather, I., 2011b. Reducing the future threat from (liver) fluke: realistic prospect or quixotic fantasy? Vet Parasitol 180, 133-143.

Fairweather, I., 2011a. Liver fluke isolates: a question of provenance. Veterinary Parasitology 176, 1-8.

Fairweather, I., Boray, J.C., 1999a. Fasciolicides: efficacy, actions, resistance and its management. Vet J 158, 81-112.

Fairweather, I., Boray, J.C., 1999b. Fasciolicides: efficacy, actions, resistance and its management. Veterinary Journal 158, 81-112.

Fairweather, I., McShane, D.D., Shaw, L., Ellison, S.E., O'Hagan, N.T., York, E.A., Trudgett, A., Brennan, G.P., 2012. Development of an egg hatch assay for

the diagnosis of triclabendazole resistance in *Fasciola hepatica*: proof of concept. Vet Parasitol 183, 249-259.

Fernando, P., Vidya, T.N., Rajapakse, C., Dangolla, A., Melnick, D.J., 2003. Reliable noninvasive genotyping: fantasy or reality? J Hered 94, 115-123.

Flanagan, A., Edgar, H.W., Gordon, A., Hanna, R.E., Brennan, G.P., Fairweather, I., 2011a. Comparison of two assays, a faecal egg count reduction test (FECRT) and a coproantigen reduction test (CRT), for the diagnosis of resistance to triclabendazole in *Fasciola hepatica* in sheep. Vet Parasitol 176, 170-176.

Flanagan, A.M., Edgar, H.W., Forster, F., Gordon, A., Hanna, R.E., McCoy, M., Brennan, G.P., Fairweather, I., 2011b. Standardisation of a coproantigen reduction test (CRT) protocol for the diagnosis of resistance to triclabendazole in *Fasciola hepatica*. Vet Parasitol 176, 34-42.

Fox, N.J., White, P.C., McClean, C.J., Marion, G., Evans, A., Hutchings, M.R., 2011. Predicting impacts of climate change on *Fasciola hepatica* risk. PLoS ONE 6, e16126.

Frömming, E., 1956. Biologie der mitteleuropäischen Süsswasserschnecken. Dunker and Humblot, Berlin.

Fuchs, M.A., Ryan, L.A., Chambers, E.L., Moore, C.M., Fairweather, I., Trudgett, A., Timson, D.J., Brennan, G.P., Hoey, E.M., 2013. Differential expression of liver fluke beta-tubulin isotypes at selected life cycle stages. Int J Parasitol 43, 1133-1139.

Gaasenbeek, C.P., Moll, L., Cornelissen, J.B., Vellema, P., Borgsteede, F.H., 2001. An experimental study on triclabendazole resistance of *Fasciola hepatica* in sheep. Vet Parasitol 95, 37-43.

Gaasenbeek, C.P.H., Over, H.J., Noorman, N., de Leeuw, W.A., 1992. An epidemiological study of *Fasciola hepatica* in the Netherlands. Veterinary Quarterly 14, 140-144.

Garcia, L.G., Nicholson, K.L., Hoffman, T.W., Lawrence, T.E., Hale, D.S., Griffin, D.B., Savell, J.W., Vanoverbeke, D.L., Morgan, J.B., Belk, K.E., Field, T.G., Scanga, J.A., Tatum, J.D., Smith, G.C., 2008. National Beef Quality Audit-2005: survey of targeted cattle and carcass characteristics related to quality, quantity, and value of fed steers and heifers. J Anim Sci 86, 3533-3543.

Gettinby, G., Byrom, W., 1991. Weather-based computer experiments on parasites. Preventive Veterinary Medicine 11, 293-308.

Gonzalez, L.C., Esteban, J.G., Bargues, M.D., Valero, M.A., Ortiz, P., Naquira, C., Mas-Coma, S., 2011. Hyperendemic human fascioliasis in Andean valleys: an altitudinal transect analysis in children of Cajamarca province, Peru. Acta Trop 120, 119-129.

Gordon, D., Zadoks, R., Skuce, P., Sargison, N., 2012a. Confirmation of triclabendazole resistance in liver fluke in the UK. Vet Rec 171, 159-160.

Gordon, D.K., Roberts, L.C., Lean, N., Zadoks, R.N., Sargison, N.D., Skuce, P.J., 2013. Identification of the rumen fluke, *Calicophoron daubney*i, in GB livestock: possible implications for liver fluke diagnosis. Vet Parasitol 195, 65-71.

Gordon, D.K., Zadoks, R.N., Stevenson, H., Sargison, N.D., Skuce, P.J., 2012b. On farm evaluation of the coproantigen ELISA and coproantigen reduction test in Scottish sheep naturally infected with *Fasciola hepatica*. Vet Parasitol 187, 436-444.

Greenfield, L., White, T.J., 1993. Sample preparation methods, In D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), Diagnostic molecular microbiology: principles and applications. American Society for Microbiology, Washington, D.C. pp 122-137.

Gregory, R.D., Woolhouse, M.E., 1993. Quantification of parasite aggregation: a simulation study. Acta Trop 54, 131-139.

Grimshaw, W.T., Hong, C., Hunt, K.R., 1996. Potential for misinterpretation of the faecal egg count reduction test for levamisole resistance in gastrointestinal nematodes of sheep. Vet Parasitol 62, 267-273.

Halferty, L., Brennan, G.P., Hanna, R.E., Edgar, H.W., Meaney, M.M., McConville, M., Trudgett, A., Hoey, L., Fairweather, I., 2008. Tegumental surface changes in juvenile *Fasciola hepatica* in response to treatment *in vivo* with triclabendazole. Vet Parasitol 155, 49-58.

Halferty, L., Brennan, G.P., Trudgett, A., Hoey, L., Fairweather, I., 2009. Relative activity of triclabendazole metabolites against the liver fluke, *Fasciola hepatica*. Vet Parasitol 159, 126-138.

Hanna, R.E., Cromie, L., Taylor, S.M., Couper, A., 2006. The effect of a parenteral ivermectin/closantel injection on the growth and reproductive development of early immature *Fasciola hepatica* in cattle. Vet Parasitol 142, 78-90.

Hanna, R.E., Edgar, H.W., McConnell, S., Toner, E., McConville, M., Brennan, G.P., Devine, C., Flanagan, A., Halferty, L., Meaney, M., Shaw, L., Moffett, D., McCoy, M., Fairweather, I., 2010. *Fasciola hepatica*: histological changes in the reproductive structures of triclabendazole (TCBZ)-sensitive and TCBZ-resistant flukes after treatment in vivo with TCBZ and the related benzimidazole derivative, Compound Alpha. Vet Parasitol 168, 240-254.

Hanna, R.E., McMahon, C., Ellison, S., Edgar, H.W., Kajugu, P.E., Gordon, A., Irwin, D., Barley, J.P., Malone, F.E., Brennan, G.P., Fairweather, I., 2015. *Fasciola hepatica*: A comparative survey of adult fluke resistance to triclabendazole, nitroxynil and closantel on selected upland and lowland

sheep farms in Northern Ireland using faecal egg counting, coproantigen ELISA testing and fluke histology. Vet Parasitol 207, 34-43.

Happich, F.A., Boray, J.C., 1969a. Quantitative diagnosis of chronic fasciolosis. 1. Comparative studies on quantitative faecal examinations for chronic *Fasciola hepatica* infection in sheep. Aust Vet J 45, 326-328.

Happich, F.A., Boray, J.C., 1969b. Quantitative diagnosis of chronic fasciolosis. 2. The estimation of daily total egg production of *Fasciola hepatica* and the number of adult flukes in sheep by faecal egg counts. Aust Vet J 45, 329-331.

Hayward, A.D., Wilson, A.J., Pilkington, J.G., Pemberton, J.M., Kruuk, L.E., 2009. Ageing in a variable habitat: environmental stress affects senescence in parasite resistance in St Kilda Soay sheep. Proc Biol Sci 276, 3477-3485.

Hecker, K.H., Roux, K.H., 1996. High and low annealing temperatures increase both specificity and yield in touchdown and stepdown PCR. Biotechniques 20, 478-485.

Hodasi, J.K., 1972. The output of cercariae of *Fasciola hepatica* by *Lymnaea truncatula* and the distribution of metacercariae on grass. Parasitology 64, 53-60.

Hodgkinson, J., Cwiklinski, K., Beesley, N.J., Paterson, S., Williams, D.J., 2013. Identification of putative markers of triclabendazole resistance by a genome-wide analysis of genetically recombinant *Fasciola hepatica*. Parasitology 140, 1523-1533.

Honer, M.R., 1965. The interpretation of faecal egg-counts. I. Daily variations in *Fasciola hepatica* egg-counts in cattle. Z Parasitenkd 26, 143-155.

Honer, M.R., Vink, L.A., 1963a. Contributions to the epidemiology of fascioliasis *hepatica* in the Netherlands. I. Studies on the dynamics of fascioliasis in lambs. Z Parasitenkd 22, 292-302.

Honer, M.R., Vink, L.A., 1963b. Contributions To The Epidemiology Of Fascioliasis *Hepatica* In The Netherlands. ii. Studies On Cattle Fascioliasis Z Parasitenkd 23, 107-120.

Howell, A., Baylis, M., Smith, R., Pinchbeck, G., Williams, D., 2015. Epidemiology and impact of *Fasciola hepatica* exposure in high-yielding dairy herds. Prev Vet Med 121, 41-48.

Hutchinson, G.W., Dawson, K., Fitzgibbon, C.C., Martin, P.J., 2009. Efficacy of an injectable combination anthelmintic (nitroxynil+clorsulon+ivermectin) against early immature *Fasciola hepatica* compared to triclabendazole combination flukicides given orally or topically to cattle. Vet Parasitol 162, 278-284.

Jones, E.M., Daniel, R., Coles, G.C., 2014. Diagnosis of resistance to triclabendazole. Vet Rec 174, 560.

Kajugu, P.E., Hanna, R.E., Edgar, H.W., McMahon, C., Cooper, M., Gordon, A., Barley, J.P., Malone, F.E., Brennan, G.P., Fairweather, I., 2015. *Fasciola hepatica*: Specificity of a coproantigen ELISA test for diagnosis of fasciolosis in faecal samples from cattle and sheep concurrently infected with gastrointestinal nematodes, coccidians and/or rumen flukes (paramphistomes), under field conditions. Vet Parasitol. *In press.*

Kalbe, M., Haberl, B., Haas, W., 1997. Miracidial host-finding in *Fasciola hepatic*a and *Trichobilharzia ocellata* is stimulated by species-specific glycoconjugates released from the host snails. Parasitol Res 83, 806-812.

Kane, H.J., Behm, C.A., Bryant, C., 1980. Metabolic studies on the new fasciolicidal drug, closantel. Mol Biochem Parasitol 1, 347-355.

Kaplan, R.M., 2002. Anthelmintic resistance in nematodes of horses. Vet Res 33, 491-507.

Kaplan, R.M., 2004. Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol 20, 477-481.

Kaplan, R.M., Nielsen, M.K., 2010. An evidence-based approach to equine parasite control: It ain't the 60s anymore. Equine Veterinary Education 22, 306-316.

Katcher, H.L., Schwartz, I., 1994. A distinctive property of Tth DNA polymerase: enzymatic amplification in the presence of phenol. Biotechniques 16, 84-92.

Keiser, J., Utzinger, J., 2009. Food-borne trematodiases. Clin Microbiol Rev 22, 466-483.

Kendall, S.B., McCullough, F.S., 1951. The Emergence of the Cercariae of *Fasciola hepatica* from the Snail *Lymnaea truncatula*. J Helminthol 25, 77-92.

Kenyon, F., Sargison, N.D., Skuce, P.J., Jackson, F., 2009. Sheep helminth parasitic disease in south eastern Scotland arising as a possible consequence of climate change. Veterinary Parasitology 163, 293-297.

Kirk, G.J.D., Bellamy, P.H., Lark, R.M., 2010. Changes in soil pH across England and Wales in response to decreased acid deposition. Global Change Biology 16, 3111-3119.

Lacey, E., 1988. The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles. Int J Parasitol 18, 885-936.

Lane, G., 1998. Anthelmintic resistance. Vet. Rec. 143, 232.

Loffert, D., 1997. PCR: Effects of template quality. Qiagen News 1: 8-10. 8-10.

Maes, L., Vanparijs, O., Lauwers, H., Deckers, W., 1990. Comparative efficacy of closantel and triclabendazole against *Fasciola hepatica* in experimentally infected sheep. Veterinary Record 127, 450-452.

Martin, K., 1987. Quantitativ-ökologische Untersuchungen zur Schneckenfauna in unterschiedlich ausgeprägten Bachuferbereichen des Mittleren Neckarraumes Veröff. Naturschutz Landschaftspflege Bad.-Württ. 62, 381-464.

Martin, K., Sommer, M., 2004. Effects of soil properties and land management on the structure of grassland snail assemblages in SW Germany. Pedobiologia 48, 193-203.

Martin, R.J., 1997. Modes of action of anthelmintic drugs. Vet J 154, 11-34.

Martinez-Perez, J.M., Robles-Perez, D., Rojo-Vazquez, F.A., Martinez-Valladares, M., 2012. Comparison of three different techniques to diagnose *Fasciola hepatica* infection in experimentally and naturally infected sheep. Vet Parasitol 190, 80-86.

Martinez-Valladares, M., Cordero-Perez, C., Castanon-Ordonez, L., Famularo, M.R., Fernandez-Pato, N., Rojo-Vazquez, F.A., 2010a. Efficacy of a moxidectin/triclabendazole oral formulation against mixed infections of *Fasciola hepatica* and gastrointestinal nematodes in sheep. Vet Parasitol 174, 166-169.

Martinez-Valladares, M., del Rosario Famularo, M., Fernandez-Pato, N., Castanon-Ordonez, L., Cordero-Perez, C., Rojo-Vazquez, F.A., 2010b. Efficacy of nitroxynil against *Fasciola hepatica* resistant to triclabendazole in a naturally infected sheep flock. Parasitol Res 107, 1205-1211.

Mas-Coma, M.S., Esteban, J.G., Bargues, M.D., 1999a. Epidemiology of human fascioliasis: a review and proposed new classification. Bull World Health Organ 77, 340-346.

Mas-Coma, S., 2005. Epidemiology of fascioliasis in human endemic areas. Journal of Helminthology 79, 207-216.

Mas-Coma, S., Angles, R., Esteban, J.G., Bargues, M.D., Buchon, P., Franken, M., Strauss, W., 1999b. The Northern Bolivian Altiplano: a region highly endemic for human fascioliasis. Tropical Medicine & International Health 4, 454-467.

Mas-Coma, S., Valero, M.A., Bargues, M.D., 2009. Chapter 2. Fasciola, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Advances in Parasitology 69, 41-146.

McCann, C.M., Baylis, M., Williams, D.J., 2010a. The development of linear regression models using environmental variables to explain the spatial

distribution of *Fasciola hepatica* infection in dairy herds in England and Wales. International Journal for Parasitology 40, 1021-1028.

McCann, C.M., Baylis, M., Williams, D.J., 2010b. Seroprevalence and spatial distribution of *Fasciola hepatica*-infected dairy herds in England and Wales. Veterinary Record 166, 612-617.

McConville, M., Brennan, G.P., Flanagan, A., Edgar, H.W., Hanna, R.E., McCoy, M., Gordon, A.W., Castillo, R., Hernandez-Campos, A., Fairweather, I., 2009. An evaluation of the efficacy of compound alpha and triclabendazole against two isolates of *Fasciola hepatica*. Vet Parasitol 162, 75-88.

McGarry, J.W., Ortiz, P.L., Hodgkinson, J.E., Goreish, I., Williams, D.J., 2007. PCR-based differentiation of *Fasciola* species (Trematoda: Fasciolidae), using primers based on RAPD-derived sequences. Ann Trop Med Parasitol 101, 415-421.

McKenna, D.R., Roebert, D.L., Bates, P.K., Schmidt, T.B., Hale, D.S., Griffin, D.B., Savell, J.W., Brooks, J.C., Morgan, J.B., Montgomery, T.H., Belk, K.E., Smith, G.C., 2002. National Beef Quality Audit-2000: survey of targeted cattle and carcass characteristics related to quality, quantity, and value of fed steers and heifers. J Anim Sci 80, 1212-1222.

Mezo, M., Gonzalez-Warleta, M., Carro, C., Ubeira, F.M., 2004. An ultrasensitive capture ELISA for detection of *Fasciola hepatica* coproantigens in sheep and cattle using a new monoclonal antibody (MM3). Journal of Parasitology 90, 845-852.

Mezo, M., Gonzalez-Warleta, M., Castro-Hermida, J.A., Muino, L., Ubeira, F.M., 2011. Association between anti-*F. hepatica* antibody levels in milk and production losses in dairy cows. Vet Parasitol 180, 237-242.

Millar, A.J., Waite, S., 1999. Molluscs in coppice woodland. Journal of Conchology 36, 25-48.

Mitchell, G., 2002. Update on fasciolosis in cattle and sheep. In Practice 24, 378-385.

Mitchell, G.B., Maris, L., Bonniwell, M.A., 1998. Triclabendazole-resistant liver fluke in Scottish sheep. Vet Rec 143, 399.

Moll, L., Gaasenbeek, C.P., Vellema, P., Borgsteede, F.H., 2000. Resistance of *Fasciola hepatica* against triclabendazole in cattle and sheep in The Netherlands. Vet Parasitol 91, 153-158.

Monteiro, L., Bonnemaison, D., Vekris, A., Petry, K.G., Bonnet, J., Vidal, R., Cabrita, J., Megraud, F., 1997. Complex polysaccharides as PCR inhibitors in feces: Helicobacter pylori model. J Clin Microbiol 35, 995-998.

Mooney, L., Good, B., Hanrahan, J.P., Mulcahy, G., de Waal, T., 2009. The comparative efficacy of four anthelmintics against a natural acquired *Fasciola hepatica* infection in hill sheep flock in the west of Ireland. Veterinary Parasitology 164, 201-205.

Morgan, E.R., Cavill, L., Curry, G.E., Wood, R.M., Mitchell, E.S., 2005. Effects of aggregation and sample size on composite faecal egg counts in sheep. Vet Parasitol 131, 79-87.

Mottier, L., Alvarez, L., Fairweather, I., Lanusse, C., 2006. Resistance-induced changes in triclabendazole transport in *Fasciola hepatica*: ivermectin reversal effect. J Parasitol 92, 1355-1360.

Mottier, L., Virkel, G., Solana, H., Alvarez, L., Salles, J., Lanusse, C., 2004. Triclabendazole biotransformation and comparative diffusion of the parent drug and its oxidized metabolites into *Fasciola hepatica*. Xenobiotica 34, 1043-1057.

Neuhaus, W., 1953. [The chemical sense of miracidia of *Fasciola hepatica*]. Z Parasitenkd 15, 476-490.

Novobilsky, A., Averpil, H.B., Hoglund, J., 2012. The field evaluation of albendazole and triclabendazole efficacy against *Fasciola hepatica* by coproantigen ELISA in naturally infected sheep. Vet Parasitol 190, 272– 276.

O'Brien, D.J., 1998. Fasciolosis: a threat to livestock. Irish Vet. J. 51, 539-541.

O'Neill, S.M., Brady, M.T., Callanan, J.J., Mulcahy, G., Joyce, P., Mills, K.H., Dalton, J.P., 2000. *Fasciola hepatica* infection downregulates Th1 responses in mice. Parasite Immunol 22, 147-155.

Oakley, G.A., Owen, B., Knapp, N.H., 1979. Production effects of subclinical liver fluke infection in growing dairy heifers. Veterinary Record 104, 503-507.

Ollerenshaw, C.B., 1959. The ecology of the liver fluke (*Fasciola hepatica*). Vet Rec 71, 591-965.

Ollerenshaw, C.B., 1967. Some observations on the epidemiology and control of fascioliasis in Wales. In: Proceedings of the Second International Liver flukeColloquium. Merck Sharp and Dohme International, Wageningen, pp. 103–125.

Ollerenshaw, C.B., 1971. Some observations on the epidemiology of fascioliasis in relation to the timing of molluscicide applications in the control of the disease. Vet Rec 88, 152-164.

Ollerenshaw, C.B., Rowlands, W.T., 1959. A method of forecasting the incidence of fascioliasis in Anglesey. Vet Rec 71, 591-598.

Ortiz, P., Scarcella, S., Cerna, C., Rosales, C., Cabrera, M., Guzman, M., Lamenza, P., Solana, H., 2013. Resistance of *Fasciola hepatica* against Triclabendazole

in cattle in Cajamarca (Peru): a clinical trial and an i*n vivo* efficacy test in sheep. Vet Parasitol 195, 118-121.

Over, H.J., 1982. Ecological basis of parasite control: trematodes with special reference to fascioliasis. Vet Parasitol 11, 85-97.

Overend, D.J., Bowen, F.L., 1995. Resistance of *Fasciola hepatica* to triclabendazole. Aust Vet J 72, 275-276.

Parnell, M.J., 1970. Determination of nitroxynil residues in sheep and calves. Pesticide Science 1, 138-143.

Pax, R.A., Bennett, J.L., 1989. Effect of closantel on intrategumental pH in *Schistosoma mansoni* and *Fasciola hepatica*. J Parasitol 75, 169-171.

Paz-Silva, A., Pedreira, J., Sanchez-Andrade, R., Suarez, J.L., Diaz, P., Panadero, R., Diez-Banos, P., Morrondo, P., 2002. Time-course analysis of coproantigens in rats infected and challenged with *Fasciola hepatica*. Parasitol Res 88, 568-573.

Prichard, R.K., Hall, C.A., Kelly, J.D., Martin, I.C., Donald, A.D., 1980. The problem of anthelmintic resistance in nematodes. Aust Vet J 56, 239-251.

Pritchard, G.C., Forbes, A.B., Williams, D.J., Salimi-Bejestani, M.R., Daniel, R.G., 2005. Emergence of fasciolosis in cattle in East Anglia. Vet Rec 157, 578-582.

Radstrom, P., Knutsson, R., Wolffs, P., Dahlenborg, M., Lofstrom, C., 2003. Pre-PCR processing of samples. Methods Mol Biol 216, 31-50.

Radstrom, P., Knutsson, R., Wolffs, P., Lovenklev, M., Lofstrom, C., 2004. Pre-PCR processing: strategies to generate PCR-compatible samples. Mol Biotechnol 26, 133-146.

Rapic, D., Dzakula, N., Cankovic, M., Stojcevic, D., 1984. Efficacy of triclabendazole against Fasciola hepatica in naturally infected sheep. Veterinarski arhiv (Yugoslavia).

Rapic, D., Dzakula, N., Sakar, D., Richards, R.J., 1988. Comparative efficacy of triclabendazole, nitroxynil and rafoxanide against immature and mature *Fasciola hepatica* in naturally infected cattle. Vet Rec 122, 59-62.

Rapsch, C., Dahinden, T., Heinzmann, D., Torgerson, P.R., Braun, U., Deplazes, P., Hurni, L., Bar, H., Knubben-Schweizer, G., 2008. An interactive map to assess the potential spread of *Lymnaea truncatula* and the free-living stages of *Fasciola hepatica* in Switzerland. Veterinary Parasitology 154, 242-249.

Rapsch, C., Schweizer, G., Grimm, F., Kohler, L., Bauer, C., Deplazes, P., Braun, U., Torgerson, P.R., 2006. Estimating the true prevalence of *Fasciola*

*hepatic*a in cattle slaughtered in Switzerland in the absence of an absolute diagnostic test. International Journal for Parasitology 36, 1153-1158.

Relf, V., Good, B., McCarthy, E., de Waal, T., 2009. Evidence of *Fasciola hepatica* infection in *Radix peregra* and a mollusc of the family Succineidae in Ireland. Vet Parasitol 163, 152-155.

Robinson, M.W., Dalton, J.P., 2009. Zoonotic helminth infections with particular emphasis on fasciolosis and other trematodiases. Philos Trans R Soc Lond B Biol Sci 364, 2763-2776.

Robinson, M.W., Lawson, J., Trudgett, A., Hoey, E.M., Fairweather, I., 2004. The comparative metabolism of triclabendazole sulphoxide by triclabendazole-susceptible and triclabendazole-resistant *Fasciola hepatica*. Parasitol Res 92, 205-210.

Robinson, M.W., Trudgett, A., Hoey, E.M., Fairweather, I., 2002. Triclabendazole-resistant *Fasciola hepatica*: beta-tubulin and response to *in vitro* treatment with triclabendazole. Parasitology 124, 325-338.

Robles-Perez, D., Martinez-Perez, J.M., Rojo-Vazquez, F.A., Martinez-Valladares, M., 2013. The diagnosis of fasciolosis in feces of sheep by means of a PCR and its application in the detection of anthelmintic resistance in sheep flocks naturally infected. Vet Parasitol 197, 277-282.

Robles-Perez, D., Martinez-Perez, J.M., Rojo-Vazquez, F.A., Martinez-Valladares, M., 2014. Development of an egg hatch assay for the detection of anthelmintic resistance to albendazole in *Fasciola hepatica* isolated from sheep. Vet Parasitol 203, 217-221.

Robles-Pérez, D., Martínez-Pérez, J.M., Rojo-Vázquez, F.A., Martínez-Valladares, M., 2013. The Diagnosis Of Fasciolosis In Faeces Of Sheep By Means Of A Pcr And Its Application In The Detection Of Anthelmintic Resistance In Sheep Flocks Naturally Infected. Vet Parasitol 197, 277-282.

Rodriguez-Perez, J., Hillyer, G.V., 1995. Detection of excretory-secretory circulating antigens in sheep infected with *Fasciola hepatica* and with S*chistosoma mansoni* and *F. hepatica*. Vet Parasitol 56, 57-66.

Rokni, M.B., Massoud, J., O'Neill, S.M., Parkinson, M., Dalton, J.P., 2002. Diagnosis of human fasciolosis in the Gilan province of Northern Iran: application of cathepsin L-ELISA. Diagn Microbiol Infect Dis 44, 175-179.

Rondelaud, D., Dreyfuss, G., 1996. Variabilite´ de l'infestation fasciolienne chez Lymnaea truncatula Mu¨ller par rapport a` la localisation de ses gıˆtes sur les re´seaux hydrographiques. Bulletin de la Socie´te´Franc¸aise de Parasitologie 14, 189–194.

Rondelaud, D., Vignoles, P., Dreyfuss, G., 2004. *Fasciola hepatica*: the developmental patterns of redial generations in naturally infected *Galba truncatula*. Parasitol Res 94, 183-187.

Rossen, L., Norskov, P., Holmstrom, K., Rasmussen, O.F., 1992. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. Int J Food Microbiol 17, 37-45.

Rowcliffe, S.A., Ollerenshaw, C.B., 1960. Observations on the bionomics of the egg of Fasciola hepatica. Ann Trop Med Parasitol 54, 172-181.

Salimi-Bejestani, M.R., Daniel, R.G., Felstead, S.M., Cripps, P.J., Mahmoody, H., Williams, D.J., 2005a. Prevalence of *Fasciola hepatica* in dairy herds in England and Wales measured with an ELISA applied to bulk-tank milk. Veterinary Record 156, 729-731.

Salimi-Bejestani, M.R., McGarry, J.W., Felstead, S., Ortiz, P., Akca, A., Williams, D.J., 2005b. Development of an antibody-detection ELISA for *Fasciola hepatica* and its evaluation against a commercially available test. Research in Veterinary Science 78, 177-181.

Sanchez-Vazquez, M.J., Lewis, F.I., 2013. Investigating the impact of fasciolosis on cattle carcase performance. Vet Parasitol 193, 307-311.

Sangster, N.C., 1999. Anthelmintic resistance: past, present and future. Int J Parasitol 29, 115-124; discussion 137-118.

Sargison, N., 2008. Fluke diseases of UK ruminant livestock Part 1: Life cycles, economic consequences and diagnosis.

Sargison, N., 2012. Diagnosis of triclabendazole resistance in *Fasciola hepatica*. Vet Rec 171, 151-152.

Sargison, N.D., Scott, P.R., 2011a. Anthelmintic resistance: potential benefits of 'over-diagnosis'. Vet Rec 168, 646-647.

Sargison, N.D., Scott, P.R., 2011b. Diagnosis and economic consequences of triclabendazole resistance in *Fasciola hepatica* in a sheep flock in south-east Scotland. Vet Rec 168, 159.

Schmid, G., 1983. Mollusken vom Mindelsee. In: Der Mindelsee bei Radolfzell. Monographie eines Naturschutzgebietes auf dem Bodanrück. Natur- und Landschaftsschutzgebiete Baden-Württembergs. 11, 409-500.

Schmidt, G.D., Roberts, L.S., 2005. Foundations of Parasitology 7. McGraw-Hill, 2006.

SCOPS, 2012. Proceedings of a Workshop To Discuss Liver Fluke Control In Cattle And Sheep (http://www.scops.org.uk/content/Proceedings-of-Liver-Fluke-Workshop-August-2012.pdf).

Scott, P.R., Sargison, N.D., Macrae, A., Rhind, S.R., 2005. An outbreak of subacute fasciolosis in Soay sheep: ultrasonographic biochemical and histological studies. Vet J 170, 325-331.

Shareef, P.A., Brennan, G.P., McVeigh, P., Khan, M.A., Morphew, R.M., Mousley, A., Marks, N.J., Saifullah, M.K., Brophy, P.M., Maule, A.G., Abidi, S.M., 2014. Time-dependent tegumental surface changes in juvenile *Fasciola gigantica* in response to triclabendazole treatment in goat. Acta Trop 136, 108-117.

Skuce, P., van Dijk, J., Smith, D., Morgan, E., 2014. Implications of extreme weather events for risk of fluke infection. Vet Rec 175, 198-200.

Skuce, P.J., Fairweather, I., 1990. The effect of the hydrogen ionophore closantel upon the pharmacology and ultrastructure of the adult liver fluke *Fasciola hepatica*. Parasitol Res 76, 241-250.

Smith, B., Li, N., Andersen, A.S., Slotved, H.C., Krogfelt, K.A., 2011. Optimising bacterial DNA extraction from faecal samples: comparison of three methods. Open Microbiol J 5, 14-17.

Smith, G., Grenfell, B.T., 1994. Modelling of parasite populations: gastrointestinal nematode models. Vet Parasitol 54, 127-143.

Stansfield, D.G., Lonsdale, B., Lowndes, P.A., Reeves, E.W., Schofield, D.M., 1987. Field trials of triclabendazole against mixed age infections of *Fasciola hepatic*a in sheep and cattle. Veterinary Record 120, 459-460.

Stear, M.J., Abuagob, O., Benothman, M., Bishop, S.C., Innocent, G., Kerr, A., Mitchell, S., 2006. Variation among faecal egg counts following natural nematode infection in Scottish Blackface lambs. Parasitology 132, 275-280.

Stear, M.J., Bairden, K., Duncan, J.L., Gettinby, G., McKellar, Q.A., Murray, M., Wallace, D.S., 1995. The distribution of faecal nematode egg counts in Scottish Blackface lambs following natural, predominantly *Ostertagia circumcincta* infection. Parasitol 110 (Pt 5), 573-581.

Stitt, A.W., Fairweather, I., 1993. *Fasciola hepatica*: tegumental surface changes in adult and juvenile flukes following treatment in vitro with the sulphoxide metabolite of triclabendazole (Fasinex). Parasitol Res 79, 529-536.

Suhardono, S., Roberts, J., Copeman, D., 2006. Variations in the survival of *Fasciola gigantica* eggs in bovine dung stored in the sun as opposed to the shade. Trop Anim Health Pro 38, 379-382.

Tansatit, T., Sahaphong, S., Riengrojpitak, S., Viyanant, V., Sobhon, P., 2012. *Fasciola gigantica*: the *in vitro* effects of artesunate as compared to triclabendazole on the 3-weeks-old juvenile. Exp Parasitol 131, 8-19.

Taylor, E.L., 1949. The epidemiology of fascioliasis in Britain. In: Proceedings of the 14th International Veterinary Conference, vol. 2, London, pp. 81-87.

Taylor, M.A., Hunt, K.R., 1989. Anthelmintic drug resistance in the UK. Vet Rec 125, 143-147.

Taylor, M.A., Hunt, K.R., Goodyear, K.L., 2002. The effects of stage-specific selection on the development of benzimidazole resistance in *Haemonchus contortus* in sheep. Vet Parasitol 109, 29-43.

Thomas, O., Coles, G.C., Duffus, K., 2000. Triclabendazole resistant *Fasciola hepatica* in south-west Wales. Veterinary Record 146.

Toner, E., Brennan, G.P., Hanna, R.E., Edgar, H.W., Fairweather, I., 2010. Tegumental surface changes in adult *Fasciola hepatica* in response to treatment *in vivo* with triclabendazole in the sheep host. Vet Parasitol 172, 238-248.

Torgerson, P.R., Claxton, J., 1999. Epidemiology and control. In: Dalton, J.P. (Ed.), Fasciolosis. CABI, Oxon, pp. 113-149.

Turner, K., Armour, J., Richards, R.J., 1984. Anthelmintic efficacy of triclabendazole against *Fasciola hepatica* in sheep. Veterinary Record 114, 41-42.

Ueno, H., Yoshihara, S., 1974. Vertical distribution of *Fasciola gigantica* metacercariae on stems of rice plant grown in a water pot. Natl Inst Anim Health Q (Tokyo) 14, 54-60.

Urquhart, G.M., Armour, J., Doyle, J., Jennings, F.W., 1970. Studies on ovine fascioliasis. 3. The comparative use of a molluscicide and an anthelmintic in the control of the disease. Vet Rec 86, 338-345.

Valero, M.A., Darce, N.A., Panova, M., Mas-Coma, S., 2001. Relationships between host species and morphometric patterns in *Fasciola hepatica* adults and eggs from the northern Bolivian Altiplano hyperendemic region. Vet Parasitol 102, 85-100.

Valero, M.A., De Renzi, M., Panova, M., Garcia-Bodelon, M.A., Periago, M.V., Ordonez, D., Mas-Coma, S., 2006. Crowding effect on adult growth, pre-patent period and egg shedding of *Fasciola hepatica*. Parasitology 133, 453-463.

Valero, M.A., Ubeira, F.M., Khoubbane, M., Artigas, P., Muino, L., Mezo, M., Perez-Crespo, I., Periago, M.V., Mas-Coma, S., 2009. MM3-ELISA evaluation of coproantigen release and serum antibody production in sheep experimentally infected with *Fasciola hepatica* and *F. gigantica*. Vet Parasitol 159, 77-81.

Valovirta, I., 1968. Land molluscs in relation to acidity on hyperite hills in central Finland. Annales Zoologici Fennici 5, 245-253.

van Dijk, J., Eagle, S.J., Gillespie, A.V., Smith, R.F., Holman, A.N., Williams, H.J., 2015. Visual weight estimation and the risk of underdosing dairy cattle. Vet Rec 177, 75.

Van Dijk, J., Sargison, N.D., Kenyon, F., Skuce, P.J., 2010. Climate change and infectious disease: helminthological challenges to farmed ruminants in temperate regions. Animal 4, 377-392.

van Leeuwen, C., 2012. Speeding up the snail's pace: Bird-mediated dispersal of aquatic organisms. PhD thesis Wageningen University, The Nerthelands.

Vercruysse, J., Claerebout, E., 2001. Treatment vs non-treatment of helminth infections in cattle: defining the threshold. Vet Parasitol 98, 195-214.

Virkel, G., Lifschitz, A., Sallovitz, J., Pis, A., Lanusse, C., 2006. Assessment of the main metabolism pathways for the flukicidal compound triclabendazole in sheep. J Vet Pharmacol Ther 29, 213-223.

von Samson-Himmelstjerna, G., Coles, G.C., Jackson, F., Bauer, C., Borgsteede, F., Cirak, V.Y., Demeler, J., Donnan, A., Dorny, P., Epe, C., Harder, A., Hoglund, J., Kaminsky, R., Kerboeuf, D., Kuttler, U., Papadopoulos, E., Posedi, J., Small, J., Varady, M., Vercruysse, J., Wirtherle, N., 2009. Standardization of the egg hatch test for the detection of benzimidazole resistance in parasitic nematodes. Parasitol Res 105, 825-834.

Waldén, H.W., 1981. Communities and diversity of land molluscs in Scandinavian woodlands. I. High diversity communities in taluses and boulder slopes in SW Sweden. J Conchol 30, 351-372.

Walker, S.M., Johnston, C., Hoey, E.M., Fairweather, I., Borgsteede, F.H., Gaasenbeek, C.P., Prodohl, P.A., Trudgett, A., 2011. Potential role of hares in the spread of liver fluke in the Netherlands. Vet Parasitol 177, 179-181.

Walker, S.M., McKinstry, B., Boray, J.C., Brennan, G.P., Trudgett, A., Hoey, E.M., Fletcher, H., Fairweather, I., 2004. Response of two isolates of *Fasciola hepatica* to treatment with triclabendazole *in vivo* and *in vitro*. Parasitol Res 94, 427-438.

Wehausen, J.D., Ramey, R.R., 2nd, Epps, C.W., 2004. Experiments in DNA extraction and PCR amplification from bighorn sheep feces: the importance of DNA extraction method. J Hered 95, 503-509.

Weyant, R.S., Edmonds, P., Swaminathan, B., 1990. Effect of ionic and nonionic detergents on the Taq polymerase. Biotechniques 9, 308-309.

Whitlock, H.V., Kelly, J.D., Porter, C.J., Griffin, D.L., Martin, I.C.A., 1980. *In vitro* field screening for anthelmintic resistance in strongyles of sheep and horses.

Widjojoatmodjo, M.N., Fluit, A.C., Torensma, R., Verdonk, G.P., Verhoef, J., 1992. The magnetic immuno polymerase chain reaction assay for direct detection of salmonellae in fecal samples. J Clin Microbiol 30, 3195-3199.

Wilkinson, R., Law, C.J., Hoey, E.M., Fairweather, I., Brennan, G.P., Trudgett, A., 2012. An amino acid substitution in *Fasciola hepatica* P-glycoprotein from triclabendazole-resistant and triclabendazole-susceptible populations. Mol Biochem Parasitol 186, 69-72.

Winkelhagen, A.J., Mank, T., de Vries, P.J., Soetekouw, R., 2012. Apparent triclabendazole-resistant human *Fasciola hepatica* infection, the Netherlands. Emerg Infect Dis 18, 1028-1029.

Wolff, K., Eckert, J., Schneiter, G., Lutz, H., 1983. Efficacy of triclabendazole against *Fasciola hepatica* in sheep and goats. Veterinary Parasitology 13, 145-150.

Wood, I.B., Amaral, N.K., Bairden, K., Duncan, J.L., Kassai, T., Malone, J.B., Jr., Pankavich, J.A., Reinecke, R.K., Slocombe, O., Taylor, S.M., et al., 1995. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) second edition of guidelines for evaluating the efficacy of anthelmintics in ruminants (bovine, ovine, caprine). Vet Parasitol 58, 181-213.

Zimmerman, G.L., Jen, L.W., Cerro, J.E., Farnsworth, K.L., Wescott, R.B., 1982. Diagnosis of *Fasciola hepatica* infections in sheep by an enzyme-linked immunosorbent assay. American Journal of Veterinary Research 43, 2097-2100.