Student Final Report No. 6310008011 **August 2020**

DNA Diagnosis of fluke infective stages in the environment

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DNA Diagnosis of liver fluke infective stages in the environment

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CONTENTS

1. Abstract

Currently, forecasting liver fluke risk to livestock is performed at a relatively crude, regional scale, based on traditional seasonal disease patterns and prevailing climatic conditions. However, farmers are concerned about fluke risk at a more local level, specific to their farm and fields, for which no detection method is currently available. The ultimate indicator of fluke infection risk is the metacercarial cyst challenge on pasture.

The overall aim of this project was to develop methods for the detection and identification of fluke infective stages (metacercariae) in pasture samples. This was a key component of the full project detailed in the thesis –' Identification of viable liver fluke metacercarial challenge to livestock' (Cuthill, 2020).

DNA testing methods offer powerful tools for the determination of liver fluke risk on pasture. In this project, DNA methods for the detection of metacercariae from pasture samples were developed and their utility demonstrated. Two methods of DNA amplification were evaluated. Polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP), respectively, were compared in their performance to amplify metacercarial DNA from grass. LAMP was able to detect metacercarial DNA concentrations x100 lower than the limit detected by PCR in the presence of grass extracts. The results of a DNA assay performed on a pasture sample from a farm would confirm *F. hepatica* presence but would not inform farmers of the viability of the metacercariae detected. Realistic application of DNA testing for pasture samples would be done as an additional test, after metacercarial recovery and viability assays.

The methods developed in this thesis have potential to give farmers information as to the quantity, viability and species identity of metacercariae on their pasture. The combined power of this knowledge will improve our understanding of fluke risk on farms and help identify disease risk with adequate time to inform avoidance strategies and facilitate targeted disease control.

2. Introduction

Fasciola hepatica, commonly known as the liver fluke, is a leaf-shaped parasitic flatworm (trematode) capable of infecting a wide range of mammalian hosts (Dalton, 1999). In the United Kingdom (UK), *F. hepatica* is responsible for most cases of fasciolosis, with significant infections occurring in sheep and cattle.

Infections of *F. hepatica* can lead to disease; fasciolosis. The destructive movement and blood-feeding habit of newly-excysted juvenile flukes and/or adult liver fluke cause significant pathology in the definitive hosts. The basis of this project focuses on the development of methods to help quantify disease risk for sheep and cattle at farm/field-scale. Other hosts include; humans, deer, goats, and pigs, but disease in these species is rare and methods developed for this project are not applicable to these types of cases.

F. hepatica, like many other trematodes, has a complicated, indirect lifecycle (Fig 1.1). Adult fluke residing in the bile ducts of their host lay eggs which are excreted onto the pasture in the host faeces. Given suitable climatic and environmental conditions, microscopic ciliated larvae (miracidia) hatch from the eggs and go on to locate and penetrate the intermediate host, the mud snail, *Galba truncatula.* There are several replicative stages within the mud snail; miracidia turn into sporocysts, sporocysts generate rediae and rediae produce cercariae (Andrews, 1999). The larvae within *G. truncatula* asexually multiply, a single sporocyst can give rise to many cloned rediae and cercariae (Dreyfuss, Vignoles & Rondelaud, 2015).

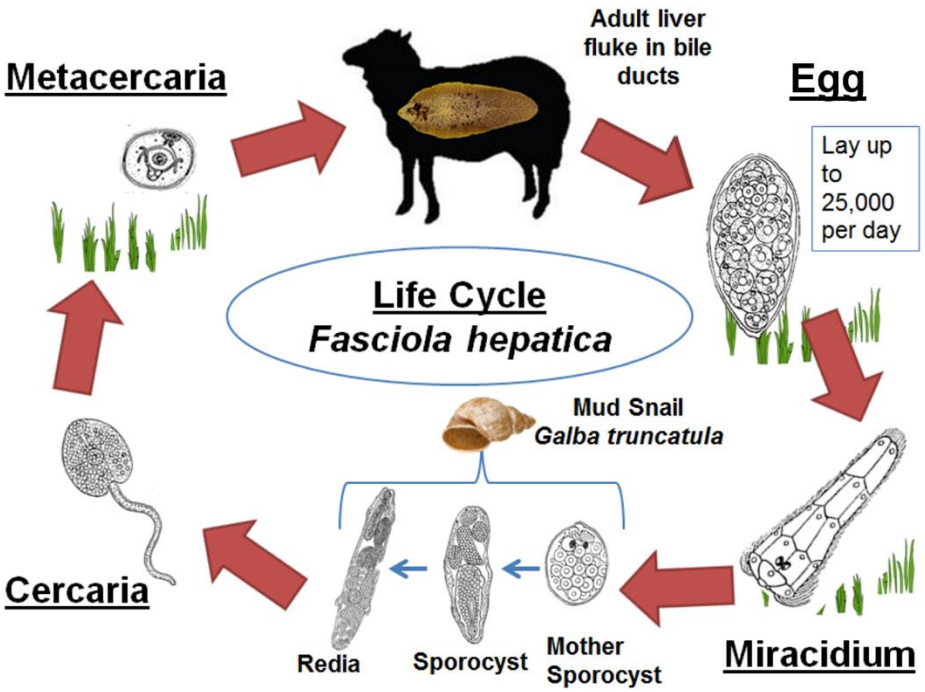

Figure 1.1 – Lifecycle of liver fluke (*Fasciola hepatica*)

Currently liver fluke risk in the UK is determined using the Ollerenshaw index (NADIS, 2019). Data of liver fluke prevalence, measurements of rainfall, the number of rain days and the potential evapotranspiration are used to produce a forecast of regional liver fluke risk (NADIS,

2019). Faecal eggs counts from livestock, numbers of liver condemnations, snail numbers, the number of infected snails and total of disease cases were used to validate the forecast index (Ollerenshaw, 1974). The climate and liver fluke epidemiology have changed since this index was first developed in the1960's (Kenyon et al., 2009). The habitat suitable for *G. truncatula* has expanded due to milder, wetter weather and now areas previously perceived to be fluke-free i.e. the eastern drier areas of the country are reporting cases of fasciolosis (Mitchell, 2002; Kenyon et al., 2009 and Howell, et al., 2015). Another drawback with this method of liver fluke forecasting is the absence of data relating to the presence of infectious metacercariae on pasture. Data relating to snails and climatic data relating to the survival of snails and eggs on the pasture can only give a crude estimation of liver fluke risk.

Sheep and cattle become infected when they ingest grass containing encysted *F. hepatica* metacercariae. **The presence and abundance of liver fluke metacercariae on pasture is the ultimate indicator of disease risk to sheep and cattle**. It is, therefore, logical that to prevent fasciolosis in their flock and herds, farmers should avoid grazing livestock on likely contaminated pasture at high risk times. Traditionally, late summer and autumn are the periods of highest risk in the UK (Ollerenshaw & Rowlands, 1959). The infectious metacercariae are likely to be at their most viable and abundant on the pasture at this time. The most challenging aspect of understanding fluke risk is determining when, where and for how long the highest risk will occur. A routine diagnostic test for the detection of *F. hepatica* metacercariae from pasture does not exist.

Traditionally, the presence of *F. hepatica*-infected *G. truncatula* in a field or in an area of a field was used as an indicator of potential fluke risk, but this is not a practical option for farmers. Most of the time, farmers already suspect fields or areas of pasture where their animals are becoming infected because high risk areas are identifiable, usually, by the presence of *Juncus sp.* reeds and waterlogged boggy ground. Indeed, in the years when the climatic conditions are favourable to the survival of *G. truncatula*, the collection of snails is fairly straightforward. However, searching for snails in fields is difficult work and often time consuming and labour-intensive (Charlier et al., 2014). Many environmental and human factors influence the success of finding snails. Identification of the correct species is obviously a key skill for this practice. Due to their size (~10mm shell length) this can be challenging (Fig 1.2). Furthermore, if *G. truncatula* snails are found this does not indicate if the snails are infected nor does it indicate if the life cycle of *F. hepatica* is supported and completed in the area. Thus collecting snails from pasture reveals very little about the risk of *F. hepatica* infection to grazing animals.

Figure 1.2 - *G. truncatula* photographed in situ, found on the edge of a man-made wader scrape in July 2017.

Despite the difficulty of detecting *G. truncatula* in the environment, detecting metacercariae on pasture poses an even greater challenge. The Ministry of Agriculture published a mechanical method for the recovery of metacercariae from pasture samples (Ministry of Agriculture, 1986). This method involves the washing of sampled grass through metal sieves, then utilises the oxidative effect of sulphuric acid to shrink any organic matter in order to improve the detection of metacercariae from other materials. This method boasts a recovery rate of up to 90% but it is not performed routinely, even by researchers because of the health and safety concerns of using large volumes of caustic acids with water.

An alternative to the time-consuming task of manual identification of metacercariae from pasture, is the detection of DNA targets from pasture samples. DNA methods are already used in research laboratories as routine diagnostic tools for the identification of *F. hepatica* from faecal samples and from snails collected from fields. Polymerase chain reaction (PCR) and loop mediated isothermal amplification (LAMP) are the main DNA-based assays used to identify parasite stages targeting the cytochrome oxidase subunit 1 (COX1). Relative to other diagnostic tests, PCR and LAMP are easy to interpret, with the appearance of corresponding DNA band(s) or fluorescence as the determination of a positive sample. These tests are also designed to be highly sensitive, detecting a particular target at very low concentrations. This is beneficial when

using environmental samples as DNA originating from life-stages of *F. hepatica* will be present in comparatively minute quantities.

COX1 is a highly conserved sequence of mitochondrial DNA. It is used widely within genetic taxonomy to determine the identity of closely related species (Hebert et al., 2003). PCR assays amplifying targets of this gene have already been established as reliable diagnostic tests for fasciolosis in sheep. *F. hepatica* DNA can be detected in the faeces of sheep 3 weeks post-infection with no cross contamination with DNA of other trematodes (Martìnez-Pèrez et al., 2012).

LAMP requires one constant temperature (~63 °C) for DNA amplification as isothermal polymerases, such as Bst, are used in LAMP reactions to displace DNA strands (Notomi et al., 2000). As a result, LAMP does not require denatured DNA template for amplification, unlike PCR and can amplify a large amount of DNA very quickly (~45 minutes) without the use of expensive equipment such as thermocyclers (Nagamine, 2002). Bst polymerases have a better tolerance than Taq polymerases to inhibitory components of biological samples such as blood and faeces (Poon et al., 2006). LAMP reactions boast strong specificity to their DNA target as this method uses 4 primers sets which can identify a total of 6 individual sequences of the DNA target (Sotiriadou & Karanis 2008). LAMP has been shown to have greater sensitivity than PCR. Ai et al., (2010) demonstrated a LAMP assay, targeting the ribosomal intergenic spacer of *F. hepatica,* that was 10^4 times sensitive than it's PCR counterpart. All of the characteristics of LAMP described indicate promise in the successful application of this method to the detection of *F. hepatica* metacercarial DNA from pasture samples.

3. Project Aims

- To demonstrate and compare the DNA techniques of PCR and LAMP in their abilities to detect *F. hepatica* metacercariae from samples of DNA extracted from grass spiked with *F. hepatica* DNA and from grass spiked with metacercariae

- Evaluate the possible application and translation of these tools for detecting fluke stages on farms and in the environment

4. Materials and methods

4.1 Sample preparation

4.1.1 Metacercariae

F. hepatica metacercariae (strain Gloucester, date shed unknown, Ridgeway Research, UK) were stored in sterile distilled water at 4°C on cellophane sheets within 10 ml capacity tubes. Maintenance involved the addition of fresh sterile water to the tubes every month.

4.1.2 Grass

Lolium perenne seeds (Homebase, UK) were sown and grown as described in Chapter 3 (section 3.2.6). This ensured that the grass was free of DNA from *F. hepatica*. The grass was harvested after a month of growth, cutting as close to the bottom of the stems without including soil in the samples.

4.1.3 Grass spiked with metacercariae

Twelve samples of freshly cut grass, 0.05g, were spiked with a single *F. hepatica* metacercaria each. These samples were used in both PCR and LAMP experiments, for the amplification of the *F. hepatica* COX1 sequence.
1g of freshly cut grass was spiked with 100 metacercariae. This mixture was processed using a blender (Philips, Netherlands) together in 100ml of water for 1 minute. The puréed contents was strained using a 100um metal sieve. The material remaining on top of the sieve was split into 12 subsamples weighing 0.05g for DNA extraction.

Samples of freshly harvested grass, cut using sterile scissors, weighing 0.05g were processed for DNA extraction separately for use as no target controls and serial dilutions.

4.1.4 DNA Extraction

All DNA samples, including *F. hepatica* metacercariae and grass samples, were extracted using the DNeasy® Blood and tissue extraction kit (Qiagen, Germany). DNA was extracted from metacercariae according to the number required for the experiment. The protocol for DNA extraction was followed as per manufacturer's instructions but DNA was eluted in 100 µl of Buffer AE rather than 200 µl. Full description of protocol can be found in Appendix 1.

4.1.5 *F. hepatica* DNA dilution series

The sensitivity of PCR and LAMP was determined using serial dilution of *F. hepatica* adult DNA diluted in water and in DNA extracted from grass. 10 µl of *F. hepatica* DNA was serially diluted 1:10 in nuclease free water to give set of samples with a concentration gradient of 1- $1x10^{-6}$

ng/µl. The same serial dilution conditions of *F. hepatica* DNA were created, 1:10 but using DNA extracted from grass.

4.1.6 DNA purification

DNA purification was performed on samples of DNA extracted from grass, DNA extracted from grass spiked with metacercariae.

Ethanol precipitation

10µl of 3M sodium acetate and 300µl of ethanol (100%) was added to DNA samples and stored at -20°C for 1 hour. Mixtures were centrifuged at 13000rpm at 4 °C for 30 minutes and the supernatant removed. The remaining pellet was washed with 500µl ethanol (70%). The mixture was centrifuged at 13000rpm for 15 minutes at 4°C and the supernatant removed. The remaining pellet was allowed to air dry on the lab bench before re-suspending in 50µl of molecular grade water. This method was provided by Seamus Stack (MAST).

QIA Quick PCR purification

Protocol was followed as per manufactures instructions (Qiagen)

4.1.7 Polymerase chain reaction (PCR)

The COX1 gene was chosen as the target for amplification. This is a highly conserved region of DNA and its use in taxonomy studies allows for identification and discern between closely related animals (Hebert et al., 2003). F. hepatica COX1 PCR was performed in 20 µl reaction volumes containing: 10x reaction buffer (Invitrogen, USA), 50mM MgCl2 (Invitrogen, USA), dNTP mix (Invitrogen, USA), Nuclease free H2O (Sigma) and 5 U Platinum Taq polymerase (Invitrogen, USA). The F. hepatica mitochondrial DNA fragment FhCox1 was amplified from metacercarial DNA using Cox 1 F 5’ –GTTGGCATATTGCGGCTTAG-3’ and Cox 1 R 5’-AGGGATCTGCACCTCAACTC-3’ primers (Martínez-Pérez et al., 2012). Cycling conditions were as follows: 2 minutes at 95°C, followed by 40 cycles of denaturing at 95°C for 30 seconds, annealing at 63°C for 30 seconds, extending at 72°C for 45 seconds and finally 10 minutes at 72°C (Biometra, Thermocycler gradient, Germany). PCR products were analysed on a 2% agarose gel prepared using Tris-acetate EDTA (TAE) and stained using GelRed™ Nucleic acid stain (Biotium). Gels were run at 100v for and visualized on AlphaImager™2200 (Alpha Innotech).

4.1.8 Loop mediated isothermal amplification (LAMP)

LAMP assays were performed using lyophilized LAMP pellets and kits provided by MAST (UK). V6.21, V6.31 and SG4.54 reagent pellets (MAST, UK) were used throughout the project. The primer mix was made up using F. h COX 1A primer set (MAST, UK) and volumes displayed in Table 1.1.

Table 1.1 – Primer sequences and volumes for *F. hepatica* LAMP assay

Primer	Sequence (5'-3')	Volume (µl)
F3	TTTGTAAAGCAGAGGTGGTT	0.5
B3	ACCGAGGAAGACCAAGAA	0.5
Fip	TCCGAATCACCTATCACAACCTGGTTTGGATTGATTGTTTCCG	4
Bip	GGCATATTGCGGCTTAGTTATGACAGAGCCACAAACGAAT	4
F Loop	AACAACCCTAAGCAAGCAAA	1
B Loop	GGTCGTTGGTAAGATCAGGATT	1

V6.21, V6.31 pellets were rehydrated in 86 µl of 0.1M Tris buffer. A master-mix was made; for every reaction, 8.6 µl of buffer and 0.4 µl of primer mix were added. 9 µl of master mix was used per reaction adding 1 µl DNA sample or 1 µl of nuclease free water for the negative control. The positive control was an in house 10ng/µl sample of adult *F. hepatica* DNA or a synthetic positive control provided by MAST within the LAMP kit.

MAST ISOPLEX® *DNA* LYO kit (MAST, UK) contained the SG4.54 pellets. These were reconstituted in 20µl 0.5M Tris and 68µl of molecular grade water. Synthetic positive controls included in the kit were dissolved in 50µl of molecular grade water for 5 minutes. Positive control primers, provided by MAST, were reconstituted in 20 µl of molecular grade water. Unless specified, assay reactions were set up as per the manufacturer's instructions.

Four LAMP assays deliberately deviated from manufacturer's instructions. These assays used SG4.54 pellets. Reconstitution of the pellets, for these four assays, was performed using 2µl 0.5M Tris and 84µl molecular grade water.

The amplification of DNA by LAMP was measured on a real-time PCR machine 7500 (Applied Biosystems, USA). Reactions were performed at 63°C and measuring the fluorescence every 33 seconds (99 cycles) on the FAM filter.

Positive detection of the *F. hepatica* COX1 (MAST) target was determined by the detection of fluorescence by dye FAM before the cut-off time of 40 minutes.

For assays that included the use of the V6.21 and V6.31 pellets the baseline was set at 3-15 cycles. For reactions using the SG4.54 reagent pellets the baseline of the assays was set at 3-5 cycles. The threshold for each assay was set individually.

5. Results

1.2. PCR

F. hepatica dilution series, as described in section (4.1.5), was used in a PCR with the *F. hepatica* COX1 primer set (Martínez-Pérez et al., 2012). Amplification of the target (~423bp) was observed in DNA concentrations 10 ng/µl to $1x10^{-4}$ ng/µl but not for DNA concentrations $1x10^{-5}$ or $1x10^{-6}$ ng/µl (Figure 5.1). The same dilution series was set up in DNA extracted from grass. The target was only detected in concentrations of 10 ng/µl, 1 ng/µl and 0.1 ng/µl *F. hepatica* DNA (Figure 5.2).

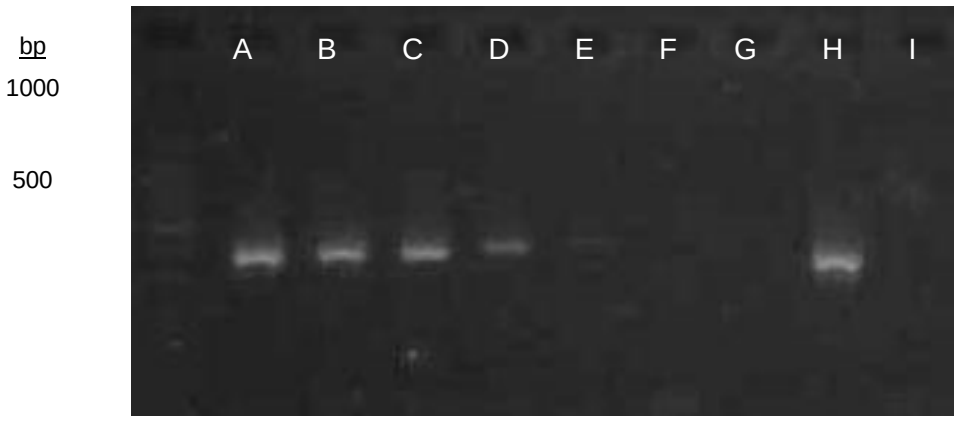

Figure 5.1 - Gel image of *F. hepatica* DNA amplified with F. h COX1 (Martìnez-Pèrez et al., 2012) primer set, ~423bp target. Wells A to G = 1- $1x10^{-6}$ ng/µl dilution series, well H = 10 ng/µl, well I = NF H_2O. 100bp DNA ladder (Invitrogen)

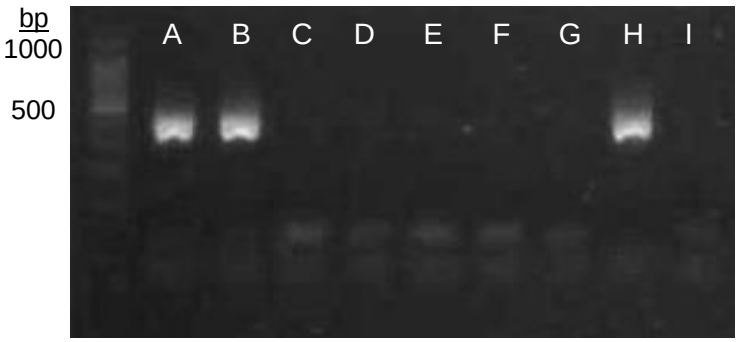

Figure 5.2 - Gel image of *F. hepatica* DNA amplification with F. h COX1 (Martìnez-Pèrez et al., 2012) primer set, ~423bp target. *F. hepatica* DNA diluted in DNA extracted from grass. Wells A to G = 1- $1x10^{-6}$ ng/µl dilution series, well H = 10 ng/µl, well I = NF H_2O. 100bp DNA ladder (Invitrogen)

1.3. LAMP

DNA extracted from 1, 10, 20, and 30 *F. hepatica* metacercariae were amplified using the LAMP assay (Fig 5.3). All blue dots presented below the red line represent positive detection of the *F. hepatica* COX1 target before the forty minute cut-off. The blue points above the red line represent negative samples for the *F. hepatica* COX1 target. The time taken to detect the target appears to be slower from DNA samples extracted from one metacercaria compared to DNA sample extracted from ten metacercariae. This trend does not continue with DNA samples extracted from larger numbers of metacercariae. Times to detection from DNA extracted from ten, twenty and thirty metacercariae cluster in similar patterns around fifteen minutes into the assay. The *F. hepatica* COX1 target was not successfully amplified from one replicate of DNA extracted from thirty metacercariae. The target was successfully detected from all replicates of the positive control; 10ng/µl of *F. hepatica* DNA, clustering around ten minutes. The target was not detected from the no target control.

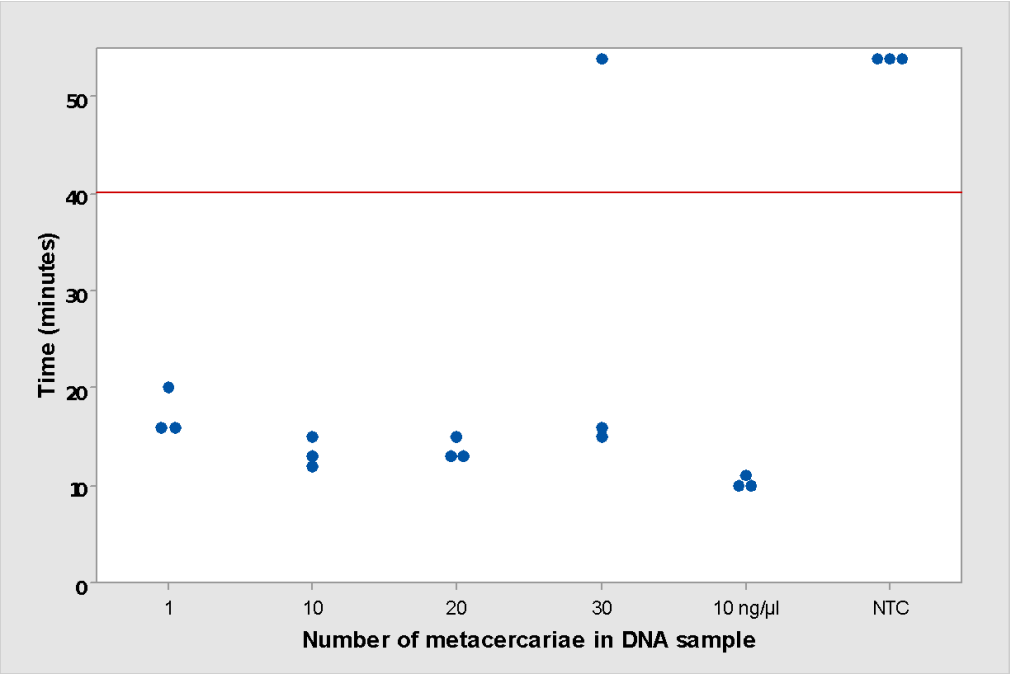

Figure 5.3 - Individual dot plot representing LAMP amplification of *F. hepatica* COX1 target from DNA extracted from different numbers of metacercariae using time to detection

Figure 5.4 depicts the limit of detection of LAMP assays performed on *F. hepatica* DNA serially diluted in water. The limit of detection of LAMP under these conditions is 0.01 ng/µl when *F. hepatica* DNA is serially diluted in water. The target is detected in two replicates of DNA concentration 0.001 ng/µl and one replicate of $1x10^{-6}$ ng/µl diluted in DNA extracted from grass (Fig 5.4). Again both assays were performed on the same plate utilising the same controls. Detection of replicates of 10 ng/µl cluster around fifteen minutes and the synthetic control

around ten minutes. False amplification is not observed in any of the grass no target controls (Fig 5.4).

The target was detected in DNA concentrations 1, 0.1, 0.01, 0.001 and 1x10 $^{-4}$ when *F. hepatica* DNA is diluted in DNA extracted from grass (Fig 5.5). Detection of the target started at around seventeen minutes and ended at around thirty three minutes (Fig 5.5). False amplification was not observed in any of the grass no target controls.

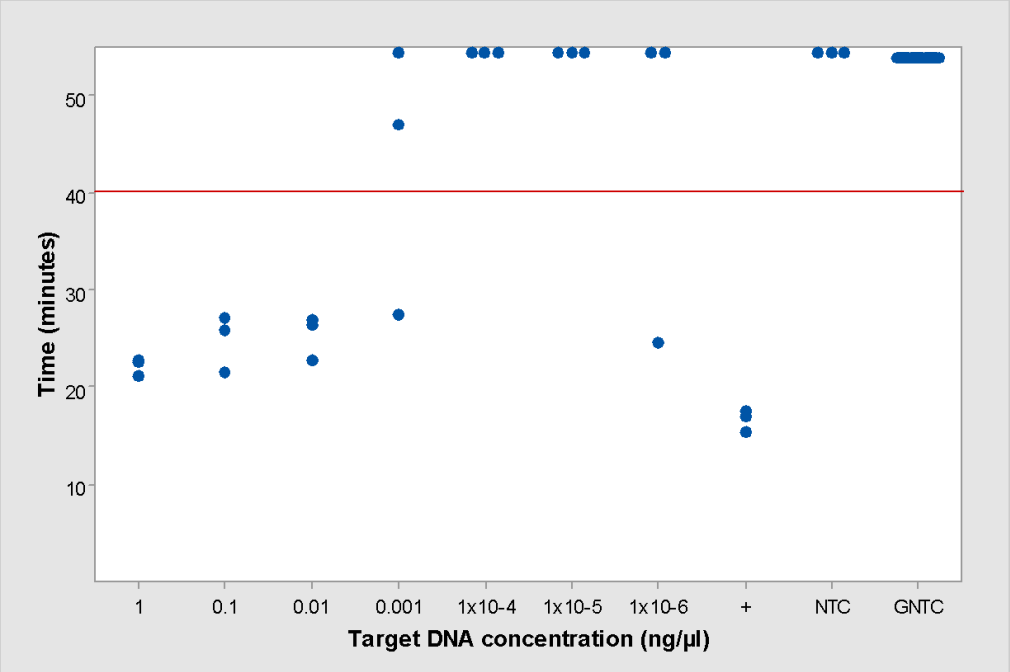

Figure 5.4- Individual dot plot representing LAMP amplification of the *F. hepatica* COX1 target from a serial dilution (1-1x10^{-6}ng/µl) of *F. hepatica* DNA in water, and from 10ng/µl of *F. hepatica* DNA. Each sample was tested in triplicate. This assay was performed using diluted Tris buffer. Each dilution was run in triplicate. + = Positive control, 10pg/µl MAST synthetic control, NTC = NF H2O. GNTC = 10 separate grass negative controls; DNA extracted from grass

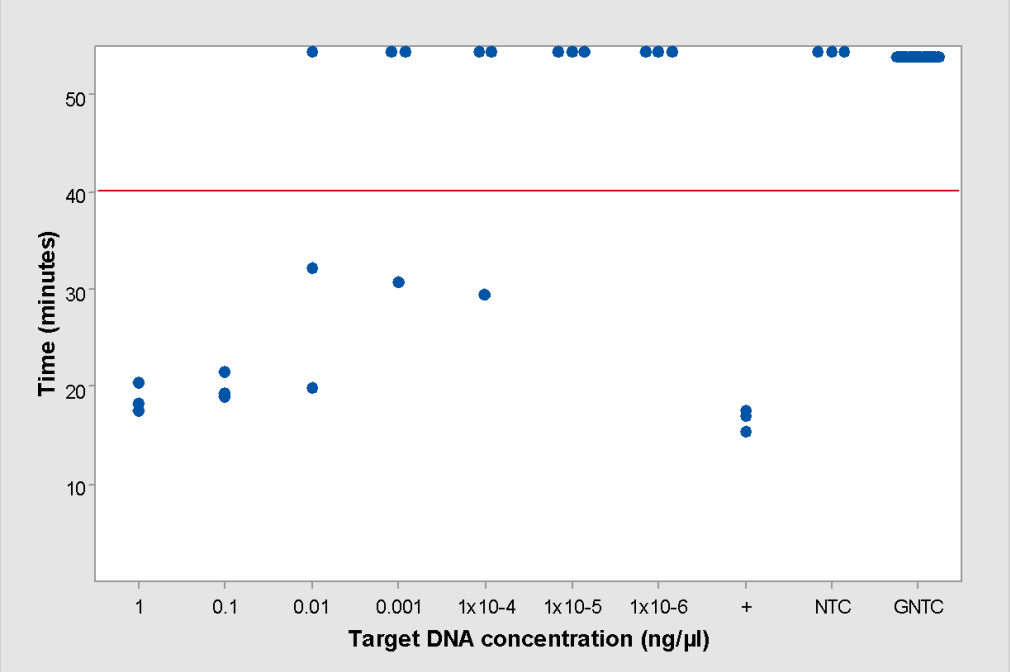

Figure 5.5 - Individual dot plot representing LAMP amplification of the *F. hepatica* COX1 target from a serial dilution (1-1x10^{-6}ng/µl) of *F. hepatica* DNA in DNA extracted from grass, and from 10ng/µl of *F. hepatica* DNA. Each sample was tested in triplicate. This assay was performed using diluted Tris buffer. + = Positive control, 10pg/µl MAST synthetic control, NTC = NF H_2O. GNTC = 10 separate grass negative control; DNA extracted from grass

6. Discussion

Overall, the results of the work carried out for this project show that it is possible to detect *F. hepatica* metacercariae from spiked grass samples using PCR and LAMP. Two sample scenarios were used to demonstrate this proof-of concept study. One sample scenario used serial dilutions of metacercarial DNA in DNA extracted from grass. This was done in order to understand the limit of detection of both PCR and LAMP when amplifying the COX1 target in the presence of grass DNA and products of its DNA extraction. In the second scenario fresh grass was spiked with metacercariae prior to DNA extraction. These samples represented pasture samples and were used in order to mimic “real farm” samples as a way of understanding how PCR and LAMP might perform on “real farm” samples but in a controlled way.

The amplification of the *F. hepatica* COX1 sequence was successfully demonstrated using PCR and LAMP in both scenarios with varied results. **Both methods of DNA amplification have the capability to identify very low concentrations of *F. hepatica* DNA, with detection sensitivity strong enough to detect DNA below the expected concentration of DNA from a single cyst (~2 ng/µl).** The variation in results centred on the inclusion of grass in samples. PCR had issues with sensitivity and LAMP had issues with false amplification when grass DNA and its extracts were included.

LAMP performed comparatively better than PCR in the presence of grass and its extracts. The limit of detection of LAMP when metacercarial DNA was diluted in DNA extracted from grass was 100 times lower than PCR (0.001ng/µl vs 0.1ng/µl). At the limit of its detection LAMP could amplify the COX1 target in under 30 minutes. By altering the concentration of Tris buffer included in the LAMP assay, it was possible to remove any false amplification caused by the inclusion of grass with minimal effect on the limit of detection.

The reduced level of sensitivity of PCR in this scenario would appear to be inconsequential when considered in the context of developing a presence/absence based test and the potential translation of this test into a tool for prediction of metacercarial challenge. A single metacercaria does not in itself equate to a high level of infective risk and one metacercaria can yield ~ 2ng/µl (personal observation) of DNA which is well within the limit of detection for PCR.

Key to identification of *F. hepatica* DNA from the environment is the ability to amplify DNA from pasture samples which, most commonly, are complex, full of detritus, and potential inhibitors. LAMP is the most suitable choice to take forward as a tool for the detection of metacercariae from pasture and as an ‘on-farm or ‘point of care’ test.

6.1 Application and translation

6.6.1. Issues with LAMP

High sensitivity in an assay is usually a desirable trait, however in the case of LAMP it can be unhelpful. Great care must be taken while setting up an assay to avoid contamination resulting in false positives. LAMP amplifies the DNA target into thousands of copies. Opening tubes or plates after a reaction is a cause for concern in terms of contamination of workspaces, especially if working in a lab space where different groups work on the same species. LAMP is also vulnerable to carry-over contamination whist setting up an assay (Kila et al. 2015). The occurrence of amplification in LAMP assay past the limit of detection is mostly likely the result of aerosol contamination during set up of the experiment. The contamination is likely to have occurred during the pipette dispensing of the dilution series of metacercarial DNA. The proximity of the wells on a 96 well plate may well have aided in the accidental dispersal of extra DNA into the wrong well. Applying commonly used practices of biosafety, such as wearing gloves, working under isolated UV hoods and using new pipette supplies with every experiment are good laboratory practices to adhere to in order to avoid cross contamination (Kwok & Higuchi, 1989).

No reactions were opened after amplification, but this also meant that LAMP products were not sequenced for validation. PCR product can be sequenced, this was used to validate the identification of single cysts from grass using LAMP.

6.2. Further work and considerations for an on-farm test

The results of the PCR and LAMP assays performed in this study highly suggest that the same methods could detect metacercarial DNA from ‘real farm’ samples. Despite this, there exist a number of barriers and considerations which need to be addressed before either test could be translated for use with pasture samples or as a ‘on farm’ test.

6.2.1. Other life stages

The most likely source of environmental DNA (eDNA) from *F. hepatica* is from eggs and miracidia (which hatch from fluke eggs), the tails of cercariae and from dead cercariae (Jones et al., 2018). It was argued that it is less likely that any eDNA would be originating from metacercariae unless the metacercariae were dead or damaged. The double cyst wall is thought to prevent any leakage of DNA into the environment (Jones et al., 2018). However, it should be noted again that the worked performed by Jones et al., (2018) was on water samples.

It is likely that the concentrations of DNA from each life stage would be reversed with pasture samples. The highest concentrations of *F. hepatica* DNA in pasture samples would most likely be from eggs and metacercariae as these life stages are designed to remain viable on the pasture for longer periods than miracidia and cercariae. Additionally, although water is important for the survival of eggs and metacercariae on pasture, it is not a direct requirement. Miracidia and cercariae require water in order to complete the life cycle. Miracidia travel through water films on surfaces in search of a snail host and cercariae use water to find a place to encyst so it is logical that their DNA would be in the highest abundance there.

Eggs, miracidia and cercariae are all life stages of *F. hepatica* that could be present on the pasture along with metacercariae. It is not possible to determine what life stage the COX1 target was amplified from the results of a PCR or LAMP assay.

6.2.2. Viability and Quantification

The results of a DNA-based test would not be powerful enough just on their own, especially in the context of developing tests for disease forecasting. The recovery of metacercariae coupled with the results of a LAMP assay would offer much more valuable information. The manual recovery of metacercariae from pasture allows extra epidemiological information to be collected, namely, the quantity and viability of metacercariae on the pasture. Recovered metacercariae can be counted and then excysted to determine the viable risk to grazing livestock. This can be applied to pasture samples that were positively identified from a LAMP assay. Or alternatively the species identity of any metacercariae can be confirmed by DNA assay after counting and excysting them.

6.2.3. Timings, presence and evaluation of risk

Presence of fluke DNA within a sample does not always correlate with the presence of a complete working lifecycle with live life stages in the sample area. The species may have been present in the past and is now no longer present because of insufficient conditions for the life cycle to complete, namely the absence of a host species in the environment. Time of year, weather and the presence of intermediate host species are other important factors when considering the risk of fluke on farms. Performing other diagnostics tests, such as faecal egg counts, snail surveys and the recovery of metacercariae from pasture would confirm a viable presence of *F. hepatica* on a farm or field.

It is important to consider the strength of a positive result in relation to the risk of fasciolosis. For example, one positive sample or replicate from an assay performed on many samples taken

from the same field is not a strong indication of high fluke risk. DNA detection can be most successfully applied through a combined approach. The level of risk must be considered on the basis of all available evidence. DNA detection can be used in the first instance to pinpoint areas of fields where liver fluke is present or as a confirmation of species identity after sample processing. But it is important to utilise all diagnostic tools at hand when deciding the level of fasciolosis risk. Considering the history of fasciolosis on a farm, assessing weather readings, performing snail surveys and manual recovery of metacercariae from pasture offer more comprehensive understanding of fluke risk on a farm. Combining data from many different farms around the country allows for the potential for greater epidemiological understanding of when and where the highest fluke risks might occur, thus leading to more accurate and reliable fasciolosis forecasts.

6.2.4. DNA extraction

The incorporation of larger sample sizes must be considered for the potential application of LAMP testing of pasture on farms. Optimal DNA extraction is key to DNA identification tests. Improper breakdown of material will result in the target of interest not being available for amplification which then can lead to false negative results. The failure to detect the target from samples of grass containing a single cyst may indicate that DNA extraction using this method is not optimal. Two out of ten grass samples spiked with a single cyst were not positive for the target when amplified with LAMP. Despite the tiny amounts of grass used in each staged sample, this may have been too much material to ensure complete breakdown of the metacercariae during DNA extraction. Single cysts may have been shielded from enzymatic breakdown by the grass, therefore, the COX1 target would not have been made available for amplification.

It is particularly challenging and expensive to extract high quality DNA from environmental samples using DNA extraction kits, because of the plant material present (Inglis , Pappas, Resende & Grattapaglia, 2018). The DNeasy (Qiagen) kit used in this study is expensive and not suited for large scale DNA extraction on plant material. The largest sample of grass from this study was 1.5g of grass. Although appearing to be a small sample size, in practice this volume of grass required blending and then subsampling before this could be processed for DNA extraction. A more suitable method of isolating the *F. hepatica* COX1 target DNA extraction from pasture samples may include the use of magnetic beads (Hawkins, 1998) or the use of affinity purification (Kadonaga & Tjia, 1986). However, even these methods might be too costly to be considered as a ‘on farm’ inexpensive test.

In order to test a farm for the presence of *F. hepatica* using LAMP, many samples of grass from one field would be required. The protocol of field sampling for pasture sample for DNA analysis might follow similar methods as those for the recovery of metacercariae. One method for the recovery of rumen fluke metacercariae, which could equally be applied to liver fluke, describes sampling grass in a 'W' format in fields at over 200 locations (O'Shaughnessy et al., 2018). This method is based on the recovery of metacercariae from herbage which suggests collection of 100g of grass per individual sample (Ministry of Agriculture, 1986) This is 100 times more grass than the largest sample used in this study. Performing DNA analysis on sample sizes this big would be impractical. Alternative methods of sample processing and DNA extraction that could handle larger samples sizes efficiently and at low cost could aid in this method of surveillance.

7. Industry messages

Determining species identity of any metacercariae isolated from pasture is important for the correct diagnosis of disease risk. Incorrect identification could lead to unnecessary and potentially damaging implementation of disease avoidance techniques and targeted treatment.

Both PCR and LAMP were demonstrated to be capable of detecting very small amounts of metacercarial DNA from spiked grass.

This is a proof-of-concept method for DNA-based detection of *F. hepatica* from the environment is achievable, the very nature of working with environmental samples is challenging.

Farmers can avoid fasciolosis in the stock by avoiding high risk pasture. Other methods of disease avoidance include the reduction of *G. truncatula* numbers through drainage of habitat. Molluscicides were previously implemented but are now banned because of the potential harm to the wider environment that they pose (Torgerson & Claxton, 1998). Reducing water from the surface and sub-surface of a field using drainage offers a less dangerous method for the removal of *G. truncatula* habitat. Where the removal of snail habitat is not possible, livestock can be prevented from grazing such areas by use of fencing and or moving them to low risk pasture.

In some west, upland areas of Scotland most of the grazing pasture could be deemed suitable habitat for *G. truncatula* and therefore identified as high risk for fasciolosis. Farmers in these areas may not have the option to graze their animals on low risk pasture and drainage may not be an economically viable option. In this situation farmers are advised to 'protect' pasture by reducing the egg output onto fields, thus reducing the risk of snails becoming infected.

LAMP has the potential to be transformed into an on farm-based test. For practicality and ease of use on farm, the current method of detecting the amplified target would need to be altered. Instead of using an intercalating fluorochrome dye (MAST), a fluorescence dye could be used to measure the amplification of the target from samples. The appearance of fluorescence within a sample tube can be detected by eye. This is beneficial for the development of a farm-based test as this does not require the use of RT-PCR thermo cyclers which are expensive and not suitable for easy determination of sample status by potential none lab based end point users.

As the origin of the amplified DNA cannot be known, any results should be considered in relation to any metacercarial recovery data, the fasciolosis status of stock on the farm, the presence and status of *G. truncatul*a on the field. The epidemiological strength of any theoretical results gathered from pasture samples would be poor, especially considering the amount of processing these methods require. Without physical confirmation of metacercariae on the pasture by their physical recovery, any DNA positive results are effectively meaningless from a *F. hepatica* risk perspective. *F. hepatica* COX 1 amplification can offer a quick, but crude pin point determination of *F. hepatica* present in a field or on a farm but does not inform the user as to the number or viable metacercariae present. However, positive and or negative confirmation of *F. hepatica* presence on fields/parts of fields does benefit some farms where confirmation of any low risk grazing would be helpful.

The amount of grass needed to determine the presence of *F. hepatica* on a field is considerable. Indeed, the MAFF (1986) method of metacercarial recovery from pasture requires 100g of grass per individual sample. Recognising that many hundreds of grams of grass would be required to survey a complete field amplifies the workload considerably.

Practical application of pasture DNA analysis to detect *F. hepatica* on this volume of grass is not realistic or appropriate for determining fasciolosis risk. Alternative methods of sample processing and DNA extraction that could handle larger samples sizes efficiently and at low cost would be the ideal solution. However, the more valuable solution would be focus on further development of efficient method for the recovery of wild metacercariae from pasture samples.

This project has highlighted that there is not a single, all-encompassing method or answer for the determination of liver fluke risk on farms. For an accurate assessment of fasciolosis risk on farms, all information must be considered, including: knowledge of the history of fasciolosis on farm, the current status of livestock, treatment plans, snail surveys and weather readings. All of this information, including the viability and quantity of metacercariae from pasture can be

combined from farms across the country to build up a more comprehensive understanding of liver fluke epidemiology and more accurate predictions of disease outbreaks on individual farms in the U.K.

8. References

Ai, L., Li, C., Elsheikha, H. M., Hong, S. J., Chen, J. X., Chen, S. H., Li, X., Cai, X. Q., Chen, M. X., and Zhu, X. Q. (2010). Rapid identification and differentiation of Fasciola hepatica and Fasciola gigantica by a loop-mediated isothermal amplification (LAMP) assay. Veterinary Parasitology. 174 (3–4), 228-233. https://doi.org/10.1016/j.vetpar.2010.09.005.

Andrews, S. J. (1999). The Life Cycle of *Fasciola hepatica*. In J. P. Dalton (Ed.), *Fasciolosis* (1-29). UK: CABI Publishing.

Charlier, J., Vercruysse, J., Morgan, E., van Dijk, J., & Williams, D. J. (2014). Recent advances in the diagnosis, impact on production and prediction of *Fasciola hepatica* in cattle. *Parasitology*, 141(3), 326-335. doi:10.1017/S0031182013001662

Choi, I. W., Kim, H. Y., Quan, J. H., Ryu, J. G., Sun, R., & Lee, Y. H. (2015). Monitoring of Fasciola Species Contamination in Water Dropwort by cox1 Mitochondrial and ITS-2 rDNA Sequencing Analysis. *Korean Journal of Parasitology*, 53(5), 641-645. doi:10.3347/kjp.2015.53.5.641

Cuthill, G. (2020). Identification of viable liver fluke metacercarial challenge to livestock. (PhD), University of Edinburgh.

Dreyfuss, G., Vignoles, P., & Rondelaud, D. (2015). The mud snail (*Galba truncatula*). Germany: LAP LAMBERT Academic Publishing. Stuttgart: Germany.

Hawkins, T. (1998). DNA purification and isolation using magnetic particles. US Patent 5,705,628, 1998

Hebert, P. D. N., Ratnaingham, S., & deWaard, J. R. (2003). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings Royal Society London* B (Sul.)(270), S96-S99

Howell, A., Baylis, M., Smith, R., Pinchbeck, G., & Williams, D. (2015). Epidemiology and impact of *Fasciola hepatica* exposure in high-yielding dairy herds. *Preventative Veterinary Medicine*, 121(1-2), 41-48. doi:10.1016/j.prevetmed.2015.05.013

Inglis , P. W., Paas, M. C. R., Resende, L. V., & Grattapaglia, D. (2018). Fast and inexpensive protocols for consistent extraction of high quality DNA and RNA from challenging plant and fungal samples for high-throughput SNP genotyping and sequencing alications*. PLoS One*, 13(10). doi:https://doi.org/10.1371/journal.

Jones, R. A., Brophy, P. M., Davis, C. N., Davies, T. E., Emberson, H., Rees Stevens, P. and Williams, H. W. (2018) Detection of Galba truncatula, Fasciola hepatica and Calicophoron daubneyi environmental DNA within water sources on pasture land, a future tool for fluke control?. *Parasite & Vectors.* 11 (1). 342. doi: 10.1186/s13071-018-2928-z.

Kadonaga, J. T., & Tjia, R. (1986). Affinity purification of sequence-specific DNA binding proteins. *Biochemistry*, 83, 5589-5893. doi:https://doiorg.ezproxy.is.ed.ac.uk/10.1073/pnas.83.16.5889

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(4), 293-297. doi:10.1016/j.vetpar.2009.03.027

Kila, E., Kima, S., Leea, Y., Kanga, E,, Leea, M., Choa, S., Kimc, M., . . . Leea, S (2015). Advanced loop-mediated isothermal amplification method for sensitive and specific detection of Tomato chlorosis virus using a uracil DNA glycosylase to control carry-over contamination. *Journal of Virological Methods*, 213, 68-74. doi:http://dx.doi.org/10.1016/j.jviromet.2014.10.020

Kwok, S., & Higuchi, A. R. (1989). Avoiding false positives with PCR. *Nature*, 339, 237-238.

Martìnez-Pèrez, J. M., Robles-Pèrez, D., Rojo-Vàzquez, F. A., & Martìnez-Valladares, M. (2012). Comparison of three different techniques to diagnose *Fasciola hepatica* infection in experimentally and naturally infected sheep. *Veterinary Parasitology*, 190, 80-86. doi: http://dx.doi.org/10.1016/j.vetpar.2012.06.002

Ministry of Agriculture, Fisheries and Food. (1986). Technique for the recovery of metacercariae from herbage. Manual of Veterinary Parasitological Laboratory Techniques Book 418 (Reference book 418 ed., . 33). London, UK.: Her Majesty's Stationary Office.

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

Nagamine, K., Hase, T., and Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. Molecular and Cellular Probes. 16. 223-229. doi:10.1006/mcpr.2002.0415

NADIS (2019). Parasite Forecast. Available at: http://www.nadis.org.uk/parasite-forecast.aspx [Accessed 20th June]

Notomi,T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, K., and Hase, T. (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Research. 28. E63. https://doi.org/10.1093/nar/28.12.e63

O'Shaughnessy, J., Garcia-Campos, A., McAloon, C. G., Fagan, S., de Waal, T., McElroy, M., . . . Zintl, A. (2018). Epidemiological investigation of a severe rumen fluke outbreak on an Irish dairy farm. *Cambridge Press Parasitology*. doi:https://doi.org/10.1017/

Ollerenshaw, C. B. (1974). Forecasting liver fluke disease. *British Society of Parasitology.* 42, 32-52

Ollerenshaw, C. B., & Rowlands, W. T. (1959). A method of forecasting the incidence of fascioliasis in Anglesey. *The The Veterinary Record*, 71(29), 591-598

Poon, L. L., Wong, B. W., Ma, E. H., Chan, K. H., Chow, L. M., Abeyewickreme, W., Tangpukdee, N., Yuen, K. Y., Guan, Y., Looareesuwan, S., and Peiris, J. S. (2006). Sensitive and inexpensive molecular test for falciparum malaria: detecting Plasmodium falciparum DNA directly from heat-treated blood by loop-mediated isothermal amplification. Clinical Chemistry. 52. 303-306. https://doi.org/10.1373/clinchem.2005.057901

Rondelaud, D., Vignoles, P., & Dreyfuss, G. (2004). *Fasciola hepatica*: the developmental patterns of redial generations in naturally infected *Galba truncatula*. *Parasitology Research*, 94, 183-187. doi:DOI 10.1007/s00436-004-1191-8

Sotiriadou, I. and Karanis, P. (2008). Evaluation of loop-mediated isothermal amplification for detection of Toxoplasma gondii in water samples and comparative findings by polymerase chain reaction and immunofluorescence test (IFT). *Diagnostic Microbiology And Infectious Disease*. 62 , 357-365

Torgerson, P., & Claxton, J. (1998). Epidemiology and control. In J. P. Dalton (Ed.), *Fasciolosis*. (113–149). Oxon: CABI Publishing

9. Research Dissemination

Proposed publications

- "Development of DNA assays for the identification of *F. hepatica* metacercariae from pasture samples"

- "Survival of *F. hepatica* metacercariae within grass silage and at pH associated with grass silage fermentation"

Presentations

Ninth International Sheep Veterinary Congress 22nd-26th May 2017;

- 8 minute presentation during session '*Global challenges due to small ruminant trematode parasites'* - "Identification of viable liver fluke and rumen fluke metacercarial challenge to grazing livestock"
- Poster presentation – *"In vitro* evaluation of the effect of pH on *F. hepatica* metacercarial viability"

World Association for the Advancement of Veterinary Parasitology (WAAVP) 2th-8th September 2017- Rapid, 2 minute, presentations;

- "*In vitro* evaluation of the impact of pH on *Fasciola hepatica* metacercarial viability"
- "Diagnosis of fluke infective stages in the environment"

AHDB

- 7/12/15; 5 minute presentation – "Identification of viable liver fluke and rumen fluke metacercarial challenge to grazing livestock"
- 15/11/16; Poster – "Diagnosis of fluke infective stages in the environment"
- 9/11/17; Poster – "Diagnosis of fluke infective stages in the environment"
- 2018; 15 minute final year presentation – "Diagnosis of fluke infective stages in the environment**"**

Co-authorship

Mitchell, G., Cuthill, G., Haine, A., Zadoks, R., Chaudhry, U., Skuce, P and Sargison, N. (2017). Evaluation of molecular methods for the field study of the natural history and changing epidemiology of *Dicrocoelium dendriticum. Veterinary Parasitology.* 235. 100-105. https://doi.org/10.1016/j.vetpar.2017.01.010.