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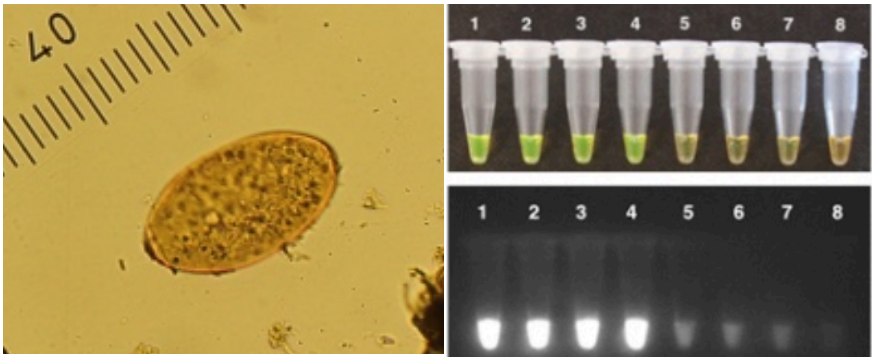
Maria Immaculata Arifin



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Development of a loop-mediated isothermal amplification (LAMP) and a polymerase chain reaction (PCR) assays for diagnosis of *Fasciola hepatica* in animal faeces and comparison with traditional diagnostic methods

Utveckling av en "loop-mediated isothermal amplification (LAMP)" och en "polymerase chain reaction (PCR)" assay för diagnos av *Fasciola hepatica* i djuravföring och jämförelser med traditionella diagnostiska metoder

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ABSTRACT

The liver fluke *Fasciola hepatica* is a parasitic trematode prevalent in mammals, primarily in sheep and cattle. There is a wide range of methods for diagnosis of *F. hepatica* infections, such as coproscopy, coproantigen ELISA, serum ELISA, PCR and Loop-mediated isothermal amplification (LAMP). As with all diagnostic methods, each presents benefits and disadvantages. Coproscopy requires no sophisticated equipment, but its robustness is limited due to difficulty of species identification and inability to detect early *F. hepatica* infections. Coproantigen ELISA can detect infections during the pre-patent period, yet its sensitivity in field applications is still debated. Serum ELISA is a good method for large herd screening, although it provides less insight to the infection status. PCR can differentiate between species using primers targeting the ITS2 region of *F. hepatica* genome. LAMP is a molecular method based on rapid amplification of target DNA under isothermal conditions. Both PCR and LAMP have only recently been attempted for *F. hepatica* identification in faeces. The aim of the study was to develop and set up LAMP and PCR methods for diagnosis of *F. hepatica* in ruminant faeces and to compare these molecular techniques with coproscopy, coproantigen detection and serology. A total of 64 faecal and blood samples were collected from 64 sheep and cattle from four farms in Sweden. Faecal samples were examined by faecal egg counts (FEC) with a sedimentation method and coproantigen ELISA using the Bio-X Bovine *Fasciola hepatica* Antigen ELISA Kit (Bio-X Diagnostics, Belgium). Serologic testing with an in-house ELISA was conducted on all serum samples. PCR and LAMP were performed with DNA extracted directly using PowerFecal® DNA isolation kit (MO BIO, USA) from faecal samples. *F. hepatica* eggs were present in 28 animals, while coproantigen and antibodies were detected in 36 and 53 animals respectively. PCR and LAMP managed to amplify only 3 and 6 samples respectively. Based on a composite reference standard, results showed that LAMP and PCR had a sensitivity of 14% and 8% respectively, which was much lower compared to the 78% sensitivity of FEC and 100% sensitivity of both coproantigen and serum ELISA. FEC, coproantigen ELISA and PCR all had 100% specificity, while LAMP and serum ELISA had 96% and 39% specificity respectively. In conclusion, FEC and coproantigen ELISA were good diagnostic tools for detection of patent *F. hepatica* infections. PCR and LAMP results could possibly improve with further development of faecal DNA extraction techniques.

Keywords: *Fasciola hepatica*, faeces, sheep, cattle, diagnosis, loop-mediated isothermal amplification, PCR, serology, coprology.

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1 Introduction

1.1 Background

The liver fluke *Fasciola hepatica* is a parasitic trematode of family Fasciolidae (Kassai, 1999). Fasciolosis, the disease caused by *F. hepatica* infection, is mostly prevalent in livestock, primarily in sheep and cattle (Robinson & Dalton, 2009; Freitas *et al.*, 2014). This liver fluke has a wide definitive host spectrum and a cosmopolitan distribution (Mas-Coma *et al.*, 2005; Taylor *et al.*, 2007). *F. hepatica* infections are of high veterinary importance, as it causes significant economic losses in livestock industry worldwide (Mezo *et al.*, 2004; Taylor *et al.*, 2007; Afshan *et al.*, 2013).

Control of fasciolosis is difficult, due to the lack of diagnostic methods that are sensitive, reliable and simple enough for inspection of large herds under field conditions (Mezo *et al.*, 2004). Identification criteria to distinguish between parasitic trematodes in livestock is mainly based on morphological characteristics of adult flukes and eggs, geographical distribution, host spectrum and pathological response of the host (Králová-Hromadová *et al.*, 2008). Coproscopy is the most widely used method for diagnosis of fasciolosis. However, use of coproscopy is limited due to difficulty of morphology-based species identification (Mage *et al.*, 2002) and inability to detect early infections with juvenile flukes (until 10-12 weeks post-infection) (Taylor *et al.*, 2007; Dusak *et al.*, 2012). Recently, coproantigen ELISA has been reported as a suitable coprological method for diagnosis of *F. hepatica*. It is sensitive in detecting the presence of *F. hepatica* antigens in faeces 6-8 weeks post-infection in cattle (Brockwell *et al.*, 2013). Some studies, however, showed that coproantigen ELISA is less sensitive compared to faecal egg count (FEC) under field conditions (Gordon *et al.*, 2012; Novobilský *et al.*, 2012). Serological tests, such as serum ELISA, are highly sensitive in detecting antibodies against *F. hepatica* within the host. These specific antibodies can be detected as early as 3-5 weeks post-infection (Mezo *et al.*, 2004), but cannot be used to determine drug efficacy as well as patent infections or re-infections (Williams *et al.*, 2014). Therefore, novel non-invasive diagnostic methods of *F. hepatica* in live animals are needed for an accurate, early detection of *F. hepatica* infection and also for the assessment of flukicide efficacy. Molecular assays, such as polymerase chain reaction (PCR), have been proven to be sensitive and with species specific primers can specifically detect *F. hepatica* infections as early as two weeks post-infection (Robles-Pérez *et al.*, 2013). Loop-mediated isothermal amplification (LAMP) assay is a molecular method based on rapid amplification of target DNA under isothermal conditions (Notomi *et al.*, 2015). This method demonstrated helminth detection in faeces with similar or

in some cases higher sensitivity compared to FEC and PCR (Melville *et al.*, 2014; Mugambi *et al.*, 2015; Martínez-Valladares & Rojo-Vázquez, 2016) and detection was as early as one week post-infection (Fernández-Soto *et al.*, 2014; Martínez-Valladares & Rojo-Vázquez, 2016). LAMP can be applied with the use of water baths or heat blocks, and simple end-point detection techniques contribute to lower diagnostic costs (Notomi *et al.*, 2000; Mori & Notomi, 2009). In addition, LAMP has been shown as an efficient tool in distinguishing different *Fasciola* species (Ai *et al.*, 2010). This method has only recently been tested for detection of *F. hepatica* in faeces (Martínez-Valladares & Rojo-Vázquez, 2016).

1.2 Aim and hypothesis

The aim of the study was to develop and set up LAMP and PCR methods for diagnosis of *F. hepatica* in ruminant faeces and to compare these molecular techniques with other conventional methods such as coproscopy, coproantigen ELISA and serology. The hypothesis was that LAMP could be more sensitive and specific in diagnosing *F. hepatica* infections in comparison to coproscopy, coproantigen ELISA, serum ELISA and PCR. Sensitivity and specificity of the different diagnostic methods used in this study for detection of *F. hepatica* infection were evaluated.

2 Literature Review

2.1 General description of *Fasciola hepatica*

F. hepatica, commonly known as the liver fluke, parasitizes in liver of ruminants, especially in sheep and cattle. *F. hepatica* are digenetic trematodes with a tegument body surface covered in spines (Taylor *et al.*, 2007; Williams *et al.*, 2014). An adult *F. hepatica* is 2-3.5 cm in length and 1 cm in width, leaf-shaped with grey-brown colouring, and possess a ventral and oral sucker (Kassai, 1999; Taylor *et al.*, 2007). *F. hepatica* is a hermaphroditic organism, meaning that each fluke possesses both male and female reproductive organs (Kassai, 1999; Williams *et al.*, 2014). *F. hepatica* eggs are ovoid with thin shells and an operculum, 130-150 μm in length. Eggs are yellowish-brown in colour and are filled with granules when excreted in faeces (Taylor *et al.*, 2007).

F. hepatica has an indirect life cycle, which means that it needs an intermediate host. In Europe, the intermediate host for *F. hepatica* is the aquatic snail *Galba truncatula* (Mas-Coma *et al.*, 2005). The pre-patent period of *F. hepatica* infections usually lasts for 10-12 weeks before the mature flukes start laying eggs in the biliary ducts, which are discharged with bile

into the digestive system and into faeces (Kaplan, 2001; Taylor *et al.*, 2007; Dusak *et al.*, 2012). In the environment, *F. hepatica* eggs hatch into miracidia which swims and penetrates the snail, develops into cercaria, which then leaves the intermediate host to find aquatic vegetation where it attaches itself and forms into the infective stage metacercaria (Kaplan, 2001; Taylor *et al.*, 2007). The whole process of *F. hepatica* development outside of the definitive host is dependent on optimal environmental conditions (Skuce & Zadoks, 2013).

An increasing prevalence of fasciolosis in Europe might be due to climate change, and farming practices that change in accordance with the conditions, e.g. longer grazing seasons. Mild winters might have also created an environment more suitable for the intermediate hosts of *F. hepatica*, thereby increasing the snail and parasite population (Fairweather, 2011; Gordon *et al.*, 2012). In Sweden, an increase in the prevalence of *F. hepatica* in Swedish livestock production has been reported (Novobilský *et al.*, 2014).

2.2 Diagnostic methods of *F. hepatica* infections

Detection of *F. hepatica* infections can be performed through a range of different diagnostic methods. Diagnosis can be determined by adult fluke recovery through post-mortem inspection of livers in abattoirs, faecal egg counts (FEC) by coproscopy methods, antibody detection in milk of lactating dairy cows and serum of animals, antigen detection in faeces by means of an enzyme-linked immunosorbent assay, liver enzyme levels, radiological imaging of the liver, and molecular methods (Mezo *et al.*, 2004; Dusak *et al.*, 2012; Robles-Pérez *et al.*, 2013; Skuce & Zadoks, 2013; Williams *et al.*, 2014; Martínez-Valladares & Rojo-Vázquez, 2016).

2.2.1 Coproscopy

Several methods have been used in previous studies for determining the FEC in *F. hepatica* infections. These methods include sedimentation, modified sedimentation with McMaster, Kato Katz and flotation methods using zinc sulphate buffer (Conceição *et al.*, 2002; Taylor *et al.*, 2007; Duthaler *et al.*, 2010; Kajugu *et al.*, 2015). Since most trematode eggs are heavy and large, detection and concentration of trematode eggs in faeces is most commonly done through the sedimentation method (Jacobs *et al.*, 2016). FEC is the most widely used method in diagnosis of *F. hepatica* infection, since it is not invasive and only requires the use of a microscope (Skuce & Zadoks, 2013). Although it detects patent infections, when the parasite burden is low no eggs might be detected (Brockwell *et al.*, 2013). However, eggs could also remain in the gall bladder for several weeks after animals have been successfully treated,

giving rise to false positive FECs (Fairweather, 2011; Gordon *et al.*, 2012). Furthermore, the number of eggs do not represent the infection intensity (Valero *et al.*, 2009a; Fairweather, 2011). Extensive differences in egg size within the *F. hepatica* species itself have previously been reported (Düwel, 1982; Mas-Coma *et al.*, 2005; Valero *et al.*, 2009a), complicating the egg identification process.

2.2.2 Serological diagnosis

The enzyme-linked immunosorbent assay, or ELISA for short, is a diagnostic method that relies on the interaction between antigen and the specific antibody against it. ELISA has a wide application, and one of the best known applications is in the field of parasitology, especially in epidemiological studies (Valero *et al.*, 2012). Serological tests, such as bulk milk tank ELISA, can be used to monitor herd infection levels in dairy herds at national level (Skuce & Zadoks, 2013; Williams *et al.*, 2014). The indirect ELISA using serum samples can detect infection of *F. hepatica* earlier than other diagnostic methods, as early as 3-5 weeks post-infection (Mezo *et al.*, 2004; Afshan *et al.*, 2013). A disadvantage of serological methods is that results do not correspond to current infections, but rather it reflects exposure to the parasite (Salimi-Bejestani *et al.*, 2005).

2.2.3 Coproantigen detection

Coproantigen ELISA detects *F. hepatica* excretory-secretory antigens, specifically the gastrodermal cells, in faeces of infected animals based on a highly sensitive monoclonal MM3 assay (Mezo *et al.*, 2004; Kajugu *et al.*, 2012). The commercial kit from Bio X Diagnostics uses a 96-well microplate sensitised with specific polyclonal antibody against *F. hepatica*. According to Flanagan *et al.* (2011b) and Martínez-Valladares & Rojo-Vázquez (2016), coproantigen ELISA can detect the presence of coproantigen in animal faeces as early as 4-5 weeks post-infection. Coproantigen ELISA values are also known to represent the fluke burden (Skuce & Zadoks, 2013). According to a study by Kajugu *et al.* (2012), this coproantigen ELISA does not cross-react with other trematode infections. As for the sensitivity of coproantigen ELISA, there have been disagreements between studies where Palmer *et al.* (2014) regards the test as being highly sensitive while other studies question the sensitivity of the test (Charlier *et al.*, 2008; Novobilský *et al.*, 2012).

2.2.4 Polymerase chain reaction (PCR)

Many parasitological researches have used PCR to identify and differentiate helminths at the molecular level. Several primers have been designed to recognize a specific region in the genome of *F. hepatica*, more specifically the internal transcribed spacer 2 (ITS2) located between the small (5.8S) and large subunit (28S) of ribosomal genes which is highly variable between different trematode classes (Ai *et al.*, 2010; Bazsalovicsová *et al.*, 2010; Choe *et al.*, 2011; Robles-Pérez *et al.*, 2013). Furthermore, two closely relative species *F. hepatica* and *F. gigantica* differ in six nucleotides with a 2.8% variation when a 213 bp long fragment of the ITS2 region of their genomes were compared (Mas-Coma *et al.*, 2005). This shows that the ITS2 region is specific to the point of distinguishing between *Fasciola* species. Majority of PCR methods in helminths have been based on DNA extraction from adult worms or eggs isolated from faeces of infected animals (Zhan *et al.*, 2001; Bazsalovicsová *et al.*, 2010; Ai *et al.*, 2011; Khademvatan *et al.*, 2013). PCR conducted directly on faecal DNA extractions (without egg isolation) could decrease the time needed for diagnosis by omitting the laborious process of helminth egg isolation and coproscopical procedures. Moreover, the PCR targets DNA from tegumental cells of flukes in the liver that are shed into faeces during liver migration, rather than eggs found in faeces (Martínez-Pérez *et al.*, 2012). Only a few studies have been published on PCR using DNA extracted directly from faecal samples without prior isolation of helminth eggs (Verweij *et al.*, 2001; Martínez-Pérez *et al.*, 2012; Robles-Pérez *et al.*, 2013; Meurs *et al.*, 2015).

2.2.5 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a relatively new nucleic acid amplification method first described by Notomi *et al.* (2000). The method uses the principle of amplification using the strand-displacement activity of a DNA polymerase and the creation of loop-structures with the help of four or six primers, resulting in a high number of amplicons within a short period of time. A major benefit of this method is that amplification occurs in isothermal conditions, therefore excluding the need of a thermocycler. Furthermore, the amplification time is considerably faster than that of PCR (Notomi *et al.*, 2015). Detection of LAMP products is relatively simple, commonly accomplished by the use of a turbidimeter which detects the magnesium pyrophosphate byproduct of LAMP or by addition of fluorescence dyes where changes are visible to the naked eye (Mori & Notomi, 2009). The use of LAMP has been widely applied in many different fields of biological research (Bakheit *et al.*, 2008; Nagdev *et al.*, 2011; Gallas-Lindemann *et al.*, 2016) including diagnosis of

Schistosoma mansoni (Fernández-Soto *et al.*, 2014), *Haemonchus contortus* (Melville *et al.*, 2014), *Necator americanus* (Mugambi *et al.*, 2015) and *F. hepatica* (Ai *et al.*, 2010; Martínez-Valladares & Rojo-Vázquez, 2016).

3 Methods

3.1 Sample collection

Samples were collected from two sheep farms Kållekärr and Töllås, and two cattle farms Henån and Binninge in Sweden during early December 2015. All four farms have a history of animals naturally infected by *F. hepatica*. Faecal and blood samples were obtained from 39 sheep (10 ewes and 29 lambs) and 25 cattle. All faecal samples were immediately frozen at -20°C for further analysis. Blood samples were centrifuged, and sera was collected and frozen at -20°C for further analysis.

3.2 Faecal egg counts

Faecal egg count (FEC) using sedimentation method was performed on all 64 faecal samples. Samples were defrosted one day before the faecal examination. Three grams of each sheep and ten grams of each cattle faecal sample were used in the sedimentation process. The sedimentation protocol was as follows: Each individual faecal sample was weighed and placed in a labelled beaker glass. The samples were suspended in water, homogenized, and filtered through a 150 μm sieve to get rid of large faecal particles. The filtrate was then subjected to washing after sedimentation for 5-6 min. Washing was done by removing filtrate from the beakers with a vacuum pump, leaving 1.5 cm (±0.5 cm) of filtrate sedimentation at the bottom and refilling the beakers with water again. This process was repeated 4-5 times depending on the clearness of the filtrate. Entire sediments were then placed on small petri discs and observation was performed using a microscope with 40x magnification (Olympus BX40). Eggs were counted and calculated as the number of eggs per gram of faeces (EPG) with the following formula:

EPG = Number of eggs counted by coproscopy / amount of faeces (g)

3.3 Serum ELISA

An indirect ELISA was performed on all 64 sera samples using an in-house protocol. Serum from cattle and sheep previously tested positive for *F. hepatica* infection were used as positive controls and serum from cattle and sheep previously tested negative for *F. hepatica* and positive for *Haemonchus contortus* infection were used as negative controls. All samples were analysed in duplicates. 96-well plates were coated with *F. hepatica* antigen with a concentration of 1:400 in coating buffer (Sodium carbonate 0.05 M in distilled water) and stored at 4°C one day before the assay was performed. Phosphate buffer saline (PBS) 10% was made using PBS 10x without calcium (SVA, code: 992442) in distilled water. PBS Tween, or washing buffer, was made by adding 0.5 ml Tween 20 into 1000 ml of the PBS solution. Blocking and dilution buffers were made using skim milk with concentrations of 10% and 5% in PBS Tween each respectively. For the assay, wells were first washed three times with washing buffer to remove any free *F. hepatica* antigens. Blocking buffer was added and plates were incubated for 30 min at 37°C. Every serum sample was diluted 1:50 in dilution buffer before being used in the assay. The plates were washed once after the 30 min incubation and 100 µl of the diluted serum was added per well. Wells were incubated for 60 min at 37°C, followed by washing three times with washing buffer. Secondary peroxidase-conjugated antibodies used were anti-bovine monoclonal antibodies (SVANOVA) for the bovine assay and anti-sheep IgG produced in donkey (Sigma-Aldrich) for the ovine assay. The secondary conjugated antibodies were diluted in a concentration of 1:10000 for bovine and 1:3000 for ovine before being used in the assay. 100 µl of the diluted secondary antibodies were added into each well and allowed to incubate for 60 min at 37°C. Following the incubation, three times washing with washing buffer was performed again. 100 µl of substrate was then added into each well. The substrate used was Tetramethylbenzidine (TMB) 20 mM (SVA, code: 382512) in Potassium citrate buffer 0.1 M (SVA, code: 381660) with a proportion of 1 TMB + 19 Potassium citrate buffer. Reaction was stopped by adding 100 µl of sulphuric acid (H_2SO_4) per well after 15 min of incubation in the dark. Absorbance was read immediately with a spectrophotometer (Multiskan FC, Thermoscientific) at 450 nm and the average OD values were calculated for each sample. Serum ELISA was considered positive when bovine samples had a sample-to-positive (S/P) ratio ≥15% and ovine samples ≥10% according to Novobilský *et al.* (2014). S/P ratios were calculated in Ms Excel with the following formulas (Novobilský *et al.*, 2014):

OD*i* = OD*ii* / OD*neg*

Sample-to-positive (S/P) ratio of sample *i* = (OD*i* / OD*pos*) x 100%

Note:

OD*ii* = mean Optical Density of sample *i* read at 450 nm (average OD of the duplicate samples).

OD*i* = Optical Density of sample *i* after adjustment with the mean OD of negative control.

OD*neg* = mean Optical Density of negative control read at 450 nm.

OD*pos* = mean Optical Density of positive control read at 450 nm.

3.4 Coproantigen ELISA

A sandwich ELISA using Bio-X Bovine *Fasciola hepatica* Antigen ELISA Kit (Bio K 201, Bio-X Diagnostics, Belgium) was performed on all faecal samples. Each individual faecal sample was homogenized with 2 ml dilution buffer (Bio K 201, Bio-X Diagnostics, Belgium) by mixing with a pipette and vortexing. For cattle 2 g of each faecal sample was used, and for sheep 0.5 g of each faecal sample was used. The homogenized samples were incubated overnight at 4°C. Supernatant was collected from all samples after centrifugation for 10 min at 1000 g and stored at -20°C. Further analysis was carried out according to the Bio-X Bovine *Fasciola hepatica* Antigen ELISA Kit (Bio K 201, Bio-X Diagnostics, Belgium) protocol with minor changes as follows: Faecal antigen extraction was done overnight. Washing buffer used was in house PBS Tween and the plates were not agitated during the first incubation step. Following the protocol completion, absorbance was read with a spectrophotometer (Multiskan FC, Thermoscientific) at 450 nm and the sample-to-positive (S/P) ratios were calculated in Ms Excel based on the optical density (OD) using formulas as previously described for serum ELISA. Coproantigen ELISA results were determined positive using a sample-to-positive ratio (S/P) cut-off from a previous research where samples with 1.6% positivity were considered positive (Novobilský & Höglund, 2015).

3.5 DNA extraction

DNA was extracted from all faecal samples using PowerFecal® DNA isolation kit (MO BIO, USA) according to manufacturer's protocol. Briefly, 250 mg of homogenized faeces was added into the dry bead tubes along with lysis buffer and incubated at 65°C for 10 min. The tubes were then subjected to severe shaking by horizontal vortexing, which was conducted by taping the dry bead tubes on a TTS 3 control shaker (Skafte MedLab) or a flat-bed vortex. After shaking, tubes were centrifuged (13,000 x g for 1 min) and supernatant was transferred into new tubes. An inhibitor removal solution (for precipitation of inorganic material, e.g. polysaccharides) was added and tubes were incubated at 4°C for 5 min. Tubes were then

centrifuged again, supernatant transferred into new tubes and salt solution was added to facilitate DNA binding to silica. Each solution mixture was then transferred to a new spin column and subjected to washing by ethanol. For the DNA elution process, 80 µl of elution buffer was added and left to incubate in the spin columns for 5 min before centrifugation. The elution process was performed twice on each spin column. DNA was stored at -20°C for further analyses. In addition, 3 randomly selected samples that tested negative by coproscopy, coproantigen ELISA and serum ELISA were taken twice and served as control for amplification inhibitors. 20 µl of DNA from adult *F. hepatica* was added to these three "spiked" samples before commencing the DNA extraction procedure mentioned above.

DNA was also extracted from an adult *F. hepatica* using QIAamp® DNA Mini Kit (Qiagen) following the manufacturer's protocol with the following modifications: 200 µl of elution buffer was used instead of 400 µl. The DNA was stored at -20°C for further analyses.

3.6 DNA concentration measurement

Concentration of *F. hepatica* DNA extracted from an adult fluke was measured using Qubit® 2.0 Fluorometer (Thermo Fisher Scientific). The DNA concentration was used to measure the sensitivity of PCR and LAMP in detecting *F. hepatica* DNA by creating serial dilutions of DNA concentrations. Quality and concentration of faecal DNA extractions was measured using Picodrop™.

3.7 Primer design

Primers for LAMP were designed to target the internal transcribed spacer 2 (ITS2) region of the *F. hepatica* genome. The primers were designed from a consensus of *F. hepatica* ITS2 sequences (Genbank accession numbers DQ683546.1, JF824668.1, KJ200622.1, AB207148.1) aligned with MUSCLE using the free open-source bioinformatics software UGENE (Okonechnikov *et al.*, 2012) and Primer Explorer v.4 (https://primerexplorer.jp/e/). The sequences of the newly designed primer (Primer 1) and the 187 bp target DNA sequence are shown in Table 3.1. and Figure 3.1, respectively. Primer 1 was also tested *in silico* for their specificity through BLAST alignment searches and comparison with other trematode's ITS2 sequences (EF534992.1, EF534993.1, EF612486.1, EU260079.1, HM026462.1, JQ966973.1, AY790883.1, KF543340.1) using UGENE.

Table 3.1. LAMP Primer 1 sequences designed and used in this study.

Primer	Length (bp)	Sequence (5' - 3')
F3	19	GCTGGCGTGATCTCCTCTA
B3	18	TAAGTGTGCCGACTAGGG
FIP (F1c-F2)	41	TCTGCCAAGACAAGGGTGCAT-GTGAGGTGCCAGATCTATGG
BIP (B1c-B2)	40	GTGCAGTGGCGGAATCGTGG-GATCGCCAAACACACTGACA

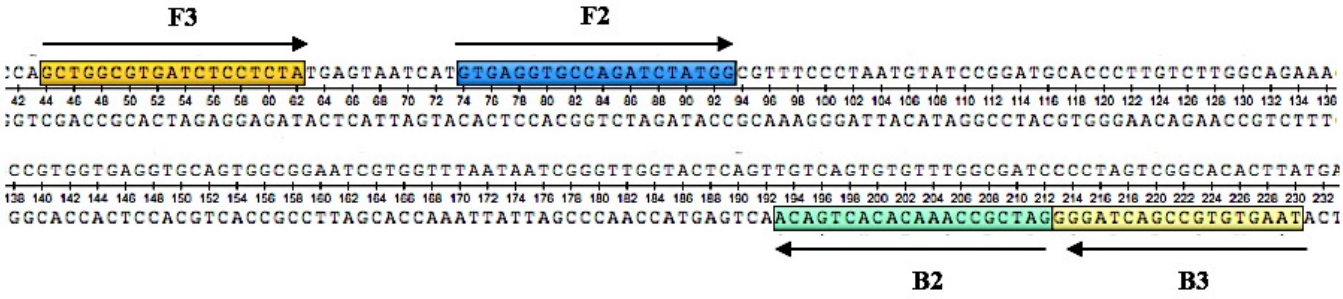

Figure 3.1. ITS2 region target sequence (187 bp) and binding sites of Primer 1 (*picture produced using UGENE bioinformatics software*).

3.8 Polymerase chain reaction (PCR)

3.8.1 PCR specificity and sensitivity test

The outer primers of the LAMP primer designed in this study (the F3 and B3 primers) were tested for their specificity in PCR. DNA from adult trematodes which comprised of *F. hepatica*, *Dicrocoelium dendriticum*, *Paramphistomum cervi*, *Calicophoron daubneyi*, *Haplometra cylindracea*, and three nematodes *Haemonchus sp.*, *Cooperia sp.* and *Ostertagia sp.* were used in a 1:10 dilution as the DNA templates for the specificity test. Each PCR reaction was based on a 25 µl volume containing 1x PCR Buffer, 2.0 mM $MgCl_2$, 10 mg/ml BSA, 0.2 mM dNTP, 0.4 µM forward and reverse primers each (or F3 and B3 outer primers from LAMP primer sets), 1.25 U AmpliTaq Gold Polymerase, and 2 µl template DNA. Cycling conditions for the PCR was as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30s, annealing at 58°C for 30s, elongation at 72°C for 45s, and a final extension at 72°C for 10 min. All products were stored at 4°C for further analyses. The sensitivity of PCR in detecting *F. hepatica* DNA was tested by performing PCR on serial ten-fold dilutions of DNA from an adult *F. hepatica* fluke (1 ng/µl to 1 ng/µl x 10^{-7}) using the same procedure as previously mentioned.

3.8.2 PCR of all samples

PCR was conducted on all faecal samples collected in the current study using LAMP Primer 1 outer primers (F3 and B3). Faecal samples with addition of *F. hepatica* DNA before the DNA extraction process ("spiked samples") were also included in the run. 2 µl template DNA from each faecal sample was used. PCR master mix composition and cycling conditions were as previously described in the PCR specificity and sensitivity test.

3.9 Loop-mediated isothermal amplification (LAMP)

3.9.1 LAMP optimization with Primer 1

Optimization of LAMP with Primer 1 was conducted using two different Mg^{2+} concentrations 8 mM and 10 mM, and three different cycling temperatures 61°C, 63°C, and 65°C. Each LAMP reaction was based on a 25 µl reaction containing 2.5 µl of 10x Isothermal Amplification Buffer (contains 2 mM $MgSO_4$) (BioLabs, England), 1 M Betaine, 1.4 mM dNTP mix, 6 mM or 8 mM $MgSO_4$, 1.6 µM of each FIP and BIP primers, 0.2 µM of each F3 and B3 primers, 8 U 2.0 Warm Start Bst-DNA Polymerase (BioLabs, England) and 2 µl template DNA from adult *F. hepatica*. The cycling conditions were amplification for 60 min at either 61°C, 63°C, or 65°C and termination of reaction at 80°C for 10 min. Another optimization was conducted using the same protocol but with two different amplification times, 60 min and 120 min, at 63°C with 8 mM Mg^{2+} concentration. All LAMP products were stored at 4°C for further analyses.

3.9.2 LAMP specificity and sensitivity test

A specificity test for Primer 1 was conducted using DNA templates from the same trematode samples previously used in the PCR specificity test. Each LAMP reaction was based on a 25 µl reaction containing 2.5µl of 10x Isothermal Amplification Buffer (BioLabs, England), 1 M Betaine, 1.4 mM dNTP mix, 8 mM $MgSO_4$, 1.6 µM of each FIP and BIP primers, 0.2 µM of each F3 and B3 primers, 8 U 2.0 Warm Start Bst-DNA Polymerase (BioLabs, England) and 2 µl template DNA. The cycling conditions were amplification for 60 min at 63°C and termination of reaction at 80°C for 10 min. All LAMP products were stored at 4°C for further analyses. Sensitivity of LAMP in detecting *F. hepatica* DNA was conducted using DNA dilutions as previously mentioned in the PCR sensitivity test, with cycling conditions as previously mentioned for LAMP specificity test.

3.9.3 LAMP of all samples

LAMP was conducted on all faecal samples collected in the current study using LAMP Primer 1 within two consecutive days. The "spiked samples" were also included in the run. 2 µl template DNA from each faecal sample was used. LAMP reaction mixture and cycling conditions were as previously described in the LAMP specificity test.

3.10 Colorimetric detection of LAMP products with fluorescence dye

Fluorescence of LAMP products was visualised by adding 2 µl of 10,000x SYBR® Green I Nucleic Acid Gel Stain (Invitrogen™, S7563) (diluted 1:10 in 0.5 x TBE buffer). Products were observed by naked eye under normal light and pictures of reaction tubes were taken using a camera (Canon EOS) on a dark background. LAMP products were considered positive when a green fluorescence was present and negative when it remained orange.

3.11 Gel electrophoresis and UV detection

All PCR products were run on a 1.5% agarose gel in 0.5 x TBE buffer for 45 min at 100 V. All LAMP products were run on a 2% agarose gel in 0.5 x TBE buffer for 45-60 min at 50-60V followed by 20-30 min at 100V in order to obtain better separation of bands with small differences in length. Gels were pre-stained with GelRed™ Nucleic Acid Gel Stain (Biotium, USA) in a concentration of 1:10000. For both PCR and LAMP, 5 µl of product with 1 µl of 6x Loading Dye (Fermentas) was loaded to the wells. Bands were visualized under UV light (Gel Doc 2000, Bio-Rad). LAMP products were run on the gel after addition of fluorescence dye to the tubes, which resulted in better band intensity when observed under UV light. LAMP tubes were also viewed under UV light (Gel Doc 2000, Bio-Rad) after addition of dye and was considered positive when a strong light was emitted.

3.12 Statistical analysis

Method sensitivity and specificity, including confidence intervals, were analysed using a Fisher's exact test (Charlier *et al.*, 2008) using Ms Excel and the GraphPad Prism statistical software. Calculations were based on the following formulas:

Sensitivity = True Positive / (True Positive + False Negative)
Specificity = True Negative / (True Negative + False Positive)

Note:

Sensitivity = The probability that a 'diseased' subject will test positive with the diagnostic method*.

Specificity = The probability that a 'healthy' subject will test negative with the diagnostic method*.

True Positive *a* = Number of samples positive for method *a*, and faecal egg count or coproantigen ELISA.

False Negative *a* = Number of samples negative for method *a*, but positive for faecal egg count or coproantigen ELISA.

True Negative *a* = Number of samples negative for method *a*, and faecal egg count or coproantigen ELISA.

False Positive *a* = Number of samples positive for method *a*, but negative for faecal egg count or coproantigen ELISA.

* 'Diseased' here refers to subjects with the target disorder/disease and 'healthy' refers to subjects without the target disorder/disease.

Formulas and definitions were adapted from Raslich *et al.* (2007) and Charlier *et al.* (2008).

Since it was not possible to slaughter animals in the current study and examine livers for presence of adult flukes, the composite reference model was used to determine the 'gold' reference for calculating the sensitivity and specificity of the diagnostic methods (Naaktgeboren *et al.*, 2013). Two diagnostic methods used as the composite reference were FEC and coproantigen ELISA, where a positive result by either one of the two methods was determined as positive for the disease. This was based on the theory that FEC and coproantigen ELISA both reflect the presence of current fluke infections by detection of eggs and fluke antigens in the faeces (Valero *et al.*, 2009b; Gordon *et al.*, 2012). Correlation between FEC, coproantigen ELISA and serum ELISA results was done with the Spearman rank correlation algorithm using the GraphPad Prism statistical software.

4 Results

4.1 Faecal Egg Count (FEC)

Out of the 64 faecal samples, 13 sheep and 15 cattle faecal samples were positive with FEC using the sedimentation method. The *Fasciola hepatica* egg counts ranged from 0.1 – 16.6 eggs per gram (EPG) with an average of 4.04 EPG for all FEC positive samples (Table 4.1; Appendix Table 1). Photo of a *F. hepatica* egg found in one of the faecal samples in this study is shown in Figure 4.1.

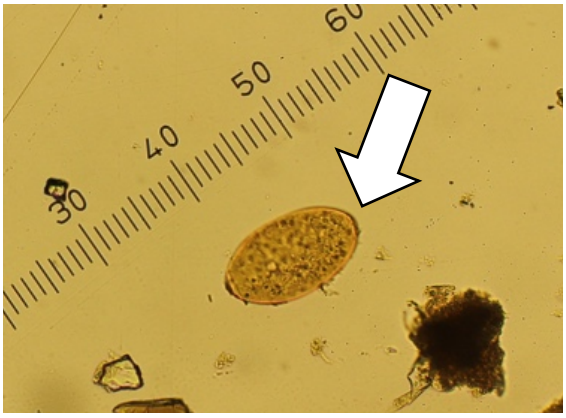

Figure 4.1. *Fasciola hepatica* egg filled with granules as seen using a microscope.

4.2 Serum ELISA

Out of the 64 serum samples, 29 sheep and 24 cattle sera were positive by serum ELISA. The range of S/P ratios were 11.7% to 140.2%, with an average of 63.5% for all serum positive samples (Table 4.1; Appendix Table 1).

4.3 Coproantigen ELISA

Out of the 64 faecal samples, 18 sheep and 18 cattle faecal samples were positive by coproantigen ELISA. The range of S/P ratios were 1.6% to 105.3%, with an average of 22.3% for all coproantigen positive samples (Table 4.1; Appendix Table 1).

4.4 DNA concentration

Concentration of *F. hepatica* DNA extracted from an adult fluke as measured by Qubit® 2.0 Fluorometer (Thermo Fisher Scientific) was 2.02 ng/µL. Concentration of faecal DNA extraction ranged from 14.30 ng/µl to 102.90 ng/µl. The 260/280 ratios of faecal DNA extractions ranged between 1.61-2.00 (Appendix Table 2).

4.5 PCR

4.5.1 PCR specificity and sensitivity test

The PCR specificity test using F3 and B3 primers of LAMP Primer 1 amplified DNA from *F. hepatica* with an amplicon length of around 200 bp (visualized by gel electrophoresis and UV detection). No amplification of DNA from any of the other trematode and nematode DNAs used in the test was observed (Appendix Figure 1). The PCR sensitivity test resulted in amplification of *F. hepatica* DNA from DNA dilutions of 1 ng/µl to 1 ng/µl x 10^{-4}. No

amplification was observed on DNA dilutions of 1 ng/µl x 10^{-5} to 1 ng/µl x 10^{-7} (Appendix Figure 2).

4.5.2 PCR of all samples

Out of the 64 samples, 3 cattle samples (Henån 2, 5 and 8) were positive by PCR. All positive controls (adult *F. hepatica* DNA) and spiked samples were also positive by PCR (Table 4.1; Appendix Table 1).

4.6 LAMP

4.6.1 LAMP optimization with Primer 1

The first LAMP optimization using two different Mg^{2+} concentrations of 6 mM and 8 mM as well as three different cycling temperatures, 61°C, 63°C and 65°C did not result in any amplification. The second LAMP optimization using two different amplification times of 60 min and 120 min at 63°C with 8 mM Mg^{2+} concentration resulted in amplification of *F. hepatica* DNA after 60 min and both *F. hepatica* and *F. magna* DNA when the LAMP was run for 120 min (Appendix Figure 3). Since the most optimum result was obtained with 8 mM Mg^{2+} and amplification for 60 min at 63°C, this cycling protocol was used for further LAMP analyses.

4.6.2 LAMP specificity and sensitivity test

LAMP using Primer 1 amplified DNA from adult *F. hepatica* fluke. No amplification was observed for the other trematode and nematode DNAs used in this specificity test (Appendix Figure 4). The LAMP sensitivity test resulted in amplification of *F. hepatica* DNA from DNA dilutions of 1 ng/µl to 1 ng/µl x 10^{-3}. No amplification was observed in DNA dilutions of 1 ng/µl x 10^{-4} to 10^{-7} (Appendix Figure 5).

4.6.3 LAMP of all samples

Out of the 64 samples, 1 sheep sample (Töllås 15015) and 5 cattle samples (Henån 2, 5, 8, 9 and 11) were positive by LAMP. All positive controls (adult *F. hepatica* DNA) and spiked samples were also positive by LAMP (Table 4.1; Appendix Table 1).

The number of positive results obtained by each diagnostic method used in the study are shown in Table 4.1.

Table 4.1. Overall result summary.

Method	Positive samples	Negative samples
FEC	28 (43.75%)	36 (56.25%)
Serum ELISA	53 (82.81%)	11 (17.19%)
Coproantigen ELISA	36 (56.25%)	28 (43.75%)
PCR	3 (4.69%)	61 (95.31%)
LAMP	6 (9.38%)	58 (90.63%)

4.7 Statistical analysis

4.7.1 Analysis of diagnostic method sensitivity and specificity

Based on the composite reference, methods with the highest sensitivity were coproantigen ELISA and serum ELISA with 100% (90.3-100%) sensitivity for both methods. The lowest sensitivity was obtained for PCR with 8% (1.8-22.5%) sensitivity. FEC, coproantigen ELISA and PCR had the highest specificity with 100% (87.7-100%) specificity for each method, and serum ELISA had the lowest specificity with 39% (21.5-59.4%) specificity. Data are shown in Table 4.2.

Table 4.2. Method sensitivity and specificity with a 95% Confidence Interval (CI).

Method	Sensitivity (95% CI)	Specificity (95% CI)
FEC	78% (60.9-89.9%)	100% (87.7-100%)
Serum ELISA	100% (90.3-100%)	39% (21.5-59.4%)
Coproantigen ELISA	100% (90.3-100%)	100% (87.7-100%)
PCR	8% (1.8-22.5%)	100% (87.7-100%)
LAMP	14% (4.7-29.5%)	96% (81.7-99.9%)

4.7.2 Relationship between FEC, coproantigen ELISA and serum ELISA data

FEC and coproantigen ELISA values correlated significantly (Spearman R-value=0.8077; $p<0.0001$). Furthermore, significant correlation (Spearman R-value=0.5502; $p<0.0001$) was also observed between FEC and serum ELISA values (Figure 4.2.).

5 Discussion

The goal of the study was to develop LAMP and PCR for detection of *Fasciola hepatica* in faeces of animals, and evaluate and compare results obtained between the different methods used. The majority of samples in this study (82.81%) were positive for antibodies against *F. hepatica*. It is generally accepted that serological tests such as serum ELISA could detect circulating antibodies against *F. hepatica* weeks before the infection becomes patent (Mezo *et al.*, 2004; Afshan *et al.*, 2013; Skuce & Zadoks, 2013). Regardless of serology's early detection ability, it does not necessarily mean that all serum ELISA-positive animals were in the pre-patent period of *F. hepatica* infection. Presence of antibodies against *F. hepatica* reflects exposure to the parasite, rather than the infection status itself (Salimi-Bejestani *et al.*, 2005). Thus there was a possibility that the 17 animals positive by serum ELISA but negative for FEC and coproantigen ELISA in this study were not currently infected, but have had previous exposure to *F. hepatica*. Moreover, it has been reported that antibodies against *F. hepatica* can remain in the circulation for several months even after treatment (Salimi-Bejestani *et al.*, 2005; Brockwell *et al.*, 2013). However, there were 10 lambs in the current study that were positive only for serum ELISA. These results reflect current infections, since the lambs were in their first grazing period and had no prior exposure to *F. hepatica* (Novobilský *et al.*, 2014). Maternal antibodies, if present at all, would have disappeared from circulation when lambs reached 11 weeks of age (Novobilský *et al.*, 2014). Hence it could be concluded that antibodies detected in this study were a result of the lambs' immunological response to current *F. hepatica* infection.

Patent infections can be diagnosed by FEC and coproantigen ELISA, as both methods detect the presence of flukes by identification of eggs and detection of *F. hepatica* antigen in faecal matter (Kajugu *et al.*, 2015). In this study, *F. hepatica* eggs and coproantigen were present in 43.75% and 56.25% of samples respectively. *F. hepatica* coproantigen can be detected in faeces as early as 4-7 weeks post-infection in sheep (Valero *et al.*, 2009b) and 6-8 weeks post-infection in cattle according to a study by Brockwell *et al.* (2013), therefore enabling detection during the pre-patent period before eggs are shed into faeces (Gordon *et al.*, 2012). Based on the information above, the 8 animals in the current study with coproantigen detected but no eggs found in their faeces were most likely in the pre-patent period of *F. hepatica* infection, or number of eggs released were too low for detection by coproscopy (Avcioglu *et al.*, 2014). However, several studies have shown coproantigen ELISA can give false negatives (Charlier *et al.*, 2008; Gordon *et al.*, 2012; Novobilský *et al.*, 2012), which may be due to egg residues

in the gall bladder resulting in false positive FECs (Fairweather, 2011) or cut-off values that were too high for detecting infections with low fluke burdens (Brockwell *et al.*, 2013).

Both PCR and LAMP in the current study did not provide results as expected. Among the 64 faecal samples, *F. hepatica* coproantigen were found in 36 of them, out of which 28 also showed the presence of eggs. PCR and LAMP were expected to be positive for these 36 samples, since both methods reflect the presence of liver flukes within the host. Moreover, the molecular methods were expected to be more sensitive with detection targeted at cellular material from the fluke's tegument that are shed into faeces during the migration phase in liver (Martínez-Pérez *et al.*, 2012). In this study, PCR and LAMP only successfully amplified 3 and 6 samples each respectively. DNA extractions from faecal samples spiked with *F. hepatica* DNA was included in the PCR and LAMP runs, and results were positive for these samples. For that reason, amplification inhibitors can be disregarded as the cause of PCR and LAMP failure to detect *F. hepatica* DNA in the current study (Schrader *et al.*, 2012). Additionally, faecal DNA concentration measurements showed that some DNA was successfully extracted from faecal samples (concentrations ranging from 14.30 ng/µl to 102.90 ng/µl) and the quality of DNA was pure enough as seen by the 260/280 ratios of 1.61-2.00 (Desjardins & Conklin, 2010). However, no relationship was observed between the DNA concentrations and success of PCR and LAMP, since the DNA concentrations did not only represent *F. hepatica* DNA but DNA from various organisms in faecal matter. A reasonable explanation to the unsuccessful PCR and LAMP is that *F. hepatica* cells and eggs were absent in the low amount of faecal material (250 mg) taken from the total amount of faeces for the DNA extraction process. In addition, the amount of *F. hepatica* DNA extracted from faeces might have been insufficient for amplification by PCR and LAMP (Desneux & Pourcher, 2014). In contrary to the less successful PCR and LAMP in the current study, Ai *et al.* (2010) and Martínez-Valladares & Rojo-Vázquez (2016) have found PCR and LAMP to be successful in detecting *F. hepatica* DNA from faecal samples. In this study, we have followed a similar PCR and LAMP protocol as described in Martínez-Valladares & Rojo-Vázquez (2016), with the only difference being the use of different primers and DNA extraction procedure. Nonetheless, these factors might have contributed to the different results obtained. However, in a recent abstract by Kamaludeen *et al.* (2015), failure of PCR in detecting *F. hepatica* DNA from faecal samples was also reported. In the current study, LAMP successfully amplified more samples than PCR, which was unexpected since PCR was able to detect DNA concentrations 100-fold lower than LAMP. In addition, LAMP amplified one faecal sample from a lamb that contained no coproantigen and eggs. Antibodies against *F. hepatica* was detected in this lamb, showing that it was undergoing patent infection. Thus, there

could have been very low numbers of *F. hepatica* cells shed in the faeces which were detectable by LAMP, since LAMP has been known to be highly sensitive and can detect very low DNA concentrations (Mugambi *et al.*, 2015). Another explanation could be that a slight contamination occurred during the LAMP process, for inconsistent results due to high contamination rates or unspecific amplifications have been reported when using LAMP (Goto *et al.*, 2009; Nagdev *et al.*, 2011). High precaution measures were applied throughout the whole process when running LAMP in the current study, including the use of different pipettes and filtered pipette tips in all stages, and different rooms for pre and post LAMP reactions. Nevertheless, unexpected contamination might have occurred and it should not be ruled out as a possibility. For an efficient and 'safe' LAMP, detection of LAMP products without of opening tubes is recommended (Goto *et al.*, 2009). Other than SYBR Green, intercalating dyes such as Eva Green and dyes that bind to magnesium (a by-product of the LAMP amplification process) such as Calcein and hydroxyl napthol blue (HNB) can be used, as they do not inhibit amplification when added before the LAMP reaction (Fischbach *et al.*, 2015). Other options of LAMP detection without opening tubes include using a turbidimeter to measure the real-time turbidity during LAMP reactions (Goto *et al.*, 2009).

Faecal and blood samples in the current study were collected in early December 2015. Infection in one of the sheep farms (Kållekärr) could be estimated to occur at different periods throughout August-October 2015, as *F. hepatica* eggs and coproantigen were detected in faeces of most ewes and lambs, while several animals were only positive for serum ELISA. In lambs from another sheep farm (Töllås) only antibodies were detected while coproantigen was negative in all but one animal and all faecal egg counts were negative, which shows that *F. hepatica* infections probably occurred in late October 2015. Infection times are in agreement to previous studies on *F. hepatica* infection periods in pasture which usually occurs between August and October (Taylor *et al.*, 2007; Novobilský *et al.*, 2014).

In this study, an animal was considered as 'diseased' (or with patent infection) when either eggs or coproantigen were detected. This so called 'any positive' composite reference was used as the 'gold standard' (Naaktgeboren *et al.*, 2013). The presence of either eggs or coproantigen indicates presence of flukes, whereas serum antibodies do not necessarily reflect ongoing infection (Salimi-Bejestani *et al.*, 2005; Kajugu *et al.*, 2015). For that reason, serum ELISA was not included in the reference standard. The hypothesis of this study was that LAMP could have higher sensitivity and specificity compared to other methods. This hypothesis was disproven, as LAMP had a sensitivity of 14%, which was much lower compared to the 78% sensitivity of FEC and 100% sensitivity of both coproantigen and serum ELISA. PCR's

sensitivity (8%) was even lower than that of LAMP. However, it cannot be directly assumed that these two molecular methods have poor sensitivity. Many factors contribute to the success of molecular methods, such as good DNA extraction techniques, sampling procedures and sample preservation (Wehausen *et al.*, 2004; Panasci *et al.*, 2011). Therefore, further development of these procedures could possibly increase the sensitivity of LAMP and PCR for *F. hepatica* detection. Serum ELISA had the lowest specificity (39%) in the current study, but it does not necessarily prove that serum ELISA is poor method. As previously mentioned, serum ELISA detects circulating antibodies and is beneficial for early diagnosis (Mezo *et al.*, 2004). Hence, when blood and faecal samples were collected prior to release of coproantigen and eggs in faeces of animals with patent infections, serum ELISA will seem to have low specificity when compared to coproantigen ELISA and FEC.

Coproantigen was detected in faecal samples where eggs were also present (28 samples) and in some where no eggs were found (8 samples). However, none of the coproantigen-negative samples (28 samples) were positive for eggs. Consequently, coproantigen ELISA data indirectly represented the composite reference standard, since animals were 'diseased' when either coproantigen or eggs were present. Naaktgeboren *et al.* (2013) mentioned in their article that composite reference is only useful when the methods incorporated cover each other's flaws, otherwise the sensitivity of the composite reference standard will not be higher than the methods comprising it. For this reason, coproantigen ELISA had 100% sensitivity and specificity in this study. Other statistical methods that have been applied in conditions where it is difficult to choose a single method as the reference standard for diagnosing infections or diseases include the Hui-Walter model, latent class models and Bayesian approaches (Rapsch *et al.*, 2006; Bronsvoort *et al.*, 2010; Louzada *et al.*, 2014). It is also important to note that a diagnostic test's sensitivity and specificity is dependent on the population where it is used, and cannot be inferred to other populations easily (Charlier *et al.*, 2008).

A positive correlation was found between FEC and sample-to-positive (S/P) ratio of coproantigen ELISA, in agreement to previous studies (Brockwell *et al.*, 2013; Hanna *et al.*, 2015; Kajugu *et al.*, 2015). In a study by Novobilský & Höglund (2015) a positive correlation between FEC and coproantigen ELISA was also seen 21 days post-treatment. Even though flukes were not recovered in this study for fluke burden estimation in the subjects, other studies showed that coproantigen ELISA correlates positively with infection intensity (Charlier *et al.*, 2008; Brockwell *et al.*, 2013). An animal in the current study with a coproantigen ELISA S/P ratio of 105.3% most likely suffered from heavier fluke burden compared to those with S/P ratios of less than 10%. Furthermore, FEC and coproantigen ELISA reduction tests for

determining drug efficacy or resistance in *F. hepatica* have shown to have good correlation in a previous study (Flanagan *et al.*, 2011a). A positive correlation between fluke burden and serum ELISA in untreated animals was also reported in a study by Brockwell *et al.* (2013). Overall, both FEC and coproantigen ELISA are good diagnostic tools for detection of *F. hepatica* patent infections.

6 Conclusion

Taking everything into account, it can be concluded that traditional methods in the current study were more sensitive in detecting *Fasciola hepatica* in faeces compared to the molecular methods. Determining method sensitivity and specificity based on a composite reference standard was reasonable, although results were highly dependent on the methods comprising it. Even though PCR and LAMP were highly specific, further development of faecal DNA extraction for diagnostic purposes is needed for better results.

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9 Appendix

Appendix Table 1. Results obtained from all diagnostic methods used in the study.

Farm	ID	Animal Group	FEC (EPG)	Serum ELISA S/P ratio (%)	Coproantigen ELISA S/P ratio (%)	PCR	LAMP
Kållekärr	9024	Ewe	0	**119.9**	**6.4**	-	-
Kållekärr	11014	Ewe	0	**24.4**	0.9	-	-
Kållekärr	11078	Ewe	**2.3**	**97.2**	**25.3**	-	-
Kållekärr	11088	Ewe	**3**	**113.0**	**38.5**	-	-
Kållekärr	12017	Ewe	0	**25.3**	**2.8**	-	-
Kållekärr	12024	Ewe	**16.6**	**109.1**	**105.3**	-	-
Kållekärr	13062	Ewe	**5.3**	**111.9**	**78.5**	-	-
Kållekärr	13077	Ewe	**2.3**	**130.3**	**60.8**	-	-
Kållekärr	13115	Ewe	**1**	**128.9**	**32.6**	-	-
Kållekärr	14030	Ewe	**4**	**78.5**	**53.8**	-	-
Kållekärr	15022	Lamb	**2.3**	**46.2**	**24.0**	-	-
Kållekärr	15023	Lamb	0	**122.1**	**9.9**	-	-
Kållekärr	15030	Lamb	0	**39.4**	**47.6**	-	-
Kållekärr	15031	Lamb	**8**	**55.0**	**69.0**	-	-
Kållekärr	15032	Lamb	**0.67**	**74.3**	**36.7**	-	-
Kållekärr	15036	Lamb	0	**12.0**	0.2	-	-
Kållekärr	15041	Lamb	0	2.5	0.9	-	-
Kållekärr	15043	Lamb	0	7.5	1.0	-	-
Kållekärr	15044	Lamb	**5**	**72.2**	**56.5**	-	-
Kållekärr	15048	Lamb	**4.67**	**52.8**	**28.2**	-	-
Kållekärr	15053	Lamb	0	**30.8**	1.1	-	-
Kållekärr	15057	Lamb	**0.67**	**88.9**	**8.6**	-	-
Kållekärr	15061	Lamb	0	7.0	1.0	-	-
Töllås	15011	Lamb	0	**51.5**	**2.2**	-	-
Töllås	15012	Lamb	0	5.7	0.6	-	-
Töllås	15014	Lamb	0	**33.5**	0.5	-	-
Töllås	15015	Lamb	0	**57.8**	-0.2	-	**X**
Töllås	15018	Lamb	0	4.7	0.9	-	-

Töllås	15021	Lamb	0	1.4	0.8	-	-
Töllås	15024	Lamb	0	**30.5**	0.8	-	-
Töllås	15025	Lamb	0	**60.5**	0.2	-	-
Töllås	15026	Lamb	0	**34.0**	1.2	-	-
Töllås	15028	Lamb	0	3.2	0.6	-	-
Töllås	15029	Lamb	0	**11.7**	0.5	-	-
Töllås	15031	Lamb	0	**16.2**	-0.2	-	-
Töllås	15034	Lamb	0	4.2	1.0	-	-
Töllås	15036	Lamb	0	**46.3**	1.4	-	-
Töllås	15041	Lamb	0	3.5	0.0	-	-
Töllås	15044	Lamb	0	2.6	-0.6	-	-
Henån	1	Cattle	**3.5**	**87.9**	**14.3**	-	-
Henån	2	Cattle	**0.3**	**62.7**	**2.0**	**X**	**X**
Henån	3	Cattle	**0.2**	**71.4**	**1.9**	-	-
Henån	4	Cattle	**1.1**	**21.1**	**11.0**	-	-
Henån	5	Cattle	**16.5**	**58.5**	**14.7**	**X**	**X**
Henån	6	Cattle	0	**28.0**	**1.8**	-	-
Henån	7	Cattle	**10.3**	**91.4**	**14.3**	-	-
Henån	8	Cattle	**4**	**54.8**	**6.3**	**X**	**X**
Henån	9	Cattle	**1.2**	**51.6**	**4.2**	-	**X**
Henån	10	Cattle	**4.2**	**59.8**	**10.7**	-	-
Henån	11	Cattle	**12.6**	**57.0**	**15.7**	-	**X**
Henån	12	Cattle	0	**26.1**	1.4	-	-
Henån	13	Cattle	**1.5**	**140.2**	**8.4**	-	-
Henån	14	Cattle	**1.6**	**46.2**	**2.9**	-	-
Henån	15	Cattle	**0.2**	**43.8**	**2.1**	-	-
Binninge	428	Cattle	0	**62.8**	**1.6**	-	-
Binninge	461	Cattle	**0.1**	**61.0**	**1.6**	-	-
Binninge	466	Cattle	0	2.3	0.8	-	-
Binninge	517	Cattle	0	**17.9**	0.2	-	-
Binninge	518	Cattle	0	**109.1**	1.3	-	-
Binninge	526	Cattle	0	**102.3**	1.3	-	-
Binninge	605	Cattle	0	**58.4**	0.9	-	-

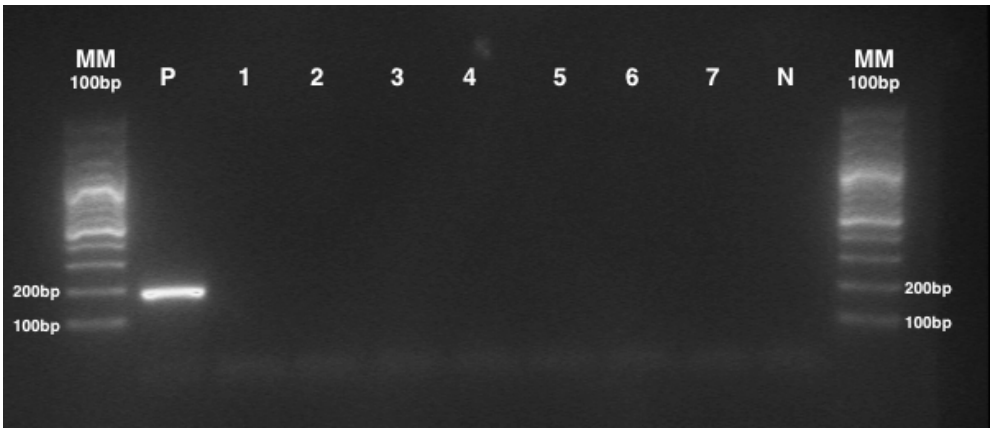
Binninge	617	Cattle	0	**63.6**	0.7	-	-
Binninge	630	Cattle	**0.1**	**93.5**	**2.6**	-	-
Binninge	631	Cattle	0	**24.5**	**1.8**	-	-

Note: Sheep (ewe and lamb) serum ELISA S/P ratio cut-off = 10.0; Cattle serum ELISA S/P ratio cut-off = 15.0; Coproantigen ELISA S/P ratio cut-off = 1.6 (Novobilský *et al.*, 2014; Novobilský & Höglund, 2015). For PCR and LAMP, an 'X' indicates positive samples and a '-' indicates negative samples. Positive samples are highlighted in bold red. EPG (eggs per gram) = number of eggs found by coproscopy / amount of faeces used (g).

Appendix Table 2. Faecal DNA concentration measurements by Picodrop™.

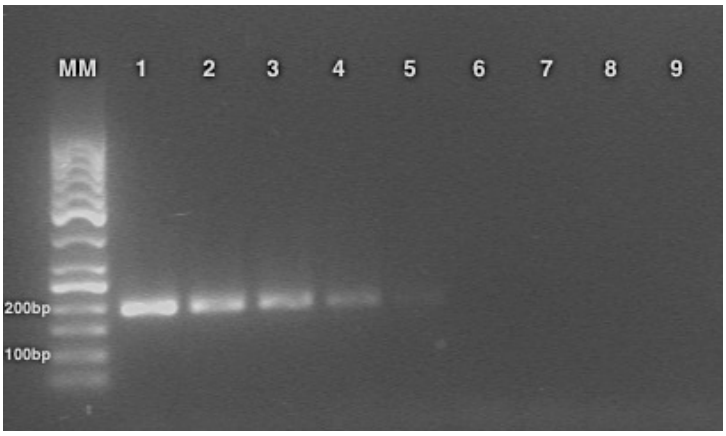
Farm	Sample ID	DNA concentration	A260/A280
Kållekärr	12024	28.3 ng/µL	1.966
Kållekärr	13062	102.9 ng/µL	1.921
Töllås	15011	70.1 ng/µL	1.938
Töllås	15012	14.3 ng/µL	1.908
Henån	1	23.0 ng/µL	2.004
Henån	5	42.1 ng/µL	2.002
Binninge	461	41.9 ng/µL	1.613
Binninge	630	31.6 ng/µL	1.78
Töllås	15012 (spiked)	24.3 ng/µL	1.697
Henån	15 (spiked)	32.0 ng/µL	1.719
Binninge	518 (spiked)	20.8 ng/µL	1.744

Appendix Figure 1. Gel electrophoresis picture of PCR specificity test.



Well loading order = MM: 100bp ladder; P: *F. hepatica*; 1: *D. dendriticum*; 2: *P.cervi*; 3: *C. daubnyei*; 4: *H. cylindracea*; 5: *Haemonchus sp.*; 6: *Cooperia sp.*; 7: *Ostertagia sp.*; N: blank; MM: 100bp ladder.

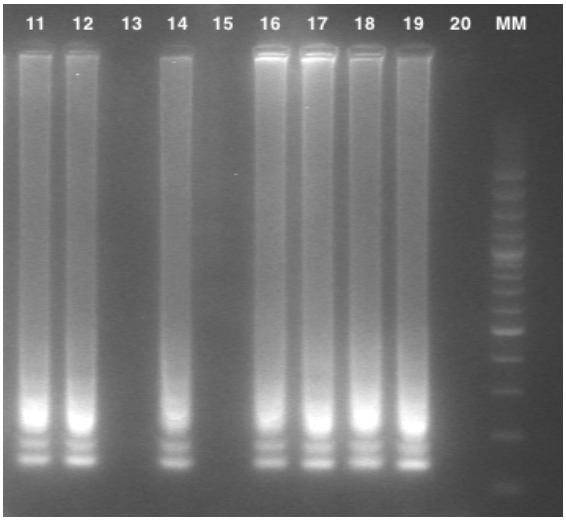
Appendix Figure 2. Gel electrophoresis picture of PCR sensitivity test.



Well loading order = MM: 100bp ladder; 1-8: *F. hepatica* DNA dilutions of 1 ng/µL to 1 ng/µL x 10^{-7} respectively; 9: blank.

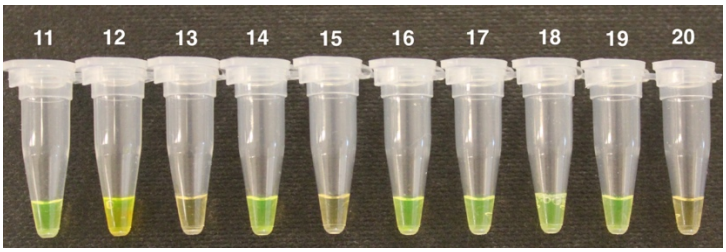
Appendix Figure 3. Gel and tube pictures of LAMP optimization.

A. Gel electrophoresis of LAMP products from optimization test.



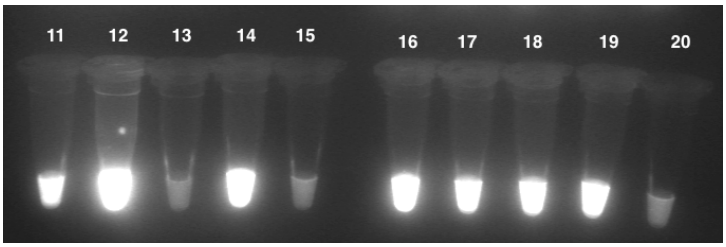
Well loading order: 11-15: 60 min; 16-20: 120 min; 11, 16: *F. hepatica* DNA, undiluted; 12, 17: *F. hepatica* DNA, diluted 1:100; 13, 18: *F. magna* DNA, diluted 1:10; 14, 19: positive cattle faecal sample (Henån 5), undiluted; 15, 20: Blank; MM: 50bp ladder.

B. LAMP reaction tubes of optimization test viewed under normal light.



Tube order: 11-15: 60 min; 16-20: 120 min; 11, 16: *F. hepatica* DNA, undiluted; 12, 17: *F. hepatica* DNA, diluted 1:100; 13, 18: *F. magna* DNA, diluted 1:10; 14, 19: positive cattle faecal sample (Henån 5), undiluted; 15, 20: Blank.

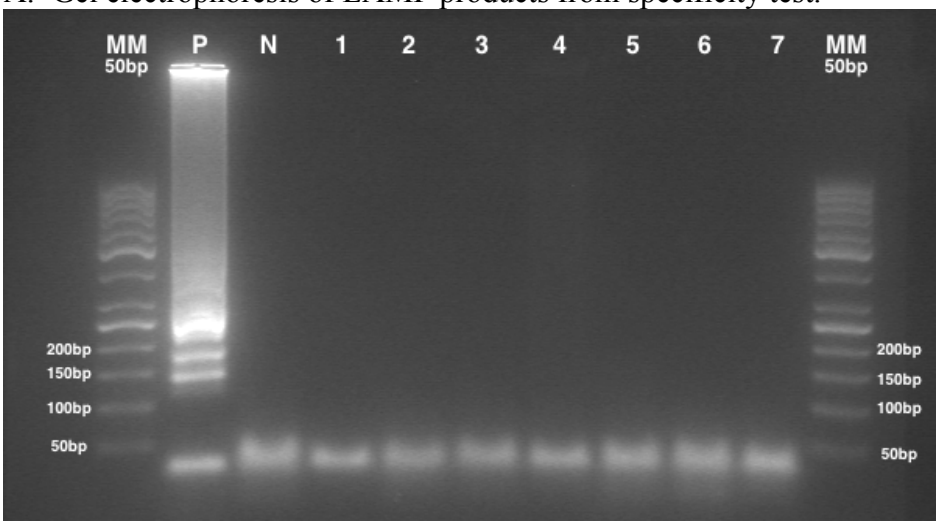
C. LAMP reaction tubes of optimization test viewed under UV light.



Tube order: 11-15: 60 min; 16-20: 120 min; 11,16: *F. hepatica* DNA, undiluted; 12,17: *F. hepatica* DNA, diluted 1:100; 13,18: *F. magna* DNA, diluted 1:10; 14,19: positive cattle faecal sample (Henån 5), undiluted; 15,20: Blank

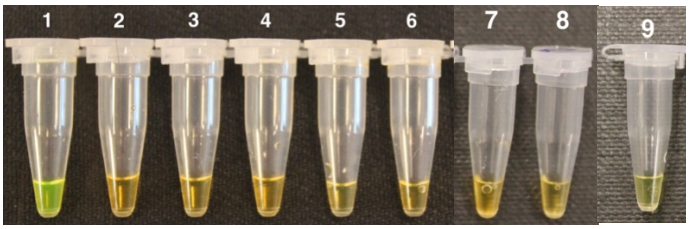
Appendix Figure 4. Gel and tube pictures of LAMP specificity test.

A. Gel electrophoresis of LAMP products from specificity test.



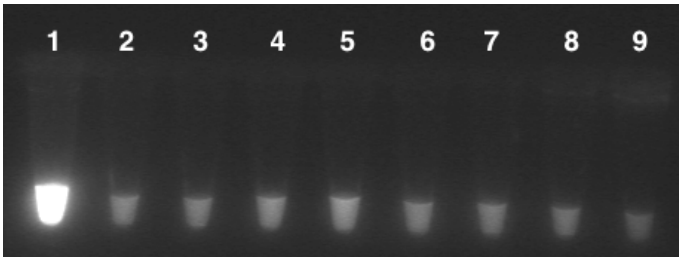
Well loading order = MM: 50bp ladder; P: *F. hepatica*; N: blank; 1: *D. dendriticum*; 2: *P.cervi*; 3: *C. daubnyei*; 4: *H. cylindracea*; 5: *Haemonchus sp.*; 6: *Cooperia sp.*; 7: *Ostertagia sp.*; MM: 50bp ladder.

B. LAMP reaction tubes of specificity test viewed under normal light.



Tube order = 1: *F. hepatica*; 2: *D. dendriticum*; 3: *P.cervi*; 4: *C. daubnyei*; 5: *H. cylindracea*; 6: *Haemonchus sp.*; 7: *Cooperia sp.*; 8: *Ostertagia sp*; 9: Blank.

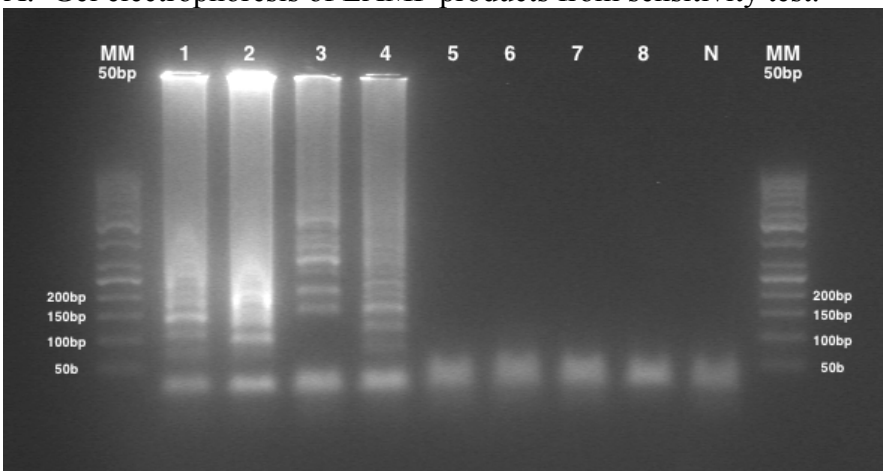
C. LAMP reaction tubes of specificity test viewed under UV light.



Tube order = 1: *F. hepatica*; 2: *D. dendriticum*; 3: *P.cervi*; 4: *C. daubnyei*; 5: *H. cylindracea*; 6: *Haemonchus sp.*; 7: *Cooperia sp.*; 8: *Ostertagia sp*; 9: Blank.

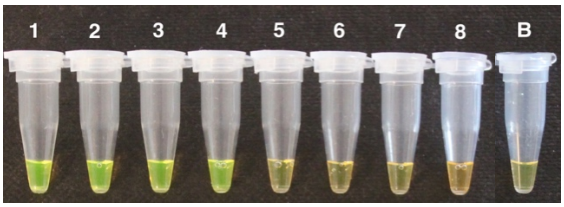
Appendix Figure 5. Gel and tube pictures of LAMP sensitivity test.

A. Gel electrophoresis of LAMP products from sensitivity test.



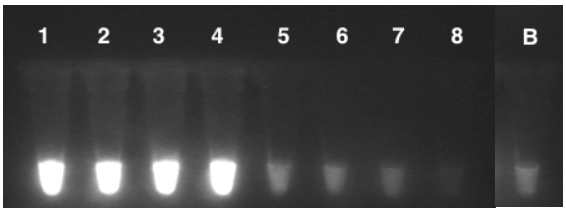
Well loading order = MM: 50bp ladder; 1-8: *F. hepatica* DNA dilutions of 1 ng/µL to 1 ng/µL x 10^{-7} respectively; N: blank; MM: 50bp ladder.

B. LAMP reaction tubes of sensitivity test viewed under normal light.



Tube order = 1-8: *Fasciola hepatica* DNA dilutions of 1 ng/µL to 1 ng/µL x 10^{-7} respectively; B: blank

C. LAMP reaction tubes of sensitivity test viewed under UV light.



Tube order = 1-8: *Fasciola hepatica* DNA dilutions of 1 ng/µL to 1 ng/µL x 10^{-7} respectively; B: blank