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Evaluation of LAMP for *Fasciola hepatica* detection from faecal samples of experimentally and naturally infected cattle

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ABSTRACT

Fasciola hepatica causes liver fluke disease in production animals and humans worldwide. Faecal egg counts (FEC) are the most common diagnostic tool for the diagnosis of liver fluke disease. However, FEC has low sensitivity and is often unreliable for the detection of patent infection. In this study, loop-mediated isothermal amplification (LAMP) was optimised and evaluated for the detection of *Fasciola hepatica* infection, with the aim of increased sensitivity and making it suitable for on-farm application. LAMP was initially conducted under laboratory conditions, optimised to enable visual detection using calcein dye. DNA extraction based on bead-beating was developed to enable on-farm application. LAMP results were compared to FEC and polymerase chain reaction (PCR). Under laboratory conditions, LAMP was conducted using two incubation methods: a conventional PCR thermocycler and a field-deployable LAMP instrument. When compared to a 'rigorous' FEC protocol consisting of multiple counts using a comparatively large volume of faeces and with infection confirmed post-mortem, LAMP was highly sensitive and specific (using silica membrane DNA extraction sensitivity 88 %, specificity 100 %; using sieving and beat-beating DNA extraction sensitivity 98.9 %, specificity 100 %). When applied on-farm, LAMP was compared to conventional FEC, which suggested high sensitivity but low specificity (sensitivity 97 %, specificity 37.5 %). However, further analysis, comparing field LAMP results to laboratory PCR, suggested that the low specificity was likely the outcome of the inability of conventional FEC to detect all true *F. hepatica* positive samples. Based on the high sensitivity and specificity of LAMP compared to a 'rigorous' FEC protocol and its ability to be used in field settings, the study demonstrates the potential of LAMP for diagnosing *F. hepatica* infection in agriculture.

1. Introduction

Fasciolosis remains one of the greatest impediments to livestock production globally. Better diagnosis of the disease could lead to improvements in animal production through better prevention and targeted control measures, including alternative parasite integrated management and the selective use of drug treatments (Bolajoko et al., 2015; Morgan et al., 2013; Roeber et al., 2013).

The faecal egg counts (FEC) is the most commonly used diagnostic test and remains the gold standard method for the diagnosis of fasciolosis in production animals. FEC allows for a relatively inexpensive diagnosis of fasciolosis, particularly for a small number of samples. However, FEC has multiple limitations as an effective diagnostic tool. The requirement for patency (egg production by mature parasites 8–10 weeks after infection) does not allow diagnosis of the immature parasites migrating in the host. Substantial physiological damage and productivity loss can occur during those 8–10 weeks (Boray, 2017). Moreover, mature parasites shed eggs only intermittently, resulting in ~30 % of

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animals not shedding detectable eggs in faeces. Consequentially, FEC for *F. hepatica* can have low sensitivity (Boray, 2017; Rojas et al., 2014).

Nucleic **a**cid **a**mplification **t**echnique**s** (NAAT**s**) such as polymerase **c**hain **r**eaction (PCR) and **l**oop **m**ediated **i**sothermal **a**mplification (LAMP) have been investigated and developed for many infectious diseases. The diagnostic capability and potential for high throughput of NAATs could overcome the shortcomings of FEC (and other diagnostic methods) for fasciolosis (Ai et al., 2010; Martínez-Pérez et al., 2012; Robles-Pérez et al., 2013). In particular, LAMP has the potential to be adequately sensitive and specific while being relatively simple to conduct (relative to FEC). LAMP assays to detect fasciolosis have been reported; however, these studies have generally involved small numbers (up to 64) of test animals (Ai et al., 2010; Arifin et al., 2016; Martínez-Valladares and Rojo-Vázquez, 2016).

Here we provide a multi-step evaluation of LAMP for the detection of *F. hepatica* in cattle faeces. Samples from a cohort of experimentally-infected cattle along with samples that were expected to be *F. hepatica* negative were used to evaluate LAMP conducted under laboratory conditions, and diagnostic capacity was compared to FEC and PCR. Thereafter, we optimised a DNA extraction protocol that was suitable for field application. Finally, we conducted an on-farm trial of LAMP compared to FEC and PCR for the detection of *F. hepatica* infection in naturally infected cattle.

2. Materials and methods

2.1. Overview of experiments

Experiment 1: Determine if the sensitivity and specificity of LAMP are comparable to FEC and conventional PCR for the detection of *F. hepatica* in faeces.

Experiment 2: Optimise a DNA extraction technique that enables the development of a field-applicable LAMP for the detection of *F. hepatica*.

Experiment 3: Determine whether a field-applicable LAMP assay (developed in the laboratory in Experiments 1 and 2, above) could be successfully implemented on-farm for diagnosis of fasciolosis in dairy cows.

2.2. Experimental design

Experiment 1 was conducted under laboratory conditions. Silica membrane column DNA extractions were used for both LAMP and PCR, and nucleic acid amplification was conducted in conventional laboratory equipment (Veriti® Thermal cycler, Life Technologies Australia Pty Ltd). A flow diagram of the experimental design is provided in the supplementary material (Fig. S1a).

For Experiment 2, a field applicable DNA extraction technique was optimised and tested again under laboratory conditions. DNA extraction was carried out using a field-applicable method (sieving and bead-beating), and then LAMP was conducted using a thermocycler (as above). These field-applicable extractions were compared to the sensitivity and specificity of LAMP using a silica membrane column DNA extraction. For these comparisons, FEC was used as the gold standard method (Fig. S1b).

For Experiment 3, the field-applicable DNA extraction technique (Experiment 2) was paired with LAMP conducted in a portable LAMP incubator, and samples were tested on-farm. Results were compared to laboratory approaches, namely FEC and PCR. PCR was conducted using a template obtained from a column DNA extraction (Fig. S1c).

2.3. Sample collection

2.3.1. Known F. hepatica positive samples (Experiments 1 and 2)

In total, 94 faecal samples from cattle collected 12 weeks post-infection were used, along with corresponding FEC data. The samples were derived from two studies approved by either the University of New England ethics committee (AEC14–043) or the CSIRO Animal Ethics Committee Approval #15/16. The adult fluke counts in these animals ranged from 21 to 171. These samples were used in Experiments 1 and 2.

2.3.2. Expected F. hepatica negative bovine faecal samples (Experiments 1 and 2)

Bovine faecal samples from a dairy farm, with no previous history of *F. hepatica* infections, were collected at Boolarra, Gippsland, Victoria. Samples were collected on a single day from 40 different fresh faecal patties from the ground. Samples were collected from at least five distinct parts of each faecal patty sampled and mixed in the sample tube. Samples were transported to the laboratory at Federation University Gippsland campus in Churchill, Victoria in an icebox, and then stored at 4 °C. FECs were performed within 48 h of collection. Samples were briefly mixed with an applicator stick when a subsample was taken for FEC. Subsamples were stored for the long term at –80 °C. These samples were (presumed) negative samples in Experiments 1 and 2.

2.3.3. Bovine faecal samples of unknown F. hepatica status from a dairy farm

Experiment 3 of this study was to evaluate LAMP as an on-farm diagnostic test. Prior to the on-farm evaluation, a suitable farm needed to be identified; i.e. where there was a likelihood of positive and negative samples. A dairy farm at Denison, Gippsland, Victoria with prior reports of *F. hepatica* infected cows was identified as a potential field site. A preliminary investigation using FEC (n = 30) was conducted to confirm the presence of *F. hepatica*. Thereafter, 66 fresh faecal samples were collected (as above) and analysed on-site for the on-farm LAMP evaluation (Experiment 3).

2.4. FEC methods

FECs were determined on the samples from the experimentally infected animals (Experiments 1 and 2) by the faecal sedimentation method. To determine patency during the infection period, FEC was performed multiple times and with larger volumes of faeces than that used in conventional FEC. In brief, 6 g cattle faecal samples were mixed with 200 ml distilled water and homogenised. Homogenized faeces were sieved into a 250 ml sedimentation flask through a 177 μm sieve, with the sieving process conducted twice. The filtrate was allowed to sediment for 3 min, after which the supernatant was discarded, leaving ~40 ml of solution (and sediment) remaining in the flask. Again, the solution was allowed to sediment, and a volume of 10 ml from the bottom of the flask (containing eggs) was transferred into a 15 ml test tube and allowed to sediment again for 3 min. The supernatant was aspirated, leaving approximately 2 ml of sediment suspension, which was then stained with two drops 1 % methylene blue. The sediment suspension was transferred to a perspex counting tray, and eggs were identified using a stereomicroscope under 15× magnification. This FEC method is referred to as the 'rigorous' FEC method, and is based on FEC conducted by Calvani et al. (2017).

For the field collected samples (samples from presumed uninfected cattle from Boolarra, Victoria (Experiments 1 and 2) and samples of unknown infection status used to evaluate LAMP compared to FEC (Experiment 3), FEC was performed using a commercial double sieve system Flukefinder® technique (www.flukefinder.com) with the aid of methylene blue stain (Elliott et al.,2015; Kelley et al.,2020). Parasites (*Fasciola* sp.) eggs present in 2 g of faeces were counted following the manufacturer's instructions.

2.5. Molecular detection of F. hepatica

2.5.1. DNA extraction

Two methods of DNA extraction from bovine faecal samples were used for subsequent *F. hepatica* DNA amplification: (i) a silica membrane column extraction method; and (ii) a sieving and bead-beating DNA

extraction method.

The silica membrane column extraction was carried out on 0.25 g of faeces using a DNeasy Power Soil Kit (Qiagen Pty Ltd., Australia) as per the manufacturer's protocol.

In the sieving and bead-beating method, the Flukefinder® system was used to separate eggs from the coarse particles of faeces. Sediment from 2 g of faeces was washed and sieved, then collected in a dry tube containing beads (Power Bead Tubes, Garnet 0.70 mm, Qiagen Pty Ltd., Australia). The tube was vortexed for 20 min (Horizontal vortex Genie® 2, Mo Bio Pty Ltd., USA). The tube was then centrifuged at ~9100 g for 1 min. The supernatant was used as a DNA template for LAMP amplification. This DNA extraction method is referred to as the sieving and bead-beating DNA extraction method.

2.5.2. PCR amplification

For Experiments 1 and 2 PCR amplification of the ITS-2 region of *F. hepatica* was performed using forward (5'-GTGCCAGATCTATGGCGTTT-3') and reverse (5'-ACCGAGGTCAGGAAGACAGA-3') primers (Robles-Pérez et al., 2013). Each PCR reaction (in a total volume of 25 µl) contained 1 µl of DNA template, 12.5 µl GoTaq Green® (Promega, Australia), 1.25 µl of each selected forward and reverse primer (0.5 µM), 1.25 µl BSA (2 mg/ml), 7.75 µl of nuclease-free water (NFW). PCR was conducted using a Veriti® thermal cycler (Life Technologies Pty Ltd., Australia) with cycling conditions: 95 °C for 2 min; 40 cycles of 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 45 s; and a final extension at 72 °C for 10 min (based on conditions used by Robles-Pérez et al., (2013)). A minimum of one positive control (DNA extracted from known *Fasciola*-infected animal faeces) and one negative control (NFW used as a template) were included per run.

2.5.3. LAMP amplification in the laboratory (Experiments 1 and 2)

LAMP amplification was conducted with calcein dye, using two different types of extracted DNA; silica membrane column extractions and sieving and bead-beating extractions.

Previously published LAMP primers for *F. hepatica* detection were used in this study (Ai et al., 2010), with amplification conditions optimised for this study. Initially, a primer pool was made up of 1.6 µM of FIP and BIP, 0.8 µM FLP and BLP, and 0.2 µM F3 and B3 with 5 µl of the primer pool used in each reaction.

Each LAMP reaction master mix was based on a 25 µl reaction volume containing 8000 U/1 µl *Bst* 2.0 WarmStart polymerase, 2.5 µl 10x isothermal buffer, 1.5 µl 100 mM $MgSO_4$, 3.5 µl 10 mM dNTP mix, 5 µl primer pool, 8 µl NFW, 1.25 µl 10 mM $MnCl_2$ and 1.25 µl 5 mM calcein. 1 µl extracted template was added to make up to a final volume of 25 µl. LAMP assays were run at 65 °C for 60 min, with a termination step at 80 °C for 2 min. Successful LAMP amplification was visually determined by colour change. Positive and negative controls were included in each run, as described above for PCR.

For both methods of faecal DNA extraction, LAMP amplification was conducted using a laboratory thermocycler as used for PCR amplification (Life Technologies Pty Ltd., Australia).

2.6. On-farm LAMP evaluation (Experiment 3)

In Experiment 3, LAMP was conducted on-site on the farm on the day of faecal sample collection. A total of 66 fresh cattle faecal samples from individual cow patties were collected from the paddock over two consecutive days; 30 on day 1 and 36 on day 2.

All the necessary equipment was transported to the farm and set up in an onsite office section of the dairy milking shed. DNA extraction (sieving and bead-beating method) and LAMP amplification were conducted on the samples on the same day of collection.

Prior to conducting the on-farm trial, some preparation was completed in the laboratory at Federation University. LAMP master-mix was prepared two days prior to fieldwork, following the reagent concentration and template volume described previously with calcein dye. The reaction mix was aliquoted into tubes with sufficient mix for ten reactions per tube and then stored at –20 °C. The prepared master-mix was taken to the farm in an icebox at ~4 °C and aliquoted into individual tubes as the assay was prepared.

On-farm, the portable T8-ISO 6 instrument (Axxin, Victoria, Australia) was used for LAMP amplification. Six test samples, one positive control (DNA extracted from known *Fasciola*-infected animal faeces) and one negative control (NFW used as a template) were included per run. The procedure for master-mix preparation and volume of the template was added the same as mentioned previously. The reaction mix was incubated for 1 h at 65 °C.

Result interpretation was conducted in two ways: 1) visual detection of colour change; and 2) spectrophotometrically by the Axxin T8-ISO.

Following LAMP detection on-farm, the remainder of each faecal sample was transported to Federation University Australia's Gippsland campus and stored at 4 °C. FECs were performed within 48 h of sample collection.

2.7. Statistical analysis

LAMP sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated using a 2 × 2 table (Banoo et al., 2008); using FEC as the gold standard and using a composite reference standard. For the composite reference standard, if a sample was positive by either FEC or PCR (or both) it was considered positive.

A cross-tabulation was prepared, and a χ^2 test was employed to test differences between the comparative variables (LAMP and FEC detection, PCR and FEC detection). The P-value was used to indicate significant differences between the comparative variables and was considered significant if the P-value was <0.05.

3. Results

3.1. Comparison of LAMP to PCR and FEC in the laboratory setting (Experiment 1)

Of 94 samples from experimentally infected cattle, 92 were positive for *F. hepatica* by FEC. The two samples negative by FEC were also negative for *F. hepatica* at post-mortem (Table S2). The number of eggs, when present, ranged from 1 to 148 EPG (median = 12, mean = 22.8). There were no FEC positive samples in the presumed negative group of cattle (n = 40) from a commercial dairy farm in Boolarra, Victoria.

Of the total 134 samples (94 experimentally infected and 40 FEC-negative samples from a farm), 80/94 experimentally infected samples were PCR positive and 81/94 experimentally infected samples were LAMP positive. None of the Boolarra farm bovine faecal samples (n = 40) was PCR or LAMP positive (Table S2). The sensitivity, specificity, PPV and NPV of PCR and LAMP compared to FEC are shown in Table 1.

Table 1
The sensitivity, specificity, PPV and NPV (with 95 % confidence intervals in parenthesis) of PCR and LAMP for the detection of *F. hepatica* from bovine faeces conducted under laboratory conditions, using FEC as the reference standard. The diagnostic outcomes of PCR and LAMP were comparable to FEC, with no statistical difference in rate of detection between PCR and FEC and between LAMP and FEC. These results correspond to Experiment 1 of the study.

	Sensitivity %	Specificity %	PPV %	NPV %	χ^2 and P-value
PCR	87.0 (80.0–94)	100 (100–100)	100 (100–100)	77.8 (77.8–77.8)	$\chi^2 = 2.337$ P = 0.1263
LAMP	88.0 (81.4–94.0)	100 (100–100)	100 (100–100)	79.2 (79.2–79.2)	$\chi^2 = 1.973$ P = 0.16012

3.2. Comparison of on-farm applicable methods to laboratory methods (Experiment 2)

Two DNA extraction methods were compared for use in LAMP. As determined in Experiment 1 of the study, LAMP conducted using DNA obtained by silica membrane column DNA extractions detected *F. hepatica* at a similar rate as PCR using the same DNA extracts. Using DNA obtained through sieving and bead-beating (a potentially field-applicable DNA extraction method), the proportion of samples positive for *F. hepatica* was higher relative to column extractions. LAMP conducted in the laboratory using sieving and bead-beating DNA extractions detected *F. hepatica* in 68 % (91/134) samples compared to 60 % (81/134) using column extractions. Of the 40 samples from expected negative faecal samples, all were negative by FEC, PCR and LAMP (using both extraction methods)(Table S2). The sensitivity, specificity, PPV and NPV (with 95 % confidence intervals) of LAMP using both extraction techniques are provided in Table 2.

3.3. On-farm trial (Experiment 3)

Of 66 samples, 53 samples (80 %) were on-farm LAMP positive compared to 34 (52 %) positive by FEC (limit of detection 0.5 EPG; median 0.5 EPG and mean 1.4 EPG) and 42 (64 %) positive by laboratory PCR (Table S3). Field LAMP results were also compared to PCR conducted in the laboratory, with a diagnostic evaluation conducted that compared LAMP to FEC and to a composite reference standard of FEC and PCR (Table 3). LAMP was highly sensitive but had low specificity relative to FEC and the composite reference standard. Statistical analysis confirmed there was a difference ($P < 0.05$) in the proportion of positives detected by LAMP relative to the proportion of positive samples detected by FEC; however, there was no statistical difference between LAMP and the composite reference standard.

4. Discussion

Currently, FEC is the mainstay of *F. hepatica* infection diagnosis in cattle and is considered the industry standard. Due to the inherent limitations of FEC for whole herd testing, a more sensitive, high throughput diagnostic test would benefit the livestock industry. This study demonstrates that LAMP has the potential as a diagnostic for the detection of fasciolosis, particularly for on-farm use.

Three advancements towards the application of LAMP for fasciolosis diagnosis were achieved in this study. First, LAMP could be an alternative to PCR, which is currently the most commonly used NAAT for *F. hepatica* detection (although limited use compared to FEC). Secondly, LAMP using a field-applicable DNA extraction method (sieving and bead-beating) yielded better sensitivity compared to silica membrane column DNA extracts. Thus, the sieving and bead-beating DNA extraction method could be an alternative to the silica membrane column DNA extraction method for *F. hepatica* detection. Most importantly, this study demonstrates the feasibility of LAMP on-farm, using appropriate DNA extractions and a field-portable incubator.

A major challenge when evaluating a new diagnostic approach is to determine whether it is better than the existing 'gold standard'. Gold standards have limitations, and it is common for new diagnostic approaches, particularly NAATs, to have a greater ability to detect true positives than the existing 'gold standard'. This is likely the case for *F. hepatica* detection, with previous studies suggesting that a conventional FEC for *F. hepatica* detection in cattle has a 'true' sensitivity of 30–70 % (Anderson et al., 1999; Charlier et al., 2014). In conducting diagnostic evaluations, it is important to be aware of such limitations to ensure new diagnostic methods are not dismissed due to an apparent lack of specificity.

With the above challenge with evaluation against an existing 'gold standard' in mind, LAMP is likely to be highly sensitive for on-farm diagnosis of fasciolosis. LAMP detected *F. hepatica* infection in 80 % of the samples, compared to 48 % by 'conventional' FEC. In conducting a conventional diagnostic evaluation (Banoo et al., 2008) this could lead to the conclusion that LAMP leads to a false positive diagnosis. However, in comparison to another NAAT (PCR, in which 79 % of samples were positive) and using a composite reference standard, it demonstrates that LAMP (and PCR) are likely detecting infection that is missed by FEC. This is consistent with other findings of this study, where it was demonstrated that LAMP and PCR are highly sensitive relative to when 'rigorous' FEC methods were used for accurate correlation with known worm burdens (Table 1). Thus, LAMP is likely accurate and specific as a field diagnostic.

It follows that the apparent poor specificity of LAMP in this field trial is likely a reflection of the shortcomings of the 'gold standard', as addressed above, and the difference in sensitivity of NAATS due to different DNA extraction methods. Supporting this supposition is that LAMP had better specificity when compared to PCR (and the composite reference standard) than when compared to FEC.

The 'rigorous' FEC used in the early phase of this study was not used in the field trial evaluation. Instead, the study design sought to compare LAMP to FEC conducted as it would likely be for diagnostic purposes. The 'rigorous' FEC is not viable when testing is performed on commercial herds. Commonly, FEC conducted for farm livestock

Table 3
The sensitivity, specificity, PPV and NPV (with 95 % confidence intervals in parenthesis) of LAMP conducted on-farm using sieving and bead-beating DNA extractions. LAMP showed high sensitivity and low specificity relative to FEC and the composite reference standard (CRS) of FEC and PCR. There was a statistical difference in rate of detection between LAMP and FEC. However, there was no difference between LAMP detection and CRS detection. These results correspond to Experiment 3 of the study.

On-farm LAMP	Sensitivity %	Specificity %	PPV %	NPV %	χ^2 and P-value
vs FEC	97.0 (91.3–102.7)	37.5 (20.7–54.2)	62.3 (49.2–75.3)	92.3 (77.8–106.7)	$\chi^2 = 12.172$ P = 0.00048
vs CRS	93.6 (86.6–100.6)	52.6 (30.2–75.1)	83.0 (72.9–93.1)	76.9 (54.0–99.8)	$\chi^2 = 1.485$ P = 0.223

Table 2
Comparison of the sensitivity, specificity, PPV and NPV (with 95 % confidence intervals in parenthesis) of LAMP for the detection of *F. hepatica* from bovine faeces using silica membrane column kit DNA extractions (data from Experiment 1) and sieving-bead-beating DNA extractions. Testing was conducted in a thermocycler under laboratory conditions, and analysis was conducted using FEC as the reference standard. LAMP conducted using DNA from both extraction techniques showed high sensitivity and specificity for *F. hepatica* detection relative to FEC, with no statistical difference in rate of detection. These results correspond to Experiment 2 of the study.

Incubator used	Type of DNA used	Sensitivity %	Specificity %	PPV %	NPV %	χ^2 and P-value
Thermocycler (laboratory)	Silica membrane column kit DNA	88.0 (81.4–94.6)	100 (100–100)	100 (100–100)	79.2 (79.2–79.2)	$\chi^2 = 1.973$ P = 0.16012
	Sieving and bead-beating DNA	98.9 (96.7–101)	100 (100–100)	100 (100–100)	97.6 (97.9–97.6)	$\chi^2 = 0.017$ P = 0.896

management purposes are performed in accordance with industry standards (e.g., Flukefinder® protocols; https://flukefinder.com/; Elliott et al., 2015; Kelly et al., 2020) which involves a small volume of faeces (1–2 g) and no replicates (single sample only). In this study, FEC was performed using the Flukefinder® protocol in line with a commercial diagnostic test. Similarly, PCR was conducted using the DNA extraction method considered best practice in the laboratory (column extractions). Based on the aforementioned limitations around FEC sensitivity, we conclude that the LAMP assay evaluated in this study is a highly sensitive approach for on-farm diagnosis of fasciolosis.

There were some limitations in the current study. To complete a diagnostic evaluation of the *F. hepatica* LAMP assay prior to the field trial, it was necessary to draw on faecal samples from two disparate locations and circumstances as matching negative control samples for the experimentally infected animal samples were not available. Ideally, samples of unknown infection status from various farms in a single region would be used in a diagnostic evaluation. However, the benefits associated with the use of faecal samples from experimentally infected animals on which 'rigorous' FEC had been conducted outweighed the limitation created by not having matching (pre-infection) negative samples (thus the need to complement the positive samples with negative samples from non-experimental cattle). In previous analyses, the 'rigorous' FEC approach had a very high sensitivity (97 %) and specificity (100 %) relative to post-mortem detection of parasites (Spithill, unpublished data). At least one other study had reported improved sensitivity of FEC when a larger faecal sample was used, and each sample was analysed in triplicate (Rapsch et al., 2006). This approach to FEC was well-suited to the laboratory analysis of LAMP conducted in this study, demonstrating the sensitivity (88 %) and specificity (100 %) of the LAMP assay. Notably, the sensitivity or specificity did not change markedly when a composite reference standard was applied to the experiment 1 data (Supplementary data, Table S1); suggesting that NAATs are likely detecting true infections that 'conventional' FEC does not detect but 'rigorous' FEC is able to detect.

Similar to PCR, conducting LAMP brings with it a risk of false positives due to carryover contamination. This can occur during nucleic acid extraction and after amplification. During the field trial, carryover contamination during the extraction process was a risk as we used the same laboratory equipment (Flukefinder) to conduct multiple egg concentrations prior to extraction. A thorough wash protocol was implemented between samples, with wash water used in the final rinse tested for presence of target DNA and not detected (data not shown). To prevent contamination of amplicons, considerable preliminary work was conducted to optimise our assay to ensure it was a closed-tube assay. Future research should consider additional measure that might decrease carryover contamination and non-specific amplicon detection, as discussed elsewhere (Moehling et al., 2021; Quyen et al., 2022).

This study demonstrated the potential of LAMP as an on-farm NAAT for the detection of *F. hepatica*. To the best of our knowledge, this research is the first on-farm demonstration of LAMP for *F. hepatica* detection in cattle faecal samples using a simplified DNA template preparation method. The LAMP protocol developed in this study provides a simple template preparation and amplification process. Therefore, this approach as a diagnostic tool for fasciolosis has the potential to be used in a location where the diagnostic capacity is restricted or the necessary laboratory equipment is limited. Further work is required to confirm the adequate specificity of the field-applicable LAMP assay and investigate the stability of enzymes in the field assay. There is potential to adapt LAMP for use in the on-farm detection of *Fasciola hepatica* (and potentially other pathogens). These results provide justification for a larger, multi-site study to further investigate the applicability of LAMP to on-farm diagnostics. While this study focuses on *Fasciola hepatica* detection, there is potential for other infectious diseases to be detected on-farm using LAMP as well.

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CRediT authorship contribution statement

Greenhill Andrew: Writing – original draft, Supervision, Project administration, Methodology, Formal analysis, Conceptualization. **Bari Tanjina:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **Mamun Md. Abdullah Al:** Writing – review & editing, Methodology, Investigation. **Toet Hayley:** Writing – review & editing, Resources, Methodology. **Rathinasamy Vignesh:** Writing – review & editing, Methodology. **Larkins Jo-Ann:** Formal analysis, Data curation. **Beddoe Travis:** Writing – review & editing, Methodology. **Spithill Terry W.:** Writing – review & editing, Resources, Methodology. **Piedrafita David:** Writing – original draft, Supervision, Project administration, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Tanjina Bari reports financial support was provided by Australian Government Department of Education.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2024.110132.

References

Ai, L., Li, C., Elsheikha, H., Hong, S., Chen, J., Chen, S., Li, X., Cai, X., Chen, M., Zhu, X., 2010. Rapid identification and differentiation of *Fasciola hepatica* and *Fasciola gigantica* by a loop-mediated isothermal amplification (LAMP) assay. Vet. Parasitol. 174, 228–233.

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

Arifin, M.I., Höglund, J., Novobilský, A., 2016. Comparison of molecular and conventional methods for the diagnosis of *Fasciola hepatica* infection in the field. Vet. Parasitol. 232, 8–11.

Banoo, S., Bell, D., Bossuyt, P., Herring, A., Mabey, D., Poole, F., Smith, P.G., Sriram, N., Wongsrichanalai, C., Linke, R., 2008. Evaluation of diagnostic tests for infectious diseases: general principles. Nat. Rev. Microbiol. 8, S16–S28.

Bolajoko, M., Rose, H., Musella, V., Bosco, A., Rinaldi, L., Van Dijk, J., Cringoli, G., Morgan, E., 2015. The basic reproduction quotient (Q0) as a potential spatial predictor of the seasonality of ovine haemonchosis. Geospat. Health 9, 333–350.

Boray, D.J.C. 2017. Liver fluke disease in sheep and cattle, New South Wales, Department of Industry, Skills and Regional Development. ⟨http://www.dpi.nsw.gov.au/_data/assets/pdf_file/0004/114691/liver-fluke-disease-in-sheep-and-cattle.pdf⟩, accessed 2 February 2021.

Calvani, N.E.D., Windsor, P.A., Bush, R.D., Slapeta, J., 2017. Scrambled eggs: a highly sensitive molecular diagnostic workflow for *Fasciola* species specific detection from faecal samples. PLoS Negl. Trop. Dis. 11, e0005931.

Charlier, J., Vercruysse, J., Morgan, E., Van Dijk, J., Williams, D., 2014. Recent advances in the diagnosis, impact on production and prediction of *Fasciola hepatica* in cattle. Parasitology 141, 326–335.

Elliott, T.P., Kelley, J.M., Rawlin, G., Spithill, T.W., 2015. High prevalence of fasciolosis and evaluation of drug efficacy against Fasciola hepatica in dairy cattle in the Maffra and Bairnsdale districts of Gippsland, Victoria, Australia. Vet. Parasitol. 209, 117–124.

Kelley, J.M., Rathinasamy, V., Elliott, T.P., Rawlin, G., Beddoe, T., Stevenson, M.A., Spithill, T.W., 2020. Determination of the prevalence and intensity of *Fasciola hepatica* infection in dairy cattle from six irrigation regions of Victoria, South-eastern Australia, further identifying significant triclabendazole resistance on three properties. Vet. Parasitol. 277, 109019.

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

Martínez-Valladares, M., Rojo-Vázquez, F.A., 2016. Loop-mediated isothermal amplification (LAMP) assay for the diagnosis of fasciolosis in sheep and its application under field conditions. Parasit. Vectors 9, 73.

Moehling, T.J., Choi, G., Dugan, L.C., Salit, M., Meagher, R.J., 2021. LAMP diagnostics at the point-of-care: emerging trends and perspectives for the developer community. Expert. Rev. Mol. Diagn. 21, 43–61.

Morgan, E.R., Charlier, J., Hendrickx, G., Biggeri, A., Catalan, D., Samson-Himmelstjerna, V., Demeler, J., Müller, E., Van Dijk, J., Kenyon, F., 2013. Global change and helminth infections in grazing ruminants in Europe: impacts, trends and sustainable solutions. Agriculture 3, 484–502.

Quyen, T.L., Vinayaka, A.C., Golabi, M., Ngoc, H.V., Bang, D.D., Wolff, A., 2022. Elimination of carryover contamination in real-time reverse transcriptase loop-mediated isothermal amplification for rapid detection of the SARS-CoV-2 virus in point-of-care testing. Front. Cell. Infect. Microbiol. 12, 856553.

Rapsch, C., Schweizer, G., Grimm, F., Kohler, L., Bauer, C., Deplazes, P., Braun, U., Torgerson, P.R., 2006. Estimating the true prevalence of *Fasciola hepatica* in cattle slaughtered in Switzerland in the absence of an absolute diagnostic test. Int. J. Parasitol. 36, 1153–1158.

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

Roeber, F., Jex, A.R., Gasser, R.B., 2013. Advances in the diagnosis of key gastrointestinal nematode infections of livestock, with an emphasis on small ruminants. Biotechnol. Adv. 31, 1135–1152.

Rojas, C.A.A., Jex, A.R., Gasser, R.B., Scheerlinck, J.Y., 2014. Techniques for the diagnosis of *Fasciola* infections in animals: room for improvement. Adv. Parasitol. 85, 65–107.