Veterinary Parasitology 232 (2016) 8–11

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Short communication

Comparison of molecular and conventional methods for the diagnosis of *Fasciola hepatica* infection in the field

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ARTICLE INFO

Article history:
Received 2 September 2016
Received in revised form 1 November 2016
Accepted 5 November 2016

Keywords:
Coproantigen
DNA extraction
Fasciolosis
Faecal egg count
LAMP
PCR
Serology

ABSTRACT

The liver fluke, *Fasciola hepatica*, is one of the major parasite threats to livestock industries world-wide. In sheep and cattle, *F. hepatica* infection is commonly diagnosed using a range of methods. Aside from conventional coprological and serological diagnostic methods, there are also several molecular methods available based on the detection of liver fluke DNA in faeces. In this study, the outcomes of faecal egg count (FEC), serology and coproantigen ELISA (cELISA) were compared with the performance of polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) in diagnosis of *F. hepatica* from naturally infected cattle and sheep. A total of 64 individual faecal and serum samples were collected from four sheep and beef cattle herds with previous histories of *F. hepatica* infection. FEC and coproantigen levels were measured in faecal samples and anti-*F.hepatica* antibody levels were measured in serum samples. DNA samples isolated from faeces were examined both by PCR and LAMP, targeting the internal transcribed spacer 2 (ITS2) region of the *F. hepatica* genome. Results showed that *F. hepatica* eggs were present in 28 animals, while coproantigen and specific anti-*F. hepatica* antibodies were detected in 36 and 53 animals, respectively. Only 3 and 6 samples were positive by PCR and LAMP, respectively. To calculate method specificity and sensitivity, a combination of FEC and cELISA was selected as the composite reference standard (CRS). When compared to the CRS, PCR had a sensitivity of 10.7% and specificity of 100%, whereas LAMP had a sensitivity and specificity of 17.9% and 97.2%, respectively. PCR and LAMP in this field study were highly specific, but both had poor sensitivity compared with FEC and cELISA. Potential reasons for PCR and LAMP failure were inadequate amounts of amplifiable *F. hepatica* DNA, possibly due to the choice of DNA extraction procedure, amount of faecal material processed, as well as different faeces consistency and composition between different animal species.

1. Introduction

The common liver fluke, *Fasciola hepatica*, is cosmopolitan parasite of significance in domestic ruminants. To date, numerous in vivo diagnostic methods have been used to detect *F. hepatica* infection in ruminants, including faecal egg counts (FEC), detection of antibodies in sera, coproantigen or DNA in faeces and several biochemical markers in blood (Fairweather, 2011). The choice of diagnostic method is affected by the purpose of the study. Serological methods provide early detection of *F. hepatica* infection, but circulating antibodies may remain in blood for several months after successful deworming (Salimi-Bejestani et al., 2005). Thus, serology measures only exposure to parasite and not always current infection. Distinguishing an animal with patent infection from a treated animal is crucial in drug efficacy/resistance diagnosis. The faecal egg count reduction test (FECRT) and coproantigen reduction test (CRT) have been suggested as suitable methods for drug efficacy/resistance diagnosis (Brockwell et al., 2013; Flanagan et al., 2011; Novobilský and Höglund, 2015). Recently, molecular methods such as polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) have been shown to diagnose *F. hepatica* infection in naturally infected sheep with the same or increased sensitivity compared with FEC and coproantigen ELISA (cELISA) (Martínez-Valladares and Rojo-Vázquez, 2016; Robles-Pérez et al., 2013). The aim of the present study was to compare sensitivity and specificity of conventional (FEC, serology, coproantigen) and two molecular diagnostic methods (PCR, LAMP) in Swedish cattle and sheep naturally infected with *F. hepatica*.

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http://dx.doi.org/10.1016/j.vetpar.2016.11.003
0304-4017/

2. Materials and methods

2.1. Animals and sampling

In total, 64 individual sheep (n = 39) and cattle (n = 25) originating from four Swedish farms all with a previous history of fasciolosis, were sampled for faeces and serum in December 2015. Samples were collected as part of routine diagnostic screening by a licensed veterinarian and no ethical permission was required under Swedish legislation (Animal Welfare Act 2009/021). In the case of the sheep samples, both ewes (n = 10) and lambs (n = 29) were randomly sampled. In cattle, only beef cattle older than 24 months were selected. Faecal samples were collected from the rectum and then stored at 4 °C during transport to the laboratory. Faecal material for coprological examination was processed immediately after arrival at the laboratory, while the remainder faecal samples for detection of *F. hepatica* coproantigen (cELISA) or DNA were frozen (−20 °C) until further use. Blood samples were collected from the jugular vein and tail vein in sheep and cattle, respectively, using Vacutainer tubes and sterile needles as per standard protocol.

2.2. Faecal egg counts, coproantigen ELISA, serology

Fresh faeces from sheep (3 g) and cattle (10 g) were taken for FEC using a sedimentation method, as previously described (Novobilský and Höglund, 2015). Coproantigen was measured in all 64 samples using the Bio-X *Fasciola hepatica* Antigen ELISA Kit (Bio K 201, batch number FASA16B23 from February 2016, Bio-X Diagnostics, Belgium), according to the manufacturer's protocol with the following modifications: overnight soaking of faecal samples in dilution buffer and application of an optimised cut-off value based on previous findings (Brockwell et al., 2013; Novobilský and Höglund, 2015). Serum samples were examined for the presence of specific anti-*F. hepatica* antibodies using an in-house ELISA with native excretory/secretory *F. hepatica* antigen according to Novobilský et al. (2014, 2012).

2.3. PCR and LAMP

Genomic DNA was extracted from 250 mg faeces using the PowerFecal® DNA isolation kit (MO BIO, USA) according to the manufacturer's protocol. As a PCR and LAMP amplification inhibitor control, 20 ng of *F. hepatica* DNA were added to 250 mg from each of three randomly selected parallel faecal samples ("spiked" samples) prior to extraction using the PowerFecal® kit. All extracted DNA samples were stored at −20 °C until further analysis.

Primers for LAMP were designed to target the internal transcribed spacer 2 (ITS2) region of the *F. hepatica* genome from a consensus of *F. hepatica* sequences (GenBank accession numbers DQ683546, JF824668, KJ200622, AB207148) and compared to that of other trematodes (*Calicophoron daubneyi, Dicrocoelium dendriticum, Fasciola gigantica*, *Fascioloides jacksoni*, *Fascioloides magna*; *Paramphistomum cervi*) to assess specificity (GenBank accession numbers EF534992, EF534993, KF543340, EU260079, EF612486, HM026462, JQ966973, AY790883), using the bioinformatics software UGENE (Okonechnikov et al., 2012) and Primer Explorer v.4 (Primer Explorer, 2016). LAMP primer sequences are shown in Table 1. LAMP was optimised using two different $MgSO_4$ concentrations of 8 mM and 10 mM and three different reaction temperatures of 61 °C, 63 °C and 65 °C and two different amplification times of 60 min and 120 min. The optimum result was obtained with 8 mM $MgSO_4$ and amplification for 60 min at 63 °C. Specificity of primers was tested using DNA from trematodes, which comprised *F. hepatica*, *D. dendriticum*, *P. cervi*, *C. daubneyi*, *Haplometra cylindracea*, and three nematodes *Haemonchus contortus*, *Cooperia oncophora* and *Ostertagia ostertagi*. The detection limit of LAMP was tested using serial 10-fold dilutions of DNA extracted from a single adult *F. hepatica* fluke (1 ng/μl to 1×10^{-7} ng/μl). Each LAMP reaction was based on a 25 μl reaction volume containing 1 x Isothermal Amplification Buffer (containing 2 mM $MgSO_4$) (BioLabs, England), 1 M Betaine, 1.4 mM dNTP mix, 6 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 with reaction conditions as follows: amplification for 60 min at 63 °C and termination of the reaction at 80 °C for 10 min. LAMP products were visualized by addition of 2 μl of 10,000× SYBR® Green I Nucleic Acid Gel Stain (Invitrogen™, S7563) diluted 1:10 in 0.5× TBE buffer. Positive reactions turned green after dye addition and negative reactions remained orange. LAMP products were also visualized by gel electrophoresis and observed under UV light.

Table 1
LAMP primer sequences.

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

PCR was performed with the outer LAMP F3 and B3 primers according to Martínez-Valladares and Rojo-Vázquez (2016) with some modifications. In brief, each PCR reaction was based on a 25 μl volume containing 1× PCR Buffer, 2.0 mM $MgCl_2$, 10 mg/ml BSA, 0.2 mM dNTP, 0.4 μM F3 and B3 LAMP primers, 1.25 U AmpliTaq Gold Polymerase and 2 μl template DNA with cycling conditions as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, elongation at 72 °C for 45 s and a final extension at 72 °C for 10 min. PCR products were also visualized by gel electrophoresis and observed under UV light. The specificity of F3 and B3 primers and the detection limit of the PCR reaction were determined using the same trematode DNA samples and *F. hepatica* DNA dilutions as for the LAMP method.

2.4. Statistical analyses

Since no gold standard method is available for diagnosis of *F. hepatica*, the composite reference model (Alonzo and Pepe, 1999) was used to calculate the sensitivity and specificity of LAMP and PCR. FEC and cELISA were selected as the composite reference standard (CRS), since the presence of both *F. hepatica* eggs and coproantigen demonstrate patent infection and are highly correlated (Brockwell et al., 2013; Flanagan et al., 2011; Novobilský and Höglund, 2015). The correlation between FEC, cELISA and serology was calculated using a Spearman correlation. All statistical analyses were performed in Microsoft Excel 2016 and GraphPad Prism version 5.0 (GraphPad Software, USA).

3. Results and discussion

Primers used in LAMP and PCR showed high specificity, resulting in amplification of *F. hepatica* DNA only (Fig. 1). Sequenced PCR products were in 100% agreement with the expected 187 bp product length *in silico* (Supplementary Fig. S1 in online version at DOI: 10.1016/j.vetpar.2016.11.003). The detection limit of PCR (1×10^{-4} ng/μl) was 10-fold higher than that of the equivalent LAMP (1×10^{-3} ng/μl) (Fig. 2). The similar detection limit and comparable sensitivity of both PCR and LAMP agrees with findings in a previous study by Martínez-Valladares and Rojo-Vázquez (2016). Other studies have reported higher sensitivity of LAMP compared