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Research paper

Comparison of early detection of *Fasciola hepatica* in experimentally infected Merino sheep by real-time PCR, coproantigen ELISA and sedimentation

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ABSTRACT

Fasciolosis due to infection with *Fasciola hepatica*, *Fasciola gigantica* or their hybrids is a significant global cause of livestock production loss. Infection is commonly diagnosed by a labour-intensive sedimentation and faecal egg count (FEC), which has limited throughput and is only applicable after completion of the 8–12 week pre-patent period (PPP). A commercially-available ELISA for the detection of coprological antigen (coproELISA) enables detection prior to the completion of the PPP and is suitable for diagnosis of larger sample sizes, although the sensitivity reported under experimental infection settings can be difficult to replicate in the field, particularly in cattle. A recently-published real-time PCR workflow for the sensitive detection of *Fasciola* spp. DNA in faecal samples provides increased sample throughput, although the point at which this technique is first able to diagnose infection remains unknown. Other tools for the molecular diagnosis of fasciolosis, such as conventional PCR and loop-mediated isothermal amplification (LAMP), have been shown to detect *F. hepatica* DNA as early as 1 week post infection (WPI). In this study, faecal samples were collected weekly from 10 experimentally-infected Merino lambs and subjected to diagnosis via traditional sedimentation, coproELISA and real-time PCR. Samples were first considered positive at 6–8 WPI by coproELISA, real-time PCR and sedimentation, respectively. At 9 WPI 100% of samples were positive by all three methods. To evaluate the capacity of the real-time PCR approach to detect infection prior to completion of the PPP, two methods of sample preparation were compared at 2 WPI: (i) 150 mg raw faecal samples and (ii) 3 g faecal starting volume prior to sedimentation and pelleting. Neither method of sample preparation yielded positive results at 2 WPI suggesting that DNA amplification by real-time PCR is associated with faecal egg load.

1. Introduction

Fasciolosis caused by infection with *Fasciola hepatica*, *Fasciola gigantica* or their hybrids is a zoonotic parasitic disease of global importance (Torgerson and Macpherson, 2011). Up to 91 million people are considered at risk of infection and production losses are estimated to exceed US$2 billion/year (Keiser and Utzinger, 2005; McManus and Dalton, 2006). The parasite has an indirect life cycle, and the pre-patent period (PPP) is generally considered to take 8–12 weeks (Andrews, 1999; Brunsdon, 1967). During this time animal production may be affected due to the migration of immature stages through the liver, causing hepatitis, without externally detectable life cycle stages (Andrews, 1999). Associated production losses include decreased weight gain, anaemia, liver condemnation, reduced reproductive performance and, importantly in the case of acute fasciolosis, the potential for significant increases in animal mortality, often with minimal warning (Andrews, 1999).

Traditionally, ante mortem diagnosis has been performed by faecal egg counts (FEC) utilising either sedimentation or flotation techniques (Happich and Boray, 1969). These techniques have limited sensitivity for animals infected with liver flukes and are only of use after the PPP has passed (Happich and Boray, 1969). Recently, a commercial ELISA for the detection of fluke antigen in faeces (coproELISA) has enabled detection prior to the completion of the PPP (Mezo et al., 2004). However, issues have been identified regarding the decreased sensitivity of this approach in lighter infections (< 10 eggs per gram, EPG), particularly in cattle (George et al., 2017b; Gordon et al., 2012; Kajugu et al., 2015; Martinez-Sernandez et al., 2016). A serological ELISA is commercially available and in naïve animals provides the earliest indication of infection (Mezo et al., 2007). However, a prolonged

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antibody response prevents this approach from detecting reinfection, limiting its application to surveillance of exposure (Mezo et al., 2007). A molecular approach utilising loop-mediated isothermal amplification (LAMP) has suggested that DNA may be detected in faeces from as early as one week post infection (WPI) in experimental studies (Martinez-Valladares and Rojo-Vazquez, 2016). Similarly, a real-time PCR-based workflow was recently published demonstrating high sensitivity in cattle faecal samples with low FECs (< 10 EPG) (Calvani et al., 2017). This approach has never been tested in an experimental infection setting and thus the point at which this technique is first able to detect *Fasciola* spp. DNA in faecal samples remains unknown (Calvani et al., 2017).

The present study aims to evaluate the earliest point of detection of *F. hepatica* in faecal samples in experimentally infected sheep using a recently described real-time PCR-based molecular workflow (for the detection of DNA from eggs), when compared to a traditional sedimentation (for the detection of eggs) and the commercially-available coproELISA (for the detection of coproantigen released by metabolically active flukes). To determine the presence of *F. hepatica* DNA in faecal samples prior to egg shedding we tested raw and sedimented faecal samples at two weeks post infection.

2. Materials and methods

2.1. Experimental infection

All animal experimentation was conducted at Yarrandoo R&D Centre (NSW, Australia). Ten commercially-sourced 10 month old Merino lambs were confirmed to be negative for *F. hepatica* prior to infection by sedimentation 12 weeks post removal from pasture. Animals were housed in group conditions in an indoor facility and fed a lucerne hay/oaten hay/straw/oats chaff mix, supplemented with a lucerne-based concentrate pellet. Water was provided *ad libitum* via the town supply system. Sheep were inspected at least daily for the duration of the sampling period. Animal ethics approval was provided by the Elanco Australasia Pty Limited Animal Ethics Committee (approval ELA170004).

Each animal was infected per os with 250 *F. hepatica* metacercariae generated at the Yarrandoo R&D Centre (New South Wales, Australia) for the purpose of strain maintenance. Weekly faecal samples were collected per rectum from 2 to 11 WPI. Four distinct Australian isolates of *F. hepatica* were used in order to maintain experimental strains; 'Oberon' (n = 2), 'Numbugga' (n = 2), 'Palmers Oaky' (n = 5) and 'Bombala' (n = 1). The 'Oberon' strain was isolated by the NSW Department of Primary Industries in 1999 and the remaining strains were isolated at the Yarrandoo R&D Centre (Fairweather, 2011; George et al., 2017a). The 'Numbugga' strain was obtained from naturally infected goats near Bega NSW and the 'Palmers Oaky' strain was obtained from sheep grazing near Oberon NSW, both in 2014. The 'Bombala' strain was obtained from sheep grazing the Monaro region of NSW in 2016.

2.2. Sedimentation and faecal egg count

Eggs per gram of faeces (EPG) were determined by a standard sedimentation method with minor modifications as described in Calvani et al. (2017) (Happich and Boray, 1969). Briefly, after initial filtering through 270 um nylon mesh, the modification consisted of three rounds of sedimentation, each three minutes in length, in successively smaller volumes of distilled water (250, 100 and 15 ml). The additional rounds of sedimentation served to remove vegetable matter and did not alter the percentage of eggs retained from the original method (Calvani et al., 2017; Happich and Boray, 1969).

Faecal samples were sedimented and counted in duplicate from 6 to 9 WPI and then once from 10 to 11 WPI. For each replication, 3 g of faeces was used. Duplicate sample EPGs are reported as the mean from two standard sedimentations. Duplicate counts during 6–9 WPI were employed to increase the sample volume to 6 g (2 × 3 g) in order to increase the sensitivity for samples with low counts (< 10 EPG). Samples were counted in duplicate backwards from 9 WPI until a minimum of two time points were negative for each animal. All morphological counts based on microscopic examination are henceforth referred to as mEPG.

2.3. DNA isolation and Fasciola hepatica real-time PCR

Faecal samples from 2 and 6–11 WPI were prepared and DNA isolated according to a previously published molecular diagnostic workflow available online at https://dx.doi.org/10.17504/protocols.io.jggcjtw (Calvani et al., 2017). Briefly, after initial sedimentation and egg-counting, the DNA in the resultant sediment was isolated using Isolate Fecal DNA kit (Bioline, Australia) following the manufacturer's recommendations. Samples were added to the 2 ml DNA isolation kit homogenisation tube with 750 µl of lysis buffer and homogenised in a high speed benchtop homogeniser at 6.0 m/s for 40 s (FastPrep-24, MP Biomedicals, Australia). For the 2 WPI samples, DNA was additionally isolated from 150 mg of raw faeces, homogenised as above and following the manufacturer's recommendations (Bioline, Australia). DNA was eluted into 100 ul elution buffer (10 mM TrisCl buffer, pH = 8.5) and stored at −20 °C prior to amplification. To monitor DNA isolation efficiency and absence of PCR inhibition, 5 ul of DNA Extraction Control 670 (Bioline, Australia) was included and samples were assayed according to manufacturer's instructions.

A TaqMan real-time PCR assay targeting *F. hepatica* ITS2 rDNA was utilised (oligonucleotides SSCP*Fa*F [S0754]/SSCP*Fa*R [S0755] and probe ProFh [S0770] FAM-BHQ1) and run in duplicate (Alasaad et al., 2011). A 10-fold dilution of the positive control of *F. hepatica* DNA served for quantification of egg estimate in EPG as previously described (Calvani et al., 2017). The EPG based on real-time PCR is referred to as qEPG.

All real-time PCR reactions were run on CFX96 Touch Real-Time PCR Detection System with the corresponding CFX Manager 3.1 software (BioRad, Australia) using SsoAdvanced Universal Probes Supermix (BioRad, Australia) according to the manufacturer's instructions and cycling condition described previously (Calvani et al., 2017). Results were considered to be positive if both replicates displayed C_T values < 36. Each batch of DNA isolation was isolated with a blank sample (ddH_2O) to detect contamination that may have occurred during the extraction process. Extraction Control samples with C_T values < 31 were considered not inhibited.

2.4. coproELISA

Faecal samples from 4 to 11 WPI were tested using a commercially available ELISA for the detection of *Fasciola* spp. coprological antigen in faeces (coproELISA) (BIOK 201, Bio-X Diagnostics S.A., Belgium, batch number FASA17A20). Briefly, samples were thoroughly mixed with the kit dilution buffer (0.5 g + 2 ml) in 15 ml centrifuge tubes and allowed to sit overnight at 4C to increase optical density (OD) readings of positive samples as recommended by Brockwell et al. (2013). Optical densities were read at 450 nm using a SpectraMax 250 plate reader (Molecular Devices, LLC., Sunnyvale CA, USA). The OD of each corresponding negative well was subtracted from the individual sample ODs (Net OD). The Scaled OD was calculated by dividing the Net OD of the sample by the Net OD of the positive coproELISA controls. Samples were considered positive for *F. hepatica* antigen if the scaled OD was > 0.08.

3. Statistical analysis and data accessibility

Data was analysed in Microsoft Excel (2013) and visualized using GraphPad Prism version 6 (GraphPad Software, USA). Positive and