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Fasciola hepatica: Specificity of a coproantigen ELISA test for diagnosis of fasciolosis in faecal samples from cattle and sheep concurrently infected with gastrointestinal nematodes, coccidians and/or rumen flukes (paramphistomes), under field conditions

P.-E. Kajugu[a], R.E.B. Hanna[a,*], H.W. Edgar[a], C. McMahon[a], M. Cooper[a], A. Gordon[c], J.P. Barley[a], F.E. Malone[a], G.P. Brennan[b], I. Fairweather[b]

[a] *Veterinary Sciences Division, Agri-Food and Biosciences Institute (AFBI), Stormont, Belfast BT4 3SD, United Kingdom*
[b] *School of Biological Sciences, The Queen's University of Belfast, Belfast BT9 7BL, United Kingdom*
[c] *Biometrics Division, Agri-Food and Biosciences Institute (AFBI), Newforge Lane, Belfast, United Kingdom*

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ABSTRACT

Chronic fasciolosis is often diagnosed by faecal egg counting (FEC), following concentration of the eggs in the sample by a zinc sulphate floatation method. However, concentration by a sedimentation technique gives improved sensitivity. Interpretation of FEC results for fasciolosis is complicated by factors such as the long pre-patent period and irregular egg shedding. Thus, FEC reduction tests (FECRT), when used alone, are not completely reliable for diagnosis of anthelmintic susceptibility or resistance in local fluke populations, especially when parasite burdens are small. A *Fasciola hepatica* coproantigen ELISA test has been introduced which more accurately reflects the presence of flukes in the host bile ducts in late pre-patent infections, and absence of flukes following successful chemotherapeutic intervention. The aim of the present study was to elucidate the specificity of the *F. hepatica* coproantigen ELISA technique, particularly regarding potential cross-reactivity with rumen fluke (paramphistome), gastrointestinal nematode and coccidian infections. The method involved parallel testing of a large battery of faecal samples from field-infected cattle and sheep using floatation and sedimentation FECs and coproantigen analysis. No evidence was found for significant false positivity in the *F. hepatica* coproantigen ELISA due to paramphistome, coccidian and/or gastrointestinal nematode co-infections. With sedimentation FECs less than 10 *F. hepatica* eggs per gram (epg), the likelihood of a positive coproantigen result for the sample progressively decreased. Diagnosis of fasciolosis should be based on consideration of both FEC and coproantigen ELISA findings, to ensure optimum sensitivity for pre-patent and low-level infections.

1. Introduction

The liver fluke, *Fasciola hepatica* causes acute, subacute and chronic fasciolosis in ruminants and imposes a significant negative economic impact on sheep and cattle production in the UK and Europe (Bennett and Ijpelaar, 2003; Schweizer et al., 2005; Charlier et al., 2007). Paramphistomosis is the infection of ruminants with paramphistomes (stomach or rumen flukes). Adult paramphistomes are found in the rumen and reticulum and usually are not considered to be associated with significant clinical disease. However, immature parasites, migrating through the upper small intestine and abomasum, are associated with outbreaks of gastroenteritis and deaths in susceptible livestock (Horak, 1971; Millar et al., 2012). *Calicophoron daubneyi*, the main paramphistome species infecting domestic stock in Europe (Abrous et al., 2000; Rinaldi et al., 2005; Díaz et al., 2006), has recently been shown, in Spain, to share a common intermediate host, *Galba truncatula*, with *F. hepatica* (Martínez-Ibeas et al., 2013). *C. daubneyi* has been identified as the most common rumen fluke in sheep and cattle in Scotland (Gordon et al., 2013), and the most common, if not the only paramphistome species infecting cattle in Ireland (Zintl et al., 2014).

Fasciolosis and paramphistomosis represent increasing threats to the welfare and productivity of sheep and cattle in the UK and Ireland (Foster et al., 2008; Murphy et al., 2008; Millar et al., 2012; Zintl et al., 2014). Factors that may contribute to the increased

* Corresponding author.
E-mail address: bob.hanna@afbini.gov.uk (R.E.B. Hanna).

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prevalence and virulence of these diseases include animal importation and movement (Pilarczyk et al., 2011), climate change (van Dijk et al., 2010), faulty treatment strategies such as routine drug dosing without accurate diagnosis and use of inappropriate chemotherapy, and anthelmintic resistance in liver fluke (Fairweather, 2011). Accurate differential diagnosis, with informed use of chemotherapy, can contribute significantly to the control of parasitic diseases such as fasciolosis and paramphistomosis, while slowing the development of anthelmintic resistance.

Faecal egg count tests (FECT) based on egg floatation or sedimentation (MAFF, 1986) are usually carried out to confirm fluke infection. In FECTs, *F. hepatica* eggs and paramphistome eggs can be distinguished by shell colour (De Waal, 2010), thus the two types of infection may be differentiated. However, certain difficulties and inaccuracies are associated with the use of FECT. First, there is no precise correlation between egg output and fluke burdens (McCaughey and Hatch, 1964). The samples examined represent only a small fraction of the animal's daily output, in which eggs may be aggregated, skewing their distribution. Second, animals with pre-patent infections will give false negative results (De Leon et al., 1981; Flanagan et al., 2011a). Finally, liver fluke eggs retained in the gall bladder after successful anthelmintic treatment and removal of flukes from bile ducts may generate false positive results (Flanagan et al., 2011a,b; Sargison, 2012).

On the other hand, detection of circulating antigens or of coproantigens in faeces may provide a reliable indication of current infection with *F. hepatica* (Kaliraj et al., 1981; Matsumura et al., 1984; Langley and Hillyer, 1989; Deplazes et al., 1991; Rojas et al., 1992). This is unlike the situation with detection of serum antibodies against the parasite, which do not necessarily reflect current infection (Hillyer, 1999). A coproantigen-based test detects and measures the levels of antigens secreted by the gut of flukes from the time they enter the host's bile ducts. Considering the ease, practicality, low cost and minimally invasive approach associated with faeces sampling, effort has been devoted to the development of coproantigen testing for fasciolosis. The potential utility of a *F. hepatica* coproantigen test has been demonstrated by its ability to detect very low fluke infestations, for example, by 1 fluke, or 5 metacercarial cysts (Mezo et al., 2004), and by the fact that it has proved more sensitive in diagnosing active fasciolosis than tests for detecting excretory/secretory antigens in serum (Almazán et al., 2001). A commercial *F. hepatica* coproantigen ELISA test has been used in experimental investigations in sheep to test coproantigen levels at several stages post-infection, and the efficacy of a coproantigen reduction test (CRT) has been confirmed for the diagnosis of TCBZ-resistant fasciolosis in sheep (Flanagan et al., 2011a,b). The utility of the same *F. hepatica* coproantigen-based test for TCBZ-resistance testing has been compared, under normal working farm conditions, with its performance in experimental studies (Gordon et al., 2012a,b; Hanna et al., 2015).

While clinical and field trials on the sensitivity of coproantigen-based diagnosis of TCBZ-resistant fasciolosis in sheep have been conducted, the specificity of coproantigen testing for fasciolosis in the presence of potentially cross-reacting parasites of ruminants such as paramphistomes, gastrointestinal nematodes, cestodes and coccidians has received less attention. Kajugu et al. (2012) found no evidence for cross-reactivity in a study involving immunocytochemical labelling of paramphistome and *Taenia hydatigena* sections with MM3 monoclonal antibody to *F. hepatica* coproantigens, and Gordon et al. (2013) indicated that *C. daubneyi* does not cross-react in a coproantigen ELISA test for fasciolosis, under field conditions.

The main aim of this project was to examine the possibility of occurrence of cross-reactivity in a commercially available coproantigen ELISA test for *F. hepatica* due to co-infection of the ruminant hosts with paramphistomes, gastro-intestinal nematodes (strongyle-type and *Nematodirus* spp.) and/or coccidians using a large battery of field-derived faecal samples submitted for routine diagnostic purposes.

2. Materials and methods

2.1. Source and storage of faecal samples

The faecal samples from naturally infected cattle and sheep used in this investigation were submitted from farms across Northern Ireland (NI) to the Veterinary Sciences Division, Agri-Food and Biosciences Institute (VSD), Stormont, for routine diagnosis of helminth infections during the period February 2010 to August 2012. In general, these were pre-treatment samples, taken from animals that had not received anthelmintic treatment in the last 12 months, and intended to inform subsequent chemotherapy. Samples accompanied by a history of recent treatment were not used for further analysis. Natural positive controls (positive *F. hepatica* faecal samples) were obtained from sheep that had died recently and were confirmed, during post-mortem examination at VSD, Stormont, to be harbouring numerous active flukes in the bile ducts. Natural negative control faeces samples were collected from shed-reared lambs, not exposed to parasite infection that had tested negative for worm eggs by sedimentation (Section 2.2.4). All faeces samples submitted were subjected to sodium chloride (NaCl) floatation (Modified McMaster Method Improved (MAFF, 1986)) and zinc sulphate ($ZnSO_4$) floatation techniques to count gastrointestinal nematode eggs, coccidian oocysts, and liver and stomach fluke eggs, respectively, within 24 h following reception at VSD, Stormont. The remainder of each sample was stored at +4 °C. For further study, samples were selected in which positive counts of one or more of coccidian oocysts, strongyle-type eggs, *Nematodirus* eggs and paramphistome eggs had been recorded, but in which no *F. hepatica* eggs were found using the $ZnSO_4$ floatation method. These samples were re-analysed using a sedimentation technique, to reveal any that were harbouring low numbers of *F. hepatica* eggs. All samples analysed by the sedimentation technique were subjected to *F. hepatica* coproantigen ELISA testing.

2.2. Examination of faecal samples

2.2.1. Preparation for floatation and sedimentation methods

From each faecal sample, 3 g were weighed and homogenised in 42 ml of water until all faecal matter was thoroughly broken down. The homogenate was passed through a 150 μm aperture wire mesh and a 15 ml sub-sample was collected in a plastic test-tube from each 45 ml homogenate.

2.2.2. NaCl floatation method (Modified McMaster method improved, for nematode eggs and coccidian oocysts (MAFF, 1986))

Test-tubes containing 15 ml homogenate (Section 2.2.1) were centrifuged for 2 min at 2000 revolutions per minute (rpm). The sediment in each was re-suspended with a fully saturated NaCl solution (specific gravity, or SG, 1.204 at 20 °C). Using a ×10 microscope objective lens, all nematode eggs and coccidian oocysts under the 1 cm^2 grid in a 0.15 ml McMaster slide chamber were counted. Each egg or oocyst counted represented 100 eggs per gram of faeces (epg) in the original sample.

2.2.3. Liver and stomach fluke egg count using zinc sulphate ($ZnSO_4$) floatation (MAFF, 1986)

Each test-tube from the NaCl floatation study (Section 2.2.2) was centrifuged for 2 min at 2000 rpm and the sediment was re-suspended with a fully saturated $ZnSO_4$ solution (SG 1.364 at 20 °C) to form a convex meniscus at the top of the tube. With re-centrifugation at 1000 rpm for 2 min, the liver and stomach fluke