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A recombinant-based ELISA evaluating the efficacy of netobimin and albendazole in ruminants with naturally acquired fascioliasis

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Abstract

The therapeutic efficacy of albendazole and netobimin in ruminants with naturally occurring fascioliasis was investigated using a recombinant-based ELISA. The variation in the IgG response against a 2.9-kDa recombinant protein (FhrAPS), termed efficacy index (EI) 1, and the egg-output changes, termed EI 2, were used to evaluate drug efficacy.

The values of EI 1 ranged between 0% and 50% in sheep, and between 0% and 30% in cattle after treatment with albendazole and netobimin. Similar EI 2 values were observed in sheep receiving albendazole or netobimin, but the highest values were found in cattle treated with netobimin. The significant reduction in the IgG response to FhrAPS found in this study shows promise in terms of developing alternative methods for evaluating the efficacy of chemotherapy against *Fasciola hepatica* in grazing ruminants.

Keywords

Fascioliasis; Ruminants; Chemoprophylaxis; Efficacy index; Recombinant protein; FhrAPS

Introduction

Although *Fasciola hepatica* infection generally results in subclinical disease, the economic consequences can be significant given the losses in animal productivity and the costs of chemoprophylaxis (Echevarria et al., 1992; Torgerson and Claxton, 1999; Elitok et al.,

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Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

2006). Most of the efforts to control fascioliasis are based on chemoprophylaxis, and several commercially available fasciolicides are highly effective against both the immature (triclabendazole) and/or mature (albendazole, closantel, netobimin) stages of liver fluke.

Fasciolicide efficacy can be evaluated by examination of the liver post mortem and by measuring faecal egg counts (Wood et al., 1995), but these methods are expensive and labour-intensive (Molloy et al., 2005). The sensitivity of faecal egg counts is also significantly reduced when small numbers of eggs are excreted (Happich and Boray, 1969). Serological tests for the diagnosis of *F. hepatica* infection have recently been developed (Ibarra et al., 1998; O'Neill et al., 1998; Sánchez-Andrade et al., 2000) and may provide an alternative, indirect method of assessing fasciolicide efficacy. In experimentally infected animals, the success of chemoprophylaxis has been measured in terms of reduced egg-output and specific IgG levels (Arias et al., 2007).

In the current study we assessed whether a recombinant-based ELISA, measuring IgG responses against a *F. hepatica* 2.9 kDa-recombinant protein (FhrAPS), could be used to evaluate the efficacy of two fasciolicides in naturally infected cattle and sheep under field conditions.

Material and methods

Sampling procedures

Blood and faecal samples were taken weekly over a 14 week period from female cattle and sheep with naturally acquired fascioliasis previously confirmed by faecal egg count. The animals were kept under field conditions and were only brought into the paddocks at night.

$$EI\ 1 = \frac{(OD_{infected\ control\ group} - OD_{treated\ group}) - OD_{uninfected\ control\ group}}{OD_{infected\ control\ group} - OD_{uninfected\ control\ group}} \times 100$$

$$EI\ 2 = \frac{(Average\ eggs\ control\ group - average\ eggs\ treated\ group)}{Average\ number\ of\ eggs\ in\ control\ group} \times 100$$

The 30 infected autochthonous Gallega sheep used (3–6 years old) were divided into three groups of 10. Sheep in group SAbz were treated with a single dose of albendazole (10 mg/kg, Valbazén, Pfizer), those in group SNb received a single dose of netobimin (15 mg/kg, Hapasil 15%, Schering-Plough), and group SC sheep were left untreated, serving as an infected control group. Sera were also obtained from an uninfected control group that consisted of 15 3-month old lambs from a farm free of fascioliasis as confirmed by faecal egg count.

The 40 infected autochthonous Rubia Gallega cattle used (3–8 years old) were divided into three groups. Group CAbz consisted of 15 animals treated with a single dose of albendazole (10 mg/kg, Zodalben-10, Laboratorios Calier). Group CNb consisted of 15 cattle that received a single dose of netobimin (20 mg/kg Hapasil 15%, Schering-Plough) and ten untreated infected cattle made up the infected control group CC. Sera were also obtained

from an uninfected control group of 12 6-month old calves from a farm free of fascioliasis as confirmed by faecal egg count.

Animals were bled by jugular venepuncture and faecal samples were collected from the rectum of each animal. These samples taken by local veterinarians as a part of collaboration with The University of Santiago de Compostela and all procedures complied with national legislation pertaining to animal health research.

Serological analysis using indirect-ELISA

Serum samples were analysed using an ELISA that detected IgG against the FhrAPS recombinant protein as previously described (Paz-Silva et al., 2005). For the analysis of the sheep sera, the FhrAPS protein concentration used to coat the wells was 3 $\mu\text{g/mL}$, sera were diluted to 1:200 in PBS–0.3% Tween 20 and 10% skimmed milk, and samples were tested in duplicate. Horseradish peroxidase conjugated with rabbit anti-bovine IgG (Nordic Immunology Laboratories) was used at a 1:1000 dilution. For the analysis of the bovine sera, the FhrAPS protein concentration used to coat the wells was 5 $\mu\text{g/mL}$, sera were diluted to 1:20 and rabbit anti-bovine IgG (Nordic Immunology Laboratories) was used at a 1:2000 dilution. Substrate of 10 mg of ortho-phenylenediamine in 12 mL of citrate buffer and 10 μL of 30% hydrogen peroxide were then added to each well and the plates incubated in the dark for 10 min at room temperature. The reaction was stopped by adding 100 μL of 3 N sulphuric acid to each well and the absorbance was read using a spectrophotometer (Titertek Multiskan) at 492 nm.

Sera from the uninfected control groups were used to calculate assay cut-off points. Positive values were determined as the mean optical density (OD) of all negative sera plus two standard deviations (Abdel-Rahman et al., 1998; Arias et al., 2007) and were calculated as P0.31 and P0.261 for sheep and cattle, respectively. Optimal serum dilutions were based on the results of previous studies (Arias et al., 2006; Arias et al., 2007).

Faecal analysis

Egg-counts were carried out on 5 g faecal samples using a sedimentation technique, and the results were presented as numbers of eggs per gram (epg) of faeces (Arias et al., 2006).

Analysis of anthelmintic efficacy

The efficacy of albendazole and netobimin treatments was determined by calculating the reduction in serum IgG and in faecal epg. These parameters were termed the efficacy indices (EI) 1 and 2, respectively, and were calculated according to the following formulae (Sánchez-Andrade et al., 2001):

Sensitivity and specificity of the tests

The sensitivity and specificity of this FhrAPS ELISA were previously calculated as 92% and 86% in sheep (Arias et al., 2007) and 86% and 88% in cattle (Paz-Silva et al., 2007), respectively.

Statistical analysis

Serological data from the FhrAPS ELISA and results of coprological assessment were analysed using the non-parametric Mann-Whitney test. The Spearman test was used to determine correlations in the data. All tests were performed using SPSS for Windows (14.1).

Results

Serological analysis using indirect-ELISA

The kinetics of the IgG response in the groups of sheep treated with each fasciolicide was similar with antibody levels decreasing 2 weeks post treatment (p.t.) and increasing from week 4 p.t. to the end of the study (Fig. 1a). Significant differences between the treated and control groups were identified from the week 2 to week 12 p.t. A positive correlation was found between the IgG values for the SABz and SNb groups ($r^2 = 0.675$, $P = 0.001$).

The IgG response curve was also very similar in both treated groups of cattle (Fig. 1b). Similar to the situation in sheep, antibody values decreased 2 weeks p.t. and then increased from 4 to 8 weeks p.t. in the CABz and CNb groups, respectively. The Mann-Whitney test revealed differences between the treated and untreated cattle at 2 and 4 weeks p.t. No correlation was found in the IgG response in the treated groups of cattle ($P > 0.05$).

Faecal examination

Mean egg counts are shown in Table 1. All animals in the infected control groups (SC and CC) shed *F. hepatica* eggs throughout the experiment. Albendazole treatment suppressed *F. hepatica* egg excretion in sheep from weeks 2 to 4 p.t. and netobimin treatment resulted in a significant reduction in excretion ($Z = -4.839$, $P = 0.001$). Significant differences in egg excretion were noted in animals in the two treated groups relative to the untreated group of sheep from weeks 2 to 8 p.t. The Spearman test identified a positive correlation between the faecal egg counts in the SABz and SNb groups ($r^2 = 0.419$, $P = 0.030$). Analysis of the egg count and IgG response indicated a significant correlation in the SABz ($r^2 = 0.577$, $P = 0.002$) and SNb groups ($r^2 = 0.720$, $P = 0.001$).

Cattle in the CNb group did not shed eggs between weeks 2 and 8 p.t. (Table 1), whereas the animals in the CABz group had significant reductions in egg counts during this time. Statistically significant differences were found between the CNb and infected control group from weeks 2 to 8 p.t., and at week 6 p.t. between the CABz and infected control group. Pearson's test found no correlation between the egg outputs of the treated groups of cattle ($P > 0.05$) and a significant positive correlation between the egg-output and IgG response of the CNb group ($r^2 = 0.670$, $P = 0.002$).

Table 2 details the percentages of animals positive on faecal egg count. No sheep in group SABz were positive from weeks 2 to 4 p.t., while the percentage of sheep shedding eggs ranged from 10% to 70% in the SNb group. In cattle group CNb, no egg shedding was recorded from weeks 2 to 8 p.t. and in the CABz group, the percentage of animals with positive faecal counts ranged from 20% to 80%.

Analysis of anthelmintic efficacy

Fig. 2 details the EI 1 values. In sheep, the highest value of 50% was recorded 4 weeks p.t. (Fig. 2a). The EI 1 values in cattle were maximal at weeks 2 and 6 in the CABz and CNb groups, respectively. Values <40% were recorded throughout (Fig. 2b).

The EI 2 values are presented in Fig. 3. A similar pattern was observed in the two treated groups of sheep, and values ranging from 80% to 100% were obtained from weeks 2 to 14 p.t. (Fig. 3a). Netobimin treatment in cattle resulted in a 100% percentage efficacy between weeks 2 and 8 p.t. In group CABz the EI 2 values ranged from 32% to 91%.

Two cattle and three sheep were pregnant during the study, and these animals were treated with albendazole. No side effects of drug treatment were reported including the treated pregnant animals.

Discussion

The use of serology to evaluate the effectiveness of chemotherapy against fascioliasis can be problematic and such evaluations are typically based on the degree to which faecal egg counts are reduced (Sánchez-Andrade et al., 2002). Although the recognition that Fasciola-specific serum IgG can persist for long periods, even following fluke removal, has limited the application of such approaches (Boulard et al., 1995; Sánchez-Andrade et al., 2002), the advantages accruing from the development of reliable serological evaluation of treatment are clear. Early chemotherapeutic intervention reduces both the tissue damage caused by the migration of the immature stages of the parasite and faecal excretion of eggs by the host (Molloy et al., 2005).

Several recombinant proteins have been used in the serological detection of *F. hepatica* infection in sheep and in cattle (O'Neill et al., 1999; Carnevale et al., 2001; Cornelissen et al., 2001; Arias et al., 2006) and a previous study indicated that an ELISA based on the FhrAPS antigen could be used to evaluate the efficacy of triclabendazole in treating experimentally infected sheep (Paz-Silva et al., 2005).

In the current study, an ELISA based on this recombinant FhrAPS protein was used to evaluate the efficacy of two fasciolicide treatments in grazing sheep and cattle. The significant reduction in the IgG responses in both species following treatment suggests that both drugs were effective in reducing the antigenic stimulus of the parasite by reducing the parasitic burden in the animals.

Wood et al. (1995) and Vera-Montenegro et al. (2003) reported that an appropriate time to evaluate the efficacy of a fasciolicide is 2–3 weeks after treatment. In the current study both fasciolicides were effective in reducing the parasitic burden in both species treated ruminants with albendazole treatment achieving 100% efficacy in sheep and netobimin treatment resulting in 100% efficacy in cattle.

The fact that antibody levels in the treated groups did not fall to the levels found in uninfected animals and increased 4 weeks after treatment, could suggest that chemoprophylaxis was unsuccessful. However, given that the animals were maintained

under field conditions throughout the experiment, treated animals were likely to have been exposed to further parasitic challenge (Sánchez-Andrade et al., 2001), which may explain the increased IgG response 4 weeks after challenge. Such a scenario might also explain why antibody levels did not fall to those found in uninfected control animals. In experimentally infected sheep, triclabendazole treatment did not result in a drop in IgG values to those of control animals in the 14 weeks after treatment (Paz-Silva et al., 2005) and in naturally infected sheep a 50–60% reduction in antibody levels against *F. hepatica*-excretory/secretory antigens was achieved following triclabendazole administration (Sánchez-Andrade et al., 2001). In our study, IgG levels were reduced by 50% in the treated animals and a significant reduction in the IgG response, rather than the given level of antibody, appears to reflect the efficacy of treatment.

Conclusions

Our results highlight the difficulty in evaluating the efficacy of fasciolicide treatments under field conditions, given the likelihood of re-infection of grazing livestock. The significant reduction in the IgG response to FhrAPS found is promising in terms of developing alternative methods for evaluating the efficacy of chemotherapy against *F. hepatica* in grazing ruminants. Other methodologies based on the detection of *F. hepatica* antigen in serum or faeces may also contribute to such assessments (Duménigo et al., 1999; Mezo et al., 2004).

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Fig. 1.

Dynamics of IgG antibody response to FhrAPS antigen in (a) sheep and (b) cattle, naturally infected with *F. hepatica*. (●) Animals treated with netobimin; (■) animals treated with albendazole; (△) infected control animals. Results are means $\pm$ SD.

Fig. 2.

Efficacy Index 1 (EI 1) of anthelmintic treatment, expressed as a percentage, as determined by the formula:

$$EI\ 1 = \frac{(OD_{infected\ control\ group} - OD_{treated\ group}) - OD_{uninfected\ control\ group}}{OD_{infected\ control\ group} - OD_{uninfected\ control\ group}} \times 100$$

in (a) sheep and (b) cattle naturally infected with *F. hepatica*. (●) Animals treated with netobimin; (■) animals treated with albendazole.

Fig. 3.

Efficacy Index 2 (EI 2) of anthelmintic treatment, expressed as a percentage, as determined by the formula: $EI\ 2 = \frac{(Average\ eggs\ control\ group - average\ eggs\ treated\ group)}{Average\ number\ of\ eggs\ in\ control\ group} \times 100$ in (a) sheep and (b) cattle naturally infected with *F. hepatica*. (●) Animals treated with netobimin; (■) animals treated with albendazole.

Fascicled hepatica egg-output in naturally infected sheep and cattle at the given time-points after treatment with albendazole (Abz) or netobimin (Nb)

Table 1

Weeks post treatment	Sheep		Cattle			
	SAbz	SNb	SC	CAbz	CNb	CC
-2	155 ± 53	227 ± 169	174 ± 134	89 ± 84	64 ± 43	120 ± 104
0	171 ± 76	236 ± 122	179 ± 71	336 ± 292	238 ± 172	273 ± 179
9	0	11 ± 18	269 ± 222	11 ± 24	0	363 ± 263
4	0	12 ± 31	187 ± 152	12 ± 32	0	459 ± 587
6	50 ± 36	11 ± 33	221 ± 218	11 ± 30	0	371 ± 292
8	9 ± 22	21 ± 37	220 ± 157	21 ± 31	0	205 ± 226
10	11 ± 14	39 ± 48	197 ± 132	35 ± 44	21 ± 26	897 ± 747
12	9 ± 16	48 ± 52	238 ± 169	54 ± 55	12 ± 19	372 ± 165
14	61 ± 27	32 ± 47	246 ± 160	29 ± 43	19 ± 34	247 ± 169

Results are expressed as means ± SD.

Table 2

Percentage of naturally infected sheep and cattle passing *F. hepatica* eggs in their faeces at the given time-points after treatment with albendazole (Abz) or netobimin (Nb)

Weeks post treatment	Sheep (% of animals)		Cattle (% of animals)	
	SAbz	SNb	CAbz	CNb
0	100	100	100	100
2	0	20	40	0
4	0	20	33	0
6	20	10	20	0
8	30	40	20	0
10	50	60	67	20
12	70	50	67	53
14	70	70	80	53