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Effect of fasciolicides on the antigenemia in sheep naturally infected with *Fasciola hepatica*

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**Abstract** A study was developed to evaluate the influence of triclabendazole (Fasinex) and netobimin (Hapasil) on the antigenemia in sheep naturally infected with *Fasciola hepatica* during 16 weeks. A sandwich-ELISA (enzyme-linked immunosorbent assay) using a rabbit polyclonal IgG antibody to *F. hepatica* antigens was employed and the data obtained were compared to those from coprological and indirect-ELISA techniques. Triclabendazole reduced the values of circulating antigens at weeks 2–4 post-treatment and faecal output at weeks 2–8 post-treatment, but antibodies showed positive values until the end of the study. Netobimin did not reduce circulating antigens of the trematode nor egg-excretion; and IgG antibodies did not decrease throughout the study.

Although a wide range of anthelmintics has been focused on eliminating immature or/adult flukes, with varying success, an important problem is to evaluate the efficacy of a treatment without killing the animals. Common diagnostic and efficacy evaluation tests consist of microscopic examination of *F. hepatica* eggs in faeces. However, this does not detect recent infections until 8–10 weeks after infection, when eggs first appear in the faeces (O’Neill et al. 2000). Likewise, the prepatent period extends beyond 10 weeks post-treatment, by which time severe liver damage has already happened (Jemli et al. 1992; Chauvin et al. 1995). Another disadvantage is that weak infections cannot be detected, becoming the source of new infections (Sánchez-Andrade et al. 1995). It has been demonstrated that most of the pathological damage takes place when flukes are migrating through the peritoneal cavity and liver parenchyma, before their establishment in the bile ducts.

**Introduction**

*Fasciola hepatica* is a liver fluke that infects many domestic mammals, especially cattle, sheep and goats. Infection occurs when metacercariae are ingested with infected vegetation and excyst in the intestine. In the acute stage, immature worms migrate through the intestine, peritoneum and liver parenchyma. The chronic stage begins when flukes reach the bile ducts and attain sexual maturity (Ikeda 1998; O’Neill et al. 2000). The significance of the economic losses led to the development and introduction of a wide variety of fasciolicides.
In the study reported here, a sandwich-ELISA was used to analyse the effect of two anthelmintics on the values of *F. hepatica* antigens circulating in sheep maintained under field conditions. We evaluated the possibility of applying the sandwich-ELISA to determine the efficacy of these two fasciolicides under the field conditions of this study.

**Material and methods**

**Animals**

Thirty female sheep from Galicia (NW Spain), naturally infected with *F. hepatica*, were used in this study. Infection was confirmed by coprological analysis 2 weeks before the beginning of the study. A group of 12 sheep were treated with a single dose of triclabendazole (10 mg kg⁻¹ bodyweight, G-T). Another group of 12 sheep were treated with a single dose of netobimin (15 mg kg⁻¹ bodyweight, G-N). One group of six naturally infected animals remained without treatment, as the control infected group (G-I).

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P.E.2 = \frac{\text{Number of eggs in control group} - \text{Number of eggs in treated group}}{\text{Number of eggs in control group}} \times 100
\]

All animals were bled weekly, by jugular venous puncture and faecal samples were collected individually, from 2 weeks prior to treatment until the 16th week post-treatment (wpt). By means of the coprological technique, faecal samples were analysed and egg counts presented as number of eggs per gram of faeces (epg).

**Sandwich-ELISA**

*F. hepatica* antigens in sera were evaluated by a polyclonal sandwich-ELISA as described by Sánchez-Andrade et al. (2000). The wells of micro-ELISA plates were coated with 100 μl of polyclonal IgG anti-*F. hepatica* (1 μg ml⁻¹ in PBS, pH 7.5) and incubated overnight at 4°C. After blocking with PBS containing 0.3% Tween 20, 10% skimmed milk and 10% horse serum, 100 μl of each serum (in duplicate) were added undiluted and incubated for 2 h. Anti-*F. hepatica* rabbit IgG (1 μg ml⁻¹) was then added to each well and incubated for 1 h. Horseradish peroxidase conjugated mouse anti-rabbit IgG (H&L chains, Nordic Immunology Laboratories, Tilburg, Netherlands) was added at 1:1500 and enzymatic reaction was revealed with substrate consisting of 10 mg of *ortho*-phenylenediamine, 12 ml citrate buffer and 10 μl of 30% hydrogen peroxide. Negative control sera were obtained from animals from a farm found by repeated coprological examinations to be free of fasciolosis for over 6 years. In order to establish the cut-off point, positive values were the mean optical density (OD) of all negative sera plus two standard deviations (Espinosa et al. 1997; Abdel-Rahman et al. 1998). Mean OD-negative sera values were 0.2464 with a standard deviation of 0.0120. Thus, positive OD values were 0.2704 or higher.

**Indirect-ELISA**

ELISAs using the excretory/secretory products from *F. hepatica* adults were performed on serum samples as previously described (Sánchez-Andrade et al. 2000). The protein concentration used to coat the wells of the polystyrene plates was 1 μg ml⁻¹, sera diluted (tested in duplicate) at 1:200 in PBS-0.3% Tween 20 and 10% skimmed milk, and horseradish peroxidase conjugated rabbit anti-ovine IgG (H&L chains, Nordic Immunology Laboratories, Tilburg, Netherlands) at 1:2000. Substrate consisting of 10 mg of *ortho*-phenylenediamine, 12 ml citrate buffer and 10 μl of 30% hydrogen peroxide was then added to each well. The plates were incubated in the dark for 10 min at room temperature. Enzymatic reaction was stopped with 100 μl well⁻¹ of 3 N sulphuric acid and OD values were read using a spectrophotometer (Titertek Multiskan) at 450 nm. Sera utilised as negative controls were the same as those used to detect specific circulating *F. hepatica* antigens by a sandwich-ELISA. In order to establish the cut-off point, positive values were the mean OD of all negative sera plus two standard deviations. Mean OD-negative sera values were 0.2730 with a standard deviation of 0.0138. Thus, positive OD values were 0.3008 or higher.

**Efficacy of the anthelmintics**

The efficacy of netobimin and triclabendazole was determined by calculating the reduction in the OD values of circulating antigens (percentage of efficacy 1, P.E.1), the epg (P.E.2) and the IgG ODs (P.E.3), by the following formulae:

\[
P.E.1 = \frac{\text{OD in control group} - \text{OD in treated group}}{\text{OD in control group}} \times 100
\]

\[
P.E.3 = \frac{\text{OD in control group} - \text{OD in treated group}}{\text{OD in control group}} \times 100
\]

**Sensitivity and specificity of the tests**

For the sandwich-ELISA, sensitivity and specificity values were determined using data from experimentally infected sheep (positive samples) and from *F. hepatica*-free sheep (controls) (Sánchez-Andrade et al. 2000). For the indirect-ELISA, these values were determined by means of positive samples obtained from infected sheep at a local abattoir, using flukes recovered from their livers. Negative samples were obtained from 3-month-old lambs from a farm found to be free of fasciolosis for over 6 years (Paz et al. 1999a).

**Statistical analysis**

Statistical analysis was performed weekly after infection. The infected groups were compared with the challenged ones and with the corresponding control group, using the non-parametric Mann–Whitney test. All tests were performed using SPSS for Windows (ver. 7.5.2S).

**Results**

**Coprological examination**

All sheep in the untreated group passed *F. hepatica* eggs throughout the experiment, with mean values of 458.68 ± 462.99 (Table 1). Mean *F. hepatica* egg counts following the administration of netobimin and triclabendazole are shown in Table 1. All G-N sheep passed eggs in faeces continuously from 1 wpt onwards, although a notable reduction was observed. In G-T