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Prevalence of natural ovine fasciolosis shown by demonstrating the presence of serum circulating antigens

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Abstract The prevalence of fasciolosis in sheep (Galicia, Northwest Spain) kept under field conditions was determined by using a sandwich-enzyme-linked immunosorbent assay (sELISA). Serum *Fasciola hepatica* circulating antigens were captured by means of a rabbit polyclonal IgG antibody to *F. hepatica* excretory/secretory products. Results were compared to those obtained by faecal sedimentation and an indirect ELISA (iELISA) and excretory/secretory antigens. Prevalences were 39.1% by sELISA, 30.4% by faecal sedimentation and 56% by iELISA; 83.3% of the sheep were positive to any one of the three tests. We observed that 59.5% of the sheep examined had active fasciolosis, 29.1% (117) had antigenaemia, 20.4% (82) passed eggs, and 40 (10%) were positive to both probes. We conclude that there is a high prevalence of fasciolosis in sheep from the studied region, and that the combination of sELISA and coprological sedimentation is extremely helpful for demonstrating current fasciolosis, so its application can be strongly recommended for epidemiological surveys.

Introduction

Fasciolosis is a parasitosis caused by the ingestion of metacercariae of *Fasciola hepatica*. Liver fluke transmission depends on a snail intermediate host. Liver flukes are enzootic primarily in regions where high

annual rainfall and large areas of poorly drained pastures provide suitable lymnaeid snail habitats, so use of irrigated pastures may increase the distribution and prevalence of liver flukes (Sánchez-Andrade et al. 2002).

Clinical disease is caused by extensive damage to the hepatic parenchyma produced during 6–8 weeks by migration of juvenile flukes. Adult liver flukes reside in the bile ducts of host animals, and eggs are passed onto the pasture in the faeces. Egg production can begin as early as 8 weeks after infection (Jemli et al. 1992), but most infections do not become patent until 11–12 weeks. Liver lesions predispose animals to infectious necrotic hepatitis and bacillary hemoglobinuria; also, host fertility can be decreased (López-Díaz et al. 1998).

Early and reliable diagnostic techniques are necessary for preventing the elevated economic losses due to the development of pathological lesions in infected animals (Kaplan 2001). Several investigations (Rodríguez-Pérez and Hillyer 1995; Duménigo et al. 1996; Espino et al. 1997; Abdel-Rahman et al. 1998; Sánchez-Andrade et al. 2001; Paz-Silva et al. 2002) have proved the suitability of the direct-enzyme-linked immunosorbent assay (ELISA) for a feasible and correct diagnostics of fasciolosis in serum or faecal samples. Duménigo et al. (1999) and Almazán et al. (2001) proved that in ovine experimental fasciolosis, antigenaemia was detected 1 week after infection, whereas positive antibody values were obtained from the second week after infection.

In previous studies carried out in Galicia (Northwest Spain), the sandwich-enzyme-linked immunosorbent assay (sELISA) has been used for the diagnosis of bovine fasciolosis (Sánchez-Andrade et al. 2000, 2002) and for evaluating anthelmintics (Sánchez-Andrade et al. 2001). Our objective was to estimate the prevalence of existing fasciolosis in ovine under field conditions. For this purpose, an sELISA was employed. Results were compared with those obtained by coprological examination and indirect-ELISA (iELISA).

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Materials and methods

Sheep

From September to December 2001, 402 blood and faecal samples were randomly collected at the same time from sheep in Galicia. Sheep were kept under field conditions. Most flocks are maintained outside and are only brought into the paddocks during the night. Lambing occurs from November to December, and lambs are weaned when approximately 1 month old. The grazing flock is generally fed on natural pastures, characterised mainly by annual grass species. All faecal and blood samples were collected before the anthelmintic treatment was administered. In this region, chemoprophylaxis is carried out after lambing ends (December).

Faecal samples were collected from the rectum of the animals. Four grams of each faecal sample were processed by the sedimentation technique, and the egg counts presented as number of eggs per gram of faeces. The sera were maintained at -25°C until their use.

To establish the sensitivity and specificity of the diagnostic probes used, samples from two groups of 15 sheep were used. These animals were kept housed since birth to avoid *F. hepatica* infections. One group was infected with 250 *F. hepatica* metacercariae, and the other remained without infection as the control group. Coprological examinations were done at weekly intervals for 14 weeks before the experimental infection to ensure that the ovine were trematode-free.

Animals were considered positive when they were positive to sELISA, to the sedimentation test, or to both probes.

F. hepatica excretory/secretory products' preparation

The use of excretory/secretory (ES) antigen from adult *F. hepatica* was based on prior reports of its successful use (Sánchez-Andrade et al. 2000). Adult liver flukes were collected from cattle bile ducts at the slaughterhouse, washed several times in phosphate-buffered saline (PBS, pH 7.5), and incubated in RPMI medium at 37°C for 3 h. Eggs were removed by sieving and centrifugation (50' at 4°C and 40,000 g), and the supernatant containing ES products was collected. The protein concentration was estimated by the bicinchoninic acid (Pierce, Madrid) technique.

Polyclonal IgG anti-*F. hepatica* preparation

Polyclonal IgG anti-*F. hepatica* antigen was obtained by inoculating two female 4-kg New Zealand White rabbits with ES trematode products (Sánchez-Andrade et al. 2000). Five-hundred micrograms of ES antigen was emulsified with 0.5 ml of Freund's complete adjuvant and was used to hyperimmunise each rabbit by giving several subcutaneous inoculations. Rabbits were given similar inoculations on three dates. Blood was drawn 21 days after the final inoculation. Serum was pooled and anti-*F. hepatica* globulins containing IgG were obtained by precipitation with 50% ammonium sulphate, dialysed exhaustively against distilled water, and then the IgG was partially purified using the MAbTrap kit (Amersham Pharmacia Biotech, Madrid) for purification of antibodies.

Sandwich-enzyme-linked immunosorbent assay

Circulating *F. hepatica* antigens in sera were evaluated by a polyclonal sELISA as described by Sánchez-Andrade et al. (2001). Briefly, wells of micro-ELISA plates were coated with 100 μl polyclonal IgG anti-*F. hepatica* ($1 \mu\text{g ml}^{-1}$ in PBS, pH 7.5) and blocked with PBS containing 0.3% Tween 20, 10% skimmed milk and 10% horse serum. One-hundred microlitres of each serum (in duplicate) was added undiluted followed by $1 \mu\text{g ml}^{-1}$ of anti-*F. hepatica* rabbit IgG. Horseradish peroxidase-conjugated mouse anti-rabbit IgG

(H&L chains; Nordic Immunology Laboratories, Tilburg, The Netherlands) was added at 1:1500, and the enzymatic reaction was revealed with substrate consisting of 10 mg *ortho*-phenylenediamine, 12 ml citrate buffer and 10 μl of 30% hydrogen peroxide.

In order to establish the cut-off point, positive values were the mean optical density (OD) of 15 negative sera plus 2 SDs (Espino et al. 1997; Abdel-Rahman et al. 1998). Thus, positive values were $\text{OD} \geq 0.31$.

Indirect-enzyme-linked immunosorbent assay

ELISAs using ES were performed on serum samples as previously described (Sánchez-Andrade et al. 2001, 2002). The protein concentration used to coat the wells of the polystyrene plates was $1 \mu\text{g ml}^{-1}$, and sera were 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) at 1:2000. After adding the substrate, the enzymatic reaction was stopped with 100 μl per well of 3 N sulphuric acid, and absorbances were read using a spectrophotometer (Titertek Multiskan) at 450 nm. Sera utilised as controls were the same as those used to detect specific circulating *F. hepatica* antigens by a sELISA. In order to establish the cut-off point, positive values were the mean OD of 15 negative control sera plus two SDs. Thus, positive absorbance values were ≥ 0.4006 .

Sensitivity and specificity of the tests

Sensitivity and sensibility were determined with sera from experimentally infected sheep, and corresponded to 100% and 100% for sedimentation, 92% and 94% for iELISA and 86% and 100% for sELISA.

Results and discussion

Sandwich-enzyme-linked immunosorbent assay

We observed 39.1% (157/402) of the sheep were positive for the presence of *F. hepatica* circulating antigens (Table 1). During their migration through the liver parenchyma (1–5 weeks after infection), immature flukes release antigens which are detectable in the serum, and once the flukes reach the bile ducts, the antigenic release ends and is not present in serum (Duménigo et al. 1999; Sánchez-Andrade et al. 2001; Almazan et al. 2001). Consequently, the demonstration of circulating antigens in sera allows the early detection of immature flukes, so an early anthelmintic treatment could be administered, which would enable a reduction in the hepatic injury due to *F. hepatica* (Sánchez-Andrade et al. 2000).

Table 1 Prevalence of fasciolosis in sheep naturally exposed to *Fasciola hepatica* by means of sandwich-enzyme-linked immunosorbent assay (sELISA) and indirect-ELISA (iELISA). Results are expressed as total numbers

	Sedimentation +	Sedimentation –	Total
sELISA + /iELISA +	16	67	83
sELISA + /iELISA –	24	50	74
sELISA – /iELISA +	46	96	142
sELISA – /iELISA –	36	67	103
Total	122	280	402

Fig. 1a, b Optical densities obtained for *Fasciola hepatica* in sheep exposed to *F. hepatica*. **a** Negative faecal examination. **b** Positive faecal examination. ▼ Animals positive to indirect-enzyme-linked immunosorbent assay (iELISA) and sandwich-ELISA (sELISA); ■ animals positive to iELISA and negative to sELISA; □ animals negative to iELISA and positive to sELISA; ○ animals negative to iELISA and sELISA. Horizontal line reflects cut-off value for detection of antibodies (iELISA). Vertical line reflects cut-off value for detection of circulating antigens (sELISA). Ab Antibodies against *F. hepatica* antigens, CAg *F. hepatica* circulating antigens

Sedimentation technique

We found that 30.4% (122/402) of sheep passed *F. hepatica* eggs in the faeces (Table 1), in agreement with previous work carried out in Northwest Spain (Ferre et al. 1995). The presence of *F. hepatica* eggs in the faeces reveals that mature flukes are located in the bile ducts and/or the gall-bladder. Most of the epidemiological studies on fasciolosis have been carried out by coproscopic analysis (Dwinger et al. 1982; Boulard et al. 1985; Bouvry and Rau 1986). Nevertheless, it has to be considered that coprological methods cannot detect fasciolosis until 8–12 weeks after infection, when most of the pathological lesions have been formed (Jemli et al. 1992; Almazan et al. 2001).

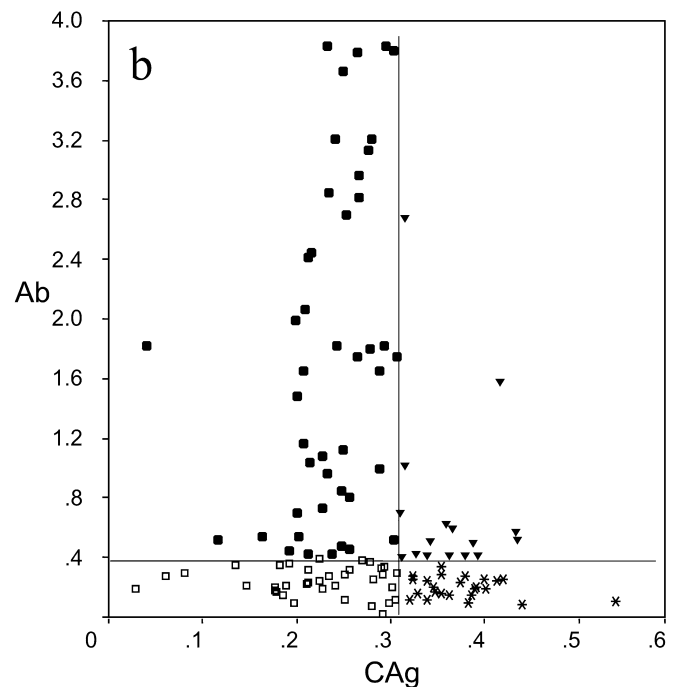
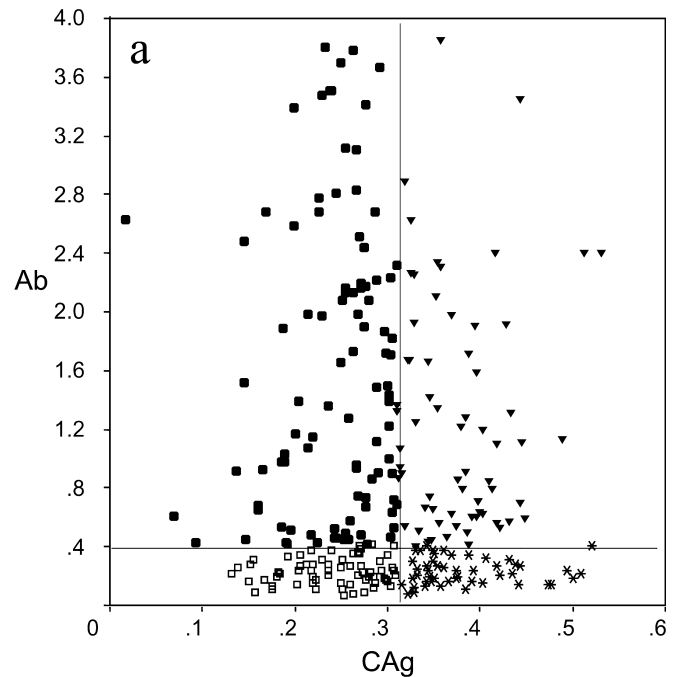
Indirect-enzyme-linked immunosorbent assay

The percentage of sheep positive for the presence of specific antibodies (IgG) against *F. hepatica* excretory/secretory antigens was 56% (225/402; Table 1). This prevalence is in agreement with data from Ferre et al. (1995). Rodríguez-Pérez and Hillyer (1995) and Duménilo et al. (1999) proved that in sheep experimentally infected with *F. hepatica* metacercariae IgG could be detected from the second week after infection. Although immunoenzymatic techniques which are focused on the detection of antibodies against parasitic antigens, such as the iELISA, have been applied to solve the disadvantages of coprological examination (Cornelissen et al. 1992; Hillyer et al. 1996), the presence of antibodies does not correlate to the existence of an active infection, and shows exposure to the parasite only (Espino et al. 1998; Sánchez-Andrade et al. 2001). This enhances the need for a feasible test for the diagnosis of fasciolosis which is capable of detecting a current infection at the moment of sampling.

Comparison between the three different diagnostic methods

Figure 1a, b summarizes the results after the comparison of the sedimentation, sELISA and iELISA tests. Only 16.7% of sheep (67/402) were negative to all three diagnostic methods, whereas the percentage of sheep positive to the three probes was 4%. Of the tested sheep 83.3% (335/402) were positive to any one of the probes employed.

In the present study, the prevalence of sheep with active fasciolosis was 59.5% (239/402), 29.1% (117) had



antigenaemia, 20.4% (82) passed eggs, and 40 (10%) were positive to both probes (Table 1, Fig. 1b), which reflects the presence of both *F. hepatica* stages, immature and mature ones. Positive antibody values to *F. hepatica* excretory/secretory antigens were observed in 50.8% (62/122) of the sheep passing eggs in the faeces.

An interesting result was that 41.8% (117/280) of the sheep negative to the sedimentation technique were positive to the sELISA, suggesting that juvenile flukes were migrating through the liver parenchyma, but had not yet reached the bile ducts/gall-bladder, so eggs could

not be observed in faeces. It has been reported that most of the notable pathological lesions during fasciolosis occur when immature flukes are migrating through the liver parenchyma (Rodríguez-Pérez and Hillyer 1995).

One group of 96 animals was positive to the iELISA and negative to the coprology test and sELISA (Table 1). This observation seems to indicate previous exposure to the liver fluke (Espino et al. 1997; Sánchez-Andrade et al. 2001), i.e. sheep had been in contact with the trematode and developed an antibody humoral response (positive to the detection of IgG), but were not infected at the moment of sampling (Espino et al. 1998). Finally, 19.7% of the sheep positive to sedimentation were negative to iELISA, which could reflect that the immunological system of these animals did not respond in a suitable way to the antigenic stimulus released by the flukes. One possible explanation could be that these sheep are very young and their immune system behaves inappropriately.

Epidemiological studies are essential because more reliable information about the distribution and determinants of a parasitic disease can be obtained. These studies are also needed to get an overview of the global actions required to protect the health of sheep, to prevent the possibility of *F. hepatica* infection or reduce its frequency. No epidemiological studies for the detection of *F. hepatica* circulating antigens have been developed in sheep maintained under field conditions. In the current work, 39% of the animals tested had antigenaemia.

One of the most employed practices in the control of fasciolosis is chemoprophylaxis. According to our results, 335 (83.3%) sheep had to be treated with a fasciolicide because they were positive to any one of the employed tests. However, active fasciolosis was only demonstrated in 59.5% of sheep, so the cost of chemoprophylaxis would be significantly reduced if treatment could be restricted to these animals.

Our results indicated a high prevalence of fasciolosis in sheep maintained under field conditions in Galicia. We conclude that the combination of the direct-ELISA and the coprological examination provides excellent knowledge about the real parasitic status of the animal, because both tests demonstrate current fasciolosis, and we suggest their application for epidemiological surveys undertaken to establish the prevalence of this trematodosis.

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References

- Abdel-Rahman SM, ÓReilly KL, Malone JB (1998) Evaluation of a diagnostic monoclonal antibody-based capture enzyme-linked immunosorbent assay coproantigen in cattle. *Am J Vet Res* 59:533–537
- Almazán C, Ávila G, Quiroz H, Ibarra F, Ochoa P (2001) Effect of parasite burden on the detection of *Fasciola hepatica* antigens in sera and feces of experimentally infected sheep. *Vet Parasitol* 97:101–112
- Boulard C, Bouvry M, Argente G (1985) Comparaison de la détection des foyers de fasciolose par test ELISA sur lactosérum et sérum et par coproscopie. *Ann Rech Vet* 16:363–368
- Bouvry M, Rau ME (1986) Seasonal variations in egg passage or *Fasciola hepatica* in dairy cows in Quebec. *Vet Parasitol* 22:263–273
- Cornelissen JB, de Leeuw WA, van der Heijden PJ (1992) Comparison of an indirect haemagglutination assay and an ELISA for diagnosing *Fasciola hepatica* in experimentally and naturally infected sheep. *Vet Q* 14:152–156
- Duménigo BE, Espino AM, Finlay CM (1996) Detection of *Fasciola hepatica* antigen in cattle faeces by a monoclonal antibody-based sandwich immunoassay. *Res Vet Sci* 60:278–279
- Duménigo BE, Espino AM, Finlay CM, Mezo M (1999) Kinetics of antibody-based antigen detection in serum and faeces of sheep experimentally infected with *Fasciola hepatica*. *Vet Parasitol* 86:23–31
- Dwinger RH, Le Riche PD, Kühne GI (1982) Fascioliasis in beef cattle in north-west Argentina. *Trop Anim Health Prod* 14:167–171
- Espino AM, Marcet R, Finlay CM (1997) *Fasciola hepatica*: detection of antigenemia and coproantigens in experimentally infected rats. *Exp Parasitol* 85:117–120
- Espino AM, Díaz A, Pérez A, Finlay CM (1998) Dynamics of antigenemia and coproantigens during a human *Fasciola hepatica* outbreak. *J Clin Microbiol* 36:2723–2726
- Ferre I, Ortega-Mora LM, Rojo-Vázquez FA (1995) Seroprevalence of *Fasciola hepatica* infection in sheep in northwestern Spain. *Parasitol Res* 81:137–142
- Hillyer GV, Soler de Galanes M, Buchón P, Buorland J (1996) Herd evaluation by enzyme-linked immunosorbent assay for the determination of *Fasciola hepatica* infection in sheep and cattle from the Altiplano of Bolivia. *Vet Parasitol* 61:211–220
- Jemli MH, Escoula L, Magnaval JF, Dorchies P (1992) Exploration de la réponse immunitaire chez l'agneau infesté expérimentalement par *Fasciola hepatica*. *Rev Med Vet* 143:355–360
- Kaplan RM (2001) *Fasciola hepatica*: a review of the economic impact in cattle and considerations for control. *Vet Therap* 2:40–50
- López-Díaz MC, Carro MC, Cadórniga C, Díez-Baños P, Mezo M (1998) Puberty and serum concentrations of ovarian steroids during prepuberal period in Friesian heifers artificially infected with *Fasciola hepatica*. *Theriogenology* 50:587–93
- Paz-Silva A, Pedreira J, Sánchez-Andrade R, Suárez JL, Díaz P, Panadero R, Díez-Baños P, Morrondo P (2002) Time-course analysis of coproantigens in rats infected and challenged with *Fasciola hepatica*. *Parasitol Res* 88:568–573
- Rodríguez-Pérez J, Hillyer GV (1995) Detection of excretory-secretory circulating antigens in sheep infected with *Fasciola hepatica* and with *Schistosoma mansoni* and *Fasciola hepatica*. *Vet Parasitol* 56:57–66
- Sánchez-Andrade R, Paz-Silva A, Suárez J, Panadero R, Díez-Baños P, Morrondo P (2000) Use of a sandwich-enzyme-linked immunosorbent assay (SEA) for the diagnosis of natural *Fasciola hepatica* infection in cattle from Galicia (NW Spain). *Vet Parasitol* 93:39–46
- Sánchez-Andrade R, Paz-Silva A, Suárez JL, Panadero R, Pedreira J, Díez-Baños P, Morrondo P (2001) Effect of fasciolicides on the antigenaemia in sheep naturally infected with *Fasciola hepatica*. *Parasitol Res* 87:609–614
- Sánchez-Andrade R, Paz-Silva A, Suárez JL, Panadero R, Pedreira J, López C, Díez-Baños P, Morrondo P (2002) Influence of age and breed on natural bovine fasciolosis in an endemic area (Galicia, NW Spain). *Vet Res Commun* 26:361–370