A sero-epidemiological study of infection by Fasciola hepatica and Schistosoma bovis in cattle and sheep in Western Spain

López-Abán, J.[a]; Ramos, S.[a]; Ramajo, V.[b]; Oleaga, A.[b]; Muro, A.[a]

[a] Laboratorio de Parasitología, Facultad de Farmacia. Universidad de Salamanca. 37007 Salamanca, Spain.
[b]Unidad de Patología Animal. Instituto de Recursos Naturales y Agrobiología de Salamanca, Consejo Superior de Investigaciones Científicas (CSIC). 37008 Salamanca, Spain

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Abstract: The present study deals with the epidemiology of *Fasciola hepatica* and *Schistosoma bovis* in Salamanca (Western Spain). The indirect ELISA was used to assess infection in 1130 bovine and 1070 ovine samples, using excretory/secretory antigens from *F. hepatica* and the somatic antigen from *S. bovis*. The relationship between geographical origin, animal age and sex, and seropositivity was as well studied. Results showed that 11.95% of bovine and 11.21% of ovine samples were *F. hepatica* positive, and 3.63% of bovine and 7.48% ovine sera were positive against *S. bovis* antigens. In addition, 19 bovine sera (1.68%) and 15 ovine sera (1.40%) were positive to both parasites. Anti-*F. hepatica* antibody detection in cattle showed medium (20.5-23.8%) and low prevalence zones. In ovine samples, we found two zones with medium prevalence for *F. hepatica* (20%) and low prevalence. Seroprevalence against *S. bovis* showed percentages of 2.3-4.9% in bovine samples and 6.4-10.2% in ovine samples, with no major differences among different areas. No significant differences were found regarding seroprevalences and age and sex of animals.

Keywords: *Schistosoma bovis*, *Fasciola hepatica*, epidemiology, cattle, sheep, ELISA.

Resumen: Este trabajo trata de la epidemiología de *Fasciola hepatica* y *Schistosoma bovis* en Salamanca (España). Se empleó un ELISA indirecto para evaluar la infección de 1130 muestras de bovino y 1070 de ovino, empleando antígenos de excreción-secreción de *F. hepatica* y antígeno somático de *S. bovis*. Se estudiaron las relaciones entre seropositividad y el origen geográfico, edad, y sexo de los animales. Los resultados mostraron que el 11,95% de las muestras de bovino y el 12,21 % de las de ovino fueron positivos a *F. hepatica* y el 3,63% de los sueros de bovino y el 7,48% de los de ovino fueron positivos frente al antígeno de *S. bovis*. Además, 19 muestras de bovino (1,68) y 15 de ovino (1,40%) fueron positivas a los dos parásitos La detección de anticuerpos frente a *F. hepatica* en bovinos mostró zonas de prevalencias medias (20,5-23,8 %) y bajas. En las muestras de ovino se encontraraon dos zonas de prevalencias medias de *F. hepatica* (20%) y el resto de prevalencias bajas. La seroprevalencia de *S. bovis* fue del 2,3-4,9% en muestras de bovino y del 6,4-10,2% en las de ovino, sin que se encontraran diferencias entre las distintas áreas. No se encontraron diferencias significativas entre las seroprevalencias por edades y sexos.

Palabras Clave: *Schistosoma bovis*, *Fasciola hepatica*, epidemiología, bovino, ovino, ELISA.

1. Introduction

Fasciola hepatica is a world-wide parasite. On the other hand, *Schistosoma bovis* is found in Africa, Middle East and some Mediterranean countries (Hussein, 1973). *F. hepatica* causes important losses on farms of both ovine and bovine cattle in the whole world and *S. bovis* is a problem limited to determined regions of Africa during certain times of the year (Genicot *et al.*, 1991; De Bont & Vercruyse, 1998). *F. hepatica* is very prevalent in areas with humid climates and *S. bovis* is more prevalent in foci of warm humid areas (Luzón Peña *et al.*, 1994; De Bont & Vercruyse, 1998). The data for prevalence and distribution together with the epidemiological data known for periods of transmission, patterns of re-infection and factors which affect the intermediary hosts are considered important for the establishment

Corresponding author:
Dr. Antonio Muro
Laboratorio de Parasitología, Facultad de Farmacia.
Universidad de Salamanca.
Avda. Campo Charro s/n 37007 Salamanca, Spain.
Tel.: +34 923 29 45 35, Fax.: +34 923 29 45 15
E-mail address: ama@usal.es

of programmes for the control of both parasites (Parr & Gray, 2000).

In this respect, coprological techniques for the detection of *S. bovis* and *F. hepatica* in infected animals are quite time consuming and thus not very useful for large scale epidemiological studies. In addition, coprology would fail to detect infected animals in the prepatent period of these diseases (Dargie, 1980; Vercruysse & Schandevyl, 1984; De Bont *et al.*, 1994, Sánchez Andrade *et al.*, 2000). On the other hand, the indirect enzyme linked immunosorbent assay (ELISA) technique using excretory/secretory proteins from *F. hepatica* (ESFh) as antigen, seems to be more useful than coprological methods. This technique allows the detection of anti-*F. hepatica* specific antibodies from the third week post-infection, and has been shown to be very sensitive for the detection of this parasite in both sheep and bovine (Hillyer *et al.*, 1996). Similarly, the indirect ELISA using somatic *S. bovis* antigens (SoSb) has been shown to detect specific antibodies from the third week of infection in experimentally infected sheep (Rodríguez-Osorio *et al.*, 1999).

Thus, the aim of this work was to determine the seroprevalence of *F. hepatica* and *S. bovis* in cattle and sheep in the province of Salamanca, a semi-arid region located in Western Spain, using ESFh and SoSb antigens in an indirect ELISA test. The area of study was selected because it represents a region where cattle and sheep are bred in extensive systems, in which 6.36% and 3.45% of total cattle and sheep in Spain are bred, respectively. Although low incidences were reported for both *S. bovis* and *F. hepatica* (Ramajo *et al.*, 1995, Ramajo *et al.*, 1996). The influence of the age and sex of the animals in seroprevalence was also evaluated as well as, the relationship between both infections.

2. Material and methods

2.1. Area of study

This study was carried out in the province of Salamanca, situated in the West of Spain an average altitude of 800 m above sea level. The climate is characterized by being hot and dry in summer, temperate in spring and autumn and cold in winter. The average maximum temperature is 20-24°C in July-August and the average minimum is 2-6°C in December-January. The rainfall varies between 300-1200 mm per year, with a very dry period in summer, which lasts from one to four months. It is considered to be a semi-arid region with a temperate climate. The study area was divided into 8 farming zones in which relative differences are found in altitude, the hydrographical network and the predominant type of lands used by the animals of this study (Figure 1).

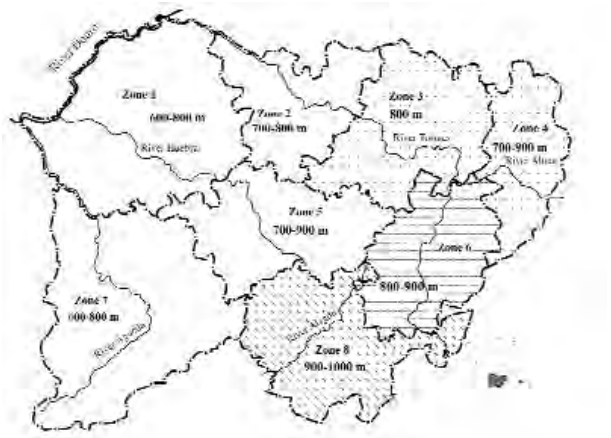

Fig. 1: Map of the geographical location of the study area (Province of Salamanca, Spain) showing the division into eight farming zones with differences regarding altitude, hydrographic network and type of land predominant on which the bovine cattle and sheep of the study graze. (dry pastures, dry pastures and dry and irrigated crops, dry and irrigated pastures and crops and mountain pastures).

2.2. Serum samples

All cattle and sheep included in this study were raised in extensive system grazing pastures throughout the year and were older than 6 month. A total of 1130 samples from bovine and 1070 sheep were collected in proportion to the cattle census in each of the eight farming zones between May and October of 2001 and were stored in aliquots at –20ºC until processed. Regarding animal ages, cattle were grouped into five groups: 0.5-2 years, 3-4 years, 5-6 years, 7-8 years and more than 8 years. Sheep were grouped into four age groups: 0.5-2 years, 3-4 years, 5-6 years and more than 6 years. The number of samples for each age group was proportional to the number of animals in that age group. The majority of

samples was originated from female animals, divided in 1,014 cows and 1,005 ewes. Sera from only 116 bulls and 65 rams were tested.

Sera used as negative controls were obtained from eight coprologically negative lambs and calves from a *S. bovis* and *F. hepatica* free area. Positive control sera were obtained from six experimentally orally infected lambs with 100 metacercariae of *F. hepatica*, three experimentally orally infected calves with 700 metacercariae of *F. hepatica*, six lambs infected experimentally with 600 cercariae of *S. bovis* percutaneously for 1 hour, and three calves infected experimentally with 1200 cercariae of *S. bovis*, for 1 hour. The sera were obtained after 12 weeks of infection and were stored in aliquots at -80ºC until used.

2.3. Serological test

Sera were analysed by indirect ELISA as described Hillyer *et al.* (1996) and Rodríguez-Osorio *et al.* (1999). Basically, flat bottom, flexible polyvinyl plates were coated with carbonate buffer (pH 9.6, 100 µl/well) containing 4 µg/ml excretory/secretory *F. hepatica* (ESFh) or 5 µg/ml somatic *S. bovis* antigens (SoSb) for 18 h at 4°C. The plates were then washed three times with phosphate buffer solution 0.05% Tween 20 (PBS-Tween). Cattle or sheep sera were tested in duplicate in a final volume of 100 µl per well at 1/100 dilution in PBS-Tween. Sera were incubated at 37°C for 1 hour and then washed as above. Subsequently, 100 µl per well of polyclonal rabbit anti-bovine or anti-sheep IgG conjugated with peroxidase (Sigma), diluted 1/2000 or 1/2500 in PBS-Tween, respectively, were incubated at 37°C for 1 hour and washed as above. Finally the plates were developed with 100 µl ortho-phenylene-diamine 0.06% and 4 µl of H_2O_2 30% in citrate buffer (pH 5) per well at room temperature for 30 minutes. The reaction was stopped with 50 µl per well 3N sulphuric acid and measured in an ELISA reader (SLT 349 ATC, Lab Instruments) at 492 nm.

Sensitivities were determined with sera from experimentally infected bovines and sheep. Using excretory/secretrory F. hepatica antigen sensitivity was of 82% for bovine cattle and 100% for sheep and with somatic antigen of *S. bovis* antigens was of 100% in sheep and bovines.

In order to establish the cut off point, the Serological Index (SI) was calculated according to the following formula: [(Optical Density (O.D.) of the negative control - O.D. of the tested problem) / (O.D. of the negative control - O.D. of the positive control)] x 100. According with Medori *et al.*, (1996) sera with a SI higher than 50 in three separate assays were considered positive.

2.4. Statistical analysis

In order to study the possible statistical differences between groups, the SI against *F. hepatica* and *S. bovis*, the two animal species and the sex groups inside each animal species were compared using the Student t- test. The differences among groups regarding geographical distribution and age were compared by analysis of variance (ANOVA). When global differences were detected, a post-ANOVA Fisher PLSD test was applied. All statistical differences were considered significant when $p<0.05$. Statistical analysis were performed using the statistical software, StatView 4.5 (Abacus Concepts, Inc.) for a Macintosh computer.

3. Results

A total of 135 cattle (11.95%) and 120 sheep (11.21%) serum samples were positive against *F. hepatica*. Thus, the overall seroprevalences against this parasite were similar in cattle and sheep. On the contrary, detection of antibodies against *S. bovis* showed a lower number of positive individuals in cattle than in sheep, with 41 cattle (3.63%) and 80 sheep (7.48%) samples positive against this trematode. The number of positive sera against both parasites was 19 (1.68%) for cattle and 15 (1.40%) for sheep. Serological Indexes (SI) are shown in Figure 2, for bovine and Figure 3, for ovine samples. The seroprevalence of *F. hepatica* was higher than *S. bovis* in bovines ($p<0.01$) but, there were no statistically significant differences between the seroprevalences of both parasites in sheep.

Distribution of positive sera against *F. hepatica* in different zones (Table 1) varied between 0% and

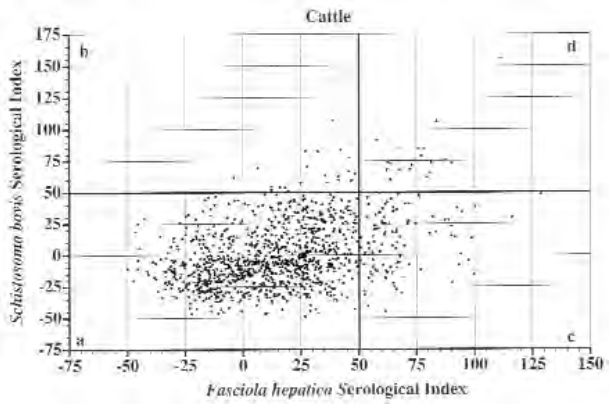

Fig. 2: Scatter of cattle serum samples showing the Serological Indexes in ELISA against *F. hepatica*/excretory secretory (ESFh) and *S. bovis.* (SoSb) antigens. Samples with Serological Index =50 were considered as positive. (a) Sera negative to ESFh and SoSb; (b) Sera positive to SoSb but negative to ESFh; (c) Sera positive to ESFh but negative to SoSb; (d) Sera positive to ESFh and to SoSb.

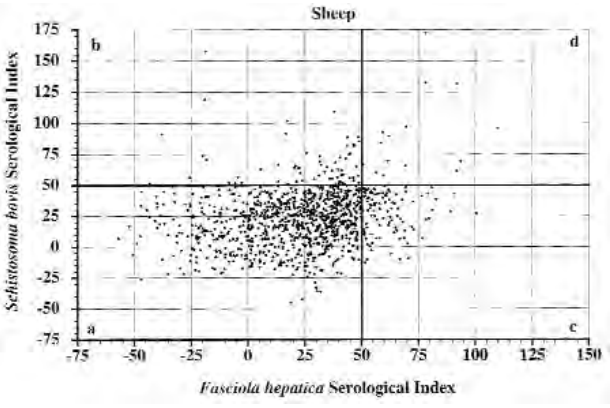

Fig. 3: Scatter of sheep serum samples showing the Serological Indexes in ELISA against *F. hepatica* excretory/secretory (ESFh) and *S. bovis.* (SoSb) antigens. Samples with Serological Index =50 were considered as positive. (a) Sera negative to ESFh and SoSb; (b) Sera positive to SoSb but negative to ESFh; (c) Sera positive to ESFh but negative to SoSb; (d) Sera positive to ESFh and to SoSb.

23.8% in cattle, with medium and low prevalence zones (statistically significant differences p<0.0001). Zones with medium prevalences were represented by Zones 3 and 8, with 20.5% and 23.8% prevalences, respectively. Within these zones, six localities of the 27 sampled showed prevalences higher than 50%. The remaining zones had prevalences lower than 14.4%, but two localities showed prevalences higher than 50%. In sheep, against *F. hepatica* showed prevalences ranged between 3.2% and 20% in the different zones, with two medium prevalence (20%) Zones 6 and 7, which were statistically different from

Table 1: Seroprevalence against *F. hepatica* excretory secretory antigen (ESFh) and *S. bovis* antigen (SoSb) in cattle and sheep in the different zones under study.

			F. hepatica		S. bovis	
	Zones	n	Positive samples	%	Positive samples	%
Cattle	Zone 1	196	16	8.2	2	1.0
	Zone 2	125	15	12.0	6	4.8
	Zone 3	105	25	23.8*	1	1.0
	Zone 4	45	0	0.0	1	2.2
	Zone 5	201	29	14.4	11	5.5
	Zone 6	136	7	5.2	8	5.9
	Zone 7	205	19	9.3	8	3.9
	Zone 8	117	24	20.5*	4	3.4
Sheep	Zone 1	343	36	10,5	22	6.4
	Zone 2	31	1	3.2	1	3.2
	Zone 3	195	11	5.6	13	6.7
	Zone 4	173	24	13.9	18	10.4
	Zone 5	162	18	11.1	12	7.5
	Zone 6	55	11	20.0*	6	10.9
	Zone 7	40	8	20.0*	5	12.5
	Zone 8	71	11	15.5	1	1.4

* Zones with medium prevalences (p<0.01)

the rest of zones (p<0.001). Nevertheless, *F. hepatica* prevalence in sheep was always lower than 50% in all localities of the study area.

Distribution of positive sera against *S. bovis* in different zones (Table 1) varied between 1% and 5.9% for cattle and 1.4%-12.5% in sheep, although no statistically significant differences were found among the different zones of the study, nor localities with more than 50% of animals infected.

Age related seroprevalences are showed in Table 2. Seroprevalences against *F. hepatica* were similar between age groups of cattle (10.4% -13.1%),

Table 2: Age related cattle and sheep seroprevalence against *F. hepatica* excretory secretory antigen (ESFh) and *S. bovis.* antigen (SoSb).

			F. hepatica		S. bovis	
	Age groups	n	Positive samples	%	Positive samples	%
Cattle	0.5-2 years	202	22	10.9	7	3.5
	3-4 years	222	28	12.6	6	2.7
	5-6 years	221	23	10.4	5	2.3
	7-8 years	217	27	12.4	10	4.6
	> 8 years	268	35	13.1	13	4.9
Sheep	0.5-2 years	227	21	9.3	17	7.5
	3-4 years	502	63	12.6	32	6.4
	5-6 years	303	27	8.9	31	10.2
	>6 years	38	9	23.7	0	0.0

whereas in sheep a greater variation was found (9.3%-23.7%). Nonetheless, the statistical study does not establish differences between any of the age groups of the two species. Seroprevalences against *S. bovis* were similar among the age groups of cattle (2.3%-4.9%) and sheep (6.4%-10.2%), and do not present statistically significant differences, either.

Sex related seroprevalences against *F. hepatica*, 12.1% for cows and 10.3% for bulls in cattle and 10.9% for ewes and 15.4% for rams in sheep, statistically significant differences were not found. Seroprevalence against *S. bovis* demonstrated prevalences of 3.5% for cows, 5.2% for bulls, 7.2% for ewes and 10.9% for rams, statistically significant differences were not found.

4. Discussion

In this study the seroprevalences reported against *Fasciola hepatica* in bovine cattle and sheep were 11.95% and 11.21%, respectively, both greater than those reported employing coprology in previous studies: 8.5% in bovines y 9.1% in sheep (Ramajo *et al.*, 1995; Ramajo *et al.*, 1996). We consider that these differences are due to the greater sensitivity of the assay used. The seropositive animals to *F. hepatica* are distributed throughout the area of study. In bovines two zones with medium prevalences are found (Zone 3 and Zone 8), where six localities are outstanding with more than 50% of the animals seropositive. In previous studies, in the same area Simón & Ramajo (1983) describe sub-acute and acute cases of fasciolosis, both in bovines and sheep in Zones 3 y 6, and now we find only mean seroprevalences in sheep in Zone 6 and bovines in Zone 3, probably because of the application of measures of control. Our data, both for cows and sheep are much lower than those obtained by serological techniques for close more humid areas of the North-East of Spain, 77.6% in sheep (Ferre *et al.*, 1995), 37.3-85.1% in bovines (Sanchez-Adrade *et al.*, 2000) and other endemic regions of fasciolosis in the world: 89% in sheep and 57-58% in bovines in Bolivia (Hillyer et al., 1996) and 60-100% in bovines in Mexico (Ibarra et al., 1998).

Regarding *Schistosoma bovis*, 3.6 % of bovines and 7.48% of sheep are seropositive. In bovines, this seroprevalence is somewhat higher than that previously reported (2.6%) by coprology (Ramajo *et al.*, 1995) and can be attributed, equally, to the greater sensitivity of the assay. Contrarily, *S. bovis* has not been reported using coprology in sheep (Ramajo *et al.*, 1996), probably because of the difficulties involved in the detection of the parasite in faeces in the initial periods and the low parasitic load, which this host habitually presents. Furthermore, in this work, *S. bovis* seropositive bovines and sheep are found in all the study zones, whereas in previous studies infection by *S. bovis* was only reported in Zones 1, 5, 7 and 8 tributaries of the Águeda, Huebra and Alagón rivers (Ramajo 1972; Simón & Ramajo, 1982). We consider that these differences are due to the greater sensitivity of the assay and the larger number of farms analysed in this study. If we compare our data with the prevalences obtained by coprology for *S. bovis* reported for endemic zones of Africa, 34% Tanzania, (Makundi *et al.*, 1998) and 37-90% in Sudan (El-Azazy & Schillhorn, 1983) in bovines and 2% in Gambia (Fritsche *et al.*, 1993) and 20-60% in Sudan (Majid *et al.*, 1983) in sheep, they are much lower. Probably, if we apply serological techniques in these study zones the prevalences would increase.

We found 1.68% of bovines and 1.40% of sheep positive to the two parasites and statistically significant differences on comparing the prevalences *F. hepatica* and *S. bovis* in cows but not in sheep. This situation could be attributed either to well-known crossed reactivity between the parasites (Haroun and Hillyer, 1986), or to infection by both parasites. We are inclinated to consider double infection, since cases have been reported of cattle farms infected by both parasites and the tests carried out to refine the technique with experimental infectious of *F. hepatica* and *S. bovis* and the results of Rodríguez-Osorio *et al.*, (1999) do not warn of significant cross reactivity between both infections.

Regarding age related infection rates, we could not detect differences in animals infected either

with *F. hepatica* or *S. bovis*, neither in cattle nor in sheep. These results agree with those regarding *F. hepatica* age-related infection rates found in cattle by Sánchez Andrade *et al.* (2000). Nevertheless, age-related differences could be found by other authors for *F. hepatica* infected sheep (Grock *et al*., 1998), although these differences were reported in age groups not represented in our study. Certain differences relative to sex are found, but they are not significant according to the statistical studies.

In summary, the present study gives an overall view about *F. hepatica* and *S. bovis* prevalence rates in bovine and ovine, using the ELISA technique, in a semi-arid region with low prevalences for both parasites. This technique could identify a higher number of infected animals than with coprology based techniques for *S. bovis*. Furthermore, we think that sheep cannot be excluded as suitable resevoir for *S. bovis.*

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