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RESEARCH NOTE

Immunodiagnosis of Fasciolosis in Horses and Pigs Using Western Blots

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Abstract—Gorman T., Aballay J., Fredes F., Silva M., Aguillón J. C. & Alcaíno H. A. 1997. Immunodiagnosis of fasciolosis in horses and pigs using Western blots. *International Journal for Parasitology* 27: 1429–1432. Crude and partially purified somatic (S) and excretory–secretory (ES) antigens of *Fasciola hepatica* were subjected to Western blot analysis in order to identify polypeptides that would enable specific and sensitive immunodiagnosis of horse and pig fasciolosis to be undertaken. Sera from 20 horses and 20 pigs with natural infections of *F. hepatica* and the same number of uninfected hosts of each species were tested, together with sera from 2 pigs with *Cysticercus cellulosae* infections. Using crude S antigens, sera from infected horses and pigs reacted specifically with a wide range of polypeptides of 14–19, 22–30, 35–37 and 42 kDa. Likewise, specific reactivity between polypeptides of 14–17, 22–30 and 40–42 kDa in crude ES antigens and sera from infected horses and pigs was obtained. Against the criteria of high sensitivity and specificity, the 22–30-kDa polypeptides would appear to be the most suitable candidate antigens for use in the immunodiagnosis of fasciolosis in horses and pigs. © **1997 Australian Society for Parasitology. Published by Elsevier Science Ltd.**

Key words: Fasciolosis; equine fasciolosis; swine fasciolosis; immunodiagnosis; *Fasciola hepatica*; antigens; Western blot.

Fasciolosis, caused by *Fasciola hepatica* and *F. gigantica*, has a worldwide distribution and causes significant economic losses among domesticated animals due to decreased productivity, mortality and liver condemnation. In some regions of Chile, the rate for bovine liver condemnation at slaughter houses is as high as 80% (Alcaíno *et al.*, 1992), and many human cases of fasciolosis have been reported in Latin America (Apt *et al.*, 1988; Hillyer *et al.*, 1992).

Diagnosis of infection is routinely based on finding the fluke eggs in faeces by coprological examination. However, this method is not sensitive, and infections where the parasite burden is low or when the host is harboring immature flukes in the liver parenchyma or the bile ducts during the prepatent phase of the infection may go undetected. Furthermore, the coprological method sometimes fails to detect all infections, and some cases are classed as false negatives (Gorman *et al.*, 1991). Moreover, in some species such as horses, an intermittent fluke egg elimination has been described (Owen, 1977). For these reasons a more effective diagnostic method is needed and immunological methods can provide a possible solution. However, it has to be noted that a possible drawback of such methods is that a positive result does not necessarily discriminate between a prior infection and a current one, and some caution needs to be exercised in interpreting the results.

Numerous immunological methods have been applied to the immunodiagnosis of fasciolosis, and enzyme-linked immunosorbent assay (ELISA), in particular, has shown adequate sensitivity and specificity for this purpose (Zimmerman *et al.*, 1982; Itagaki *et al.*, 1989). However, the complex nature of antigenic preparations used in such methods can lead to problems of cross reactivity, indicating the need for more purified antigen fractions. Furthermore, there is lack of information regarding fasciolosis in some animal

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species such as horses and pigs, which in some countries may be important reservoir hosts of infection but are usually disregarded when treatment strategies are implemented.

As there is scant information regarding the immune response of horses and pigs to antigenic components of *F. hepatica*, this study was carried out in order to evaluate the pattern of recognition of crude somatic (S) and excretory–secretory (ES) antigens, as well as their respective semipurified chromatographic fractions, by sera from naturally infected horses and pigs from endemic areas. The principal objective was to evaluate these antigens in terms of their application for the immunodiagnosis of animal fasciolosis, emphasising its sensitivity and specificity.

Live adult flukes were collected from the livers of infected animals at a local slaughter house, and washed by rinsing 8–10 times in physiological saline (0.9% NaCl); the first saline rinse contained 0.01% of merthiolate. In the case of S antigens, flukes were homogenised (1 fluke ml^{-1} physiological saline) in a tissue homogeniser on an ice bath. The homogenate was delipidised by incubation overnight at 36°C in a 20% v/v ether solution. The resulting aqueous phase was reconstituted to its initial volume with physiological saline and then centrifuged at 7602 *g* for 20 min at 4°C. The supernatant fluid was dialysed using distilled water for 24 h at 4°C.

Crude ES antigens were prepared by incubating previously washed flukes in Hedon–Fleig solution (1 fluke/0.5 ml solution) at 37°C for 15–18 h (Morilla & Bautista, 1986). The supernatant fluid obtained following centrifugation of the incubation fluid at 4°C for 20 min at 7602 *g* was used. The protein content of S and ES antigens was determined (Bradford, 1976) to be 2.35 and 3.4 $\mu g\,\mu l^{-1}$, respectively. The antigens were aliquoted and kept at −70°C until required.

The crude preparations described above were filtered through nitrocellulose membranes (0.20-μm pore size) and 10-ml samples were loaded on to molecular sieving chromatographic columns (2.5 cm diameter × 90 cm) packed with Sephacryl S 300 (Sigma, MO, USA). The optical densities of the eluates were monitored at 280 nm by a continuous flow rate detector (Pharmacia, Uppsala, Sweden). Phosphate buffered saline (PBS; 0.01 M), pH 7.2 containing 0.03% w/v azide, was used as eluant. Fraction volumes were collected automatically and those corresponding to the fourth protein peak, which contained components with the best diagnostic potential (Gorman *et al.*, 1996), were pooled and used in the present study. They are referred to as partially purified antigens.

Serum samples were obtained from 20 pigs and 20 horses with liver-fluke infection examined at the slaughter houses. Additional serum samples from 20 pigs and 20 horses from a fasciolosis-free area (XII Region, Chile) were also collected as negative controls. Two serum samples from pigs with *Cysticercus cellulosae* were also included in the study to test for cross reactivity with *F. hepatica* antigens. All serum samples were analysed individually by Western blot.

The *F. hepatica* S and ES crude antigens and their respective partially purified fractions were processed by polyacrylamide gel electrophoresis (12%) under reducing conditions using minigels (BioRad Laboratories, CA, USA). Guided by preliminary titrations, 180 μg protein of crude S and ES antigens or 150 μg of partially purified antigens were loaded on to gels. Western blots were performed as described by Tsang *et al.* (1983) and carried out for 20 min at 25 V (Semidry Transblot, BioRad Laboratories). The strips were incubated for 1 h with a horseradish peroxidase-conjugated IgG fraction of an affinity purified antiserum raised in goats against either horse IgG or pig IgG (Sigma) as appropiate, diluted 1:5000.

Western blot procedures using crude antigenic S or ES preparations from *F. hepatica* and sera from fluke-infected horses and pigs highlighted many polypeptide bands. However, some of these had no immunodiagnostic potential due to their cross reactivity with sera from uninfected animals. It was also noted that ES antigens gave rise to less non-specific staining than S antigens. When using crude S and ES antigens 4 groups of specific polypeptides ranging from 14 and 42 kDa were recognised by sera from infected horses and pigs. With S antigens and sera from infected horses these corresponded to 16–19; 25–30; 35–39 kDa and a band of 42 kDa (Table 1). With minor variations, similar results were obtained using crude ES antigens (Table 1). Sera from infected pigs specifically recognised polypeptides of 14 and 42 kDa and groups of 22–30 and 35–37 kDa in crude S antigens. Polypeptides of 22–30, 33 and 40–42 kDa (Table) were recognised in crude ES antigens by the same sera. A group of bands ranging from 46 to 59 kDa cross-reacted with sera from pigs infected with *C. cellulosae* (results not shown). The most frequently recognised polypeptides belonged to the 22–30-kDa group; they were recognised by sera from all infected horses and pigs (Table 1). Groups of polypeptides in the 35–37-kDa range (S antigens) or 35–39-kDa range (ES antigens) were also recognised by sera from more than half the infected horses and pigs (Table 1).

The partially purified chromatographic fractions obtained from S and ES antigens used in the present study were originally tested against sera from sheep infected with *F. hepatica*. Those studies indicated that the antigens contained polypeptides of 14 kDa and 22–30 kDa that reacted specifically with infected sheep

Table 1—Immunoreactive bands detected by Western blot in *Fasciola hepatica*-infected horses and pigs, using crude somatic (S) and excretory–secretory (ES) antigens

Antigens				ES Antigens			
Horses		Pigs		Horses		Pigs	
kDa	Freq.[a]	kDa	Freq.[a]	kDa	Freq.[a]	kDa	Freq.[a]
42	6	42	13	40–42	2	40–42	11
35–39	11	35–37	15	35–39	14	33	6
25–30	20	22–30	20	25–30	20	22–30	20
16–19	6	14	12	14–17	9	—	

[a] Frequency of reactive antigenic groups detected by 20 test sera.

sera (Gorman *et al.*, 1996). The results obtained with these fractions using horse and pig sera indicate that the polypeptides ranging between 22 and 30 kDa were also detected by all infected animals, as shown in Table 1 and Figs 1 and 2 but not by sera from uninfected animals. Therefore, the 22–30-kDa antigen group contains polypeptides which warrant further purification for use in immunodiagnostic assays. There are other promising polypeptides as well, such as the band of 14 kDa which was detected by all

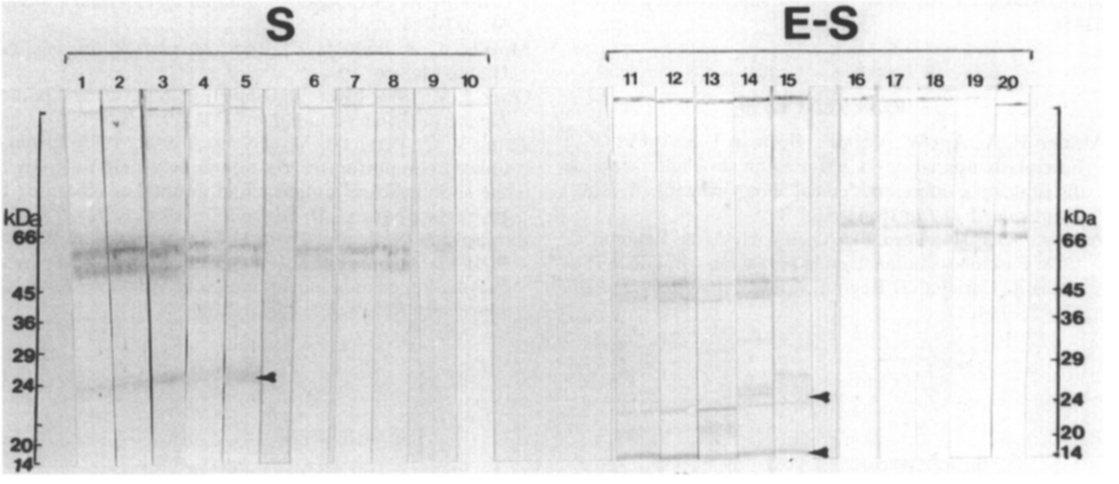

Fig. 1. *Fasciola hepatica* Western blots with somatic (S, left) and excretory–secretory (ES, right) chromatographic fractions, using sera from infected horses and from non-infected horses. S fraction: lanes 1–5, *F. hepatica*-infected horses; lanes 6–10, non-infected horses. ES fraction: lanes 11–15, *F. hepatica*-infected horses; lanes 16–20, non-infected horses.

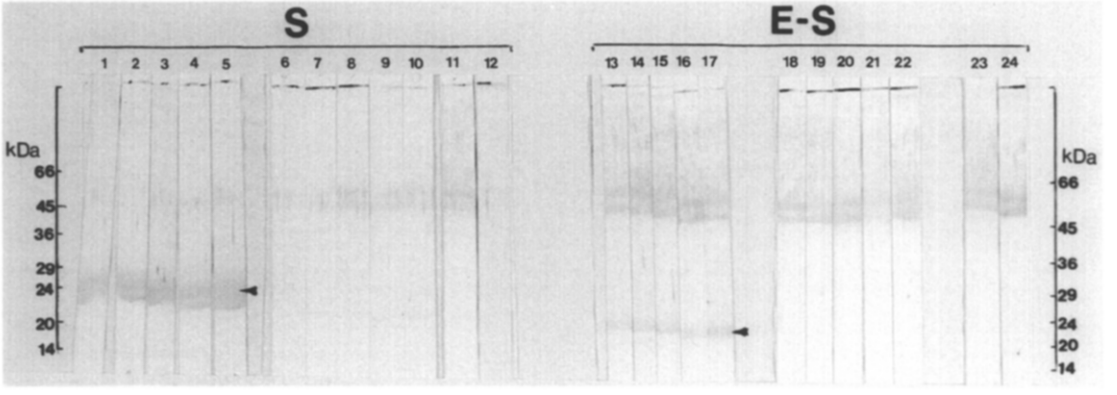

Fig. 2. *Fasciola hepatica* Western blots with somatic (S, left) and excretory–secretory (ES, right) chromatographic fractions, using sera from infected pigs, from non-infected pigs and from non-infected pigs with *Cysticercus cellulosae*. S fraction: lanes 1–5, *F. hepatica*-infected pigs; lanes 6–10, non-infected pigs; lanes 11–12, infected with *C. cellulosae*. ES fraction: lanes 13–17, *F. hepatica*-infected pigs; lanes 18–22, non-infected pigs; lanes 23–24, infected with *C. cellulosae*.

infected sera but not by pig sera when using the ES partially purified antigen fraction (Table 1, Figs 1 and 2).

In summary, this study has demonstrated that there are a number of antigenic components of *F. hepatica* that could be selected for further purification and application for the diagnosis of fasciolosis in horses and pigs. Among these the 22–30-kDa antigenic group is particularly promising, as it is recognised specifically by sera from all infected animals of both species.

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REFERENCES

Alcaíno H. A., Apt W., Vega F., Gorman T. & Apt P. 1992. Fasciolasis animal en la VII Región de Chile: areas de distribución e infecciones en caballos y conejos silvestres. *Parasitología al Día* **16:** 11–16.

Apt W., Klein P., Vega F., Alcaíno H. A. & Retamal C. 1988. Fasciolasis humana en la población rural de la Provincia de Curicó (VII Región), Chile. *Parasitología al Día* **12:** 155–164.

Bradford M. 1976. A rapid and sensitive method for microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry* **72:** 248–254.

Gorman T., Jaque S., Fredes F., Silva M. & Aguillón J. C. 1996. Relevant immunodiagnostic *Fasciola hepatica* antigenic fractions separated by molecular sieving chromatography, analyzed by immunoelectrotransfer blot under native and reduced conditions. *Parasitología al Día* **20:** 38–44.

Gorman T., Moreno P., Lorca M., Ibarra L. & Alcaíno H. A. 1991. Inmunodiagnóstico de las fasciolasis animal mediante una prueba inmunodiagnóstica (ELISA). *Parasitología al Día* **15:** 87–93.

Hillyer G. V., Soler de Galanes M., Rodriguez-Pérez J., Jorlan J., Silva de la Grava M., Ramírez Guzmán S. & Bryan R. T. 1992. Use of the Falcon assay screening test, enzyme-linked immunosorbent assay (FAST-ELISA) and the enzyme-linked immunotransfer blot (EITB) to determine the prevalence of human fascioliasis in the Bolivian Altiplano. *American Journal of Tropical Medicine Hygiene* **46:** 603–609.

Itagaki T., Ohta Y., Iso H., Konishi M., Chinone S. & Itagaki H. 1989. Diagnosis of *Fasciola* sp. infections in cattle by ELISA. *Japanese Journal of Veterinary Science* **51:** 757–764.

Morilla A. & Bautista C. 1986. *Manual de Inmunología.* Diana, México.

Owen J. M. 1977. Liver fluke infection in horses and ponies. *Equine Veterinary Journal* **9:** 29–31.

Tsang V. C., Peralta J. M. & Simons A. R. 1983. Enzyme-linked immunotransfer blot technique (EITB) for studying the specificities of antigens and antibodies separated by gel electrophoresis. *Methods in Enzymology* **92:** 377–391.

Zimmerman G., Jen L., Cerro J., Farnsworth K. & Wescott R. 1982. Diagnosis of *F. hepatica* infections in sheep by an enzyme-linked immunosorbent assay. *American Journal of Veterinary Research* **3:** 2.097–2.100.