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Determination of exposure to *Fasciola hepatica* in horses from Uruguay using a recombinant-based ELISA

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ABSTRACT: The risk of exposure to *Fasciola hepatica* in horses from Uruguay was evaluated using ELISA and a recombinant surface protein (FhrAPS). Blood samples were collected from 368 horses from different districts. Detection of antibodies and the seasonal IgG pattern against the trematode was also established. The overall seroprevalence was 54% (ranging 23% November to 93% December). Significantly higher values were observed in the Anglo-Arab horses (86%) and in those older than xix year (63%). No sex- or origin-differences were observed. Two primary risk periods for infection in Uruguay have been identified, the end of spring and autumn. Exposure to the fluke should be taken into account among those horses feeding on pastures grazed by ruminants. Using FhrAPS-ELISA, helpful information concerning the risk of horses to the infection by trematode can be obtained. This test allows the opportunity to compare the results achieved in different laboratories by minimising specific regional effects.

Keywords: *Fasciola hepatica*; equine; Uruguay; sensitisation; FhrAPS

Fasciolosis (*Fasciola hepatica*) is a widespread trematodosis among grazing animals, which become infected by consuming forage or drinking water contaminated by metacercariae, the infective stages that are released by amphibious snails (*Lymnaea* spp.) acting as their intermediate hosts (Marcos et al. 2008; Sanchis et al. 2011). The effective control of infection in ruminants is based on accurate diagnosis and successful deworming. Currently, the detection of *Fasciola*-infected animals is based on clinical observations, copromicroscopical tests and/or immunoenzymatic probes (Arias et al. 2012b; Rojo-Vazquez et al. 2012). The majority of studies performed on horses experimentally infected with *F. hepatica* metacercariae showed that patency (adult flukes) is only reached by a small proportion of the infective stages (Grelck et al. 1977), whereas faecal egg-output has been observed after a period longer than 14–15 weeks post-infection (Soule et al. 1989). This limits the usefulness of faecal examination in the diagnosis of equine fasciolosis, which is therefore habitually discarded.

Infection by *Fasciola* compromises liver function, but clinical signs, such as low-level jaundice, anaemia, and intermittent diarrhoea are not often observed and well-interpreted in equine disease; therefore, liver fluke infection remain undiagnosed

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unless serological methods of detection are used (Haridy et al. 2007; Awad et al. 2009). Because horses fail to develop experimental infections, they are frequently considered resistant to infection with *Fasciola* (Nansen et al. 1975; Alves et al. 1988; Soykan and Oge 2012). The identification of horses exposed to *F. hepatica* using immunoenzymatic probes has previously been proposed (Acici et al. 2013). In a recent study, an ELISA with the FhrAPS recombinant surface antigen was used to demonstrate the high seroprevalence of fasciolosis among horses feeding on grasslands formerly pastured by ruminants (Arias et al. 2012a). Because horses and cattle regularly share the same pastures in Uruguay, exposure to the liver trematode in equines from this country has been evaluated in this study.

MATERIAL AND METHODS

Area of study. This study was performed from March 2011 to March 2012 in Uruguay (30°05'00'' to 34°58'00'' S, 53°12'00'' to 58°43'40'' W), where cattle breeding is the most important agrarian economic activity. This country has a moderate climate, characterised by significant rainfall in all seasons and small annual variations in temperature, with more than four months averaging above 10 °C (Figure 1). Under these conditions, forage growth occurs throughout the year, and ruminants, including cattle and sheep, are extensively reared (Sanchis et al. 2014). Because of the absence of geographical barriers, no differences in the climate conditions are expected throughout the country (Eleftheratos et al. 2011).

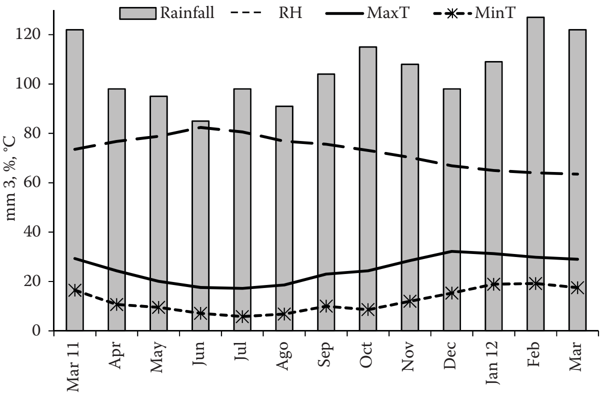

Figure 1. Monthly changes in climate parameters in Uruguay. MaxT = maximal temperature (°C); MinT = minimal temperature (°C); RH = relative humidity (%)

Analysis of the seroprevalence of horse fasciolosis. A total of 368 horses representing the Anglo-Arab Breed, Uruguay Creole, Arabian Pure Breed and English Pure Breed from 10 out of 19 districts were bled by veterinary clinicians during equestrian festivals (Table 1). Several horses were bled prior to purchase. The horses were grouped based on their age as follows: G1 (six year) (Table 2). According to their housing, two categories were established: indoors, which included the Anglo-Arabs and English Pure Breed, and outdoors, which included the Uruguay Creoles and Arabians.

FhrAPS-ELISA. The presence of IgG antibodies against the liver trematode *F. hepatica* was evaluated using an ELISA with the FhrAPS recombinant surface protein. The ELISA was 83% sensitive and 86% specific (Arias et al. 2012a). In brief, 1 μg/ml FhrAPS was added to the wells of microtitre plates containing sera (tested in duplicate) diluted 1/100 in 10% PTL (PBS, 0.3% Tween 20 and 10% skimmed milk) and horseradish peroxidase conjugated to rabbit anti-horse IgG (Nordic Immunology Laboratories, The Netherlands) at a 1/1000 dilution. Substrates consisting of 10 mg of ortho-phenylenediamine in 12 ml of citrate buffer and 10 μl of 30% H_2O_2 were then added to each well. The plates were incubated in the dark for 10 min at room temperature. Finally, the absorbances at 492 nm were measured using a spectrophotometer (Titertek Multiskan). Positive and negative controls were added to each ELISA plate. Serum samples from horses passing *F. hepatica* by faeces were utilised as positive controls, while the samples collected from three-month-old foals (n = 44) without access to grass or fresh forage served as negative controls. The cut-off value was taken as the mean of the absorbances belonging to the negative controls plus three standard deviations, which was calculated to be 0.347.

Statistical analysis. All tests were performed using SPSS for Windows (20.0, SPSS Inc., Chicago, IL, USA). The true prevalence was calculated by taking the sensitivity, specificity and apparent prevalence values into account. The percentages of the true seroprevalence were expressed as the value and the 95% confidence interval and were analysed using the χ^2 test. Differences were considered significant at $P < 0.05$. Correlations among the different parameters were assessed using Pearson's correlation test. The possible relationship between breed, age, and seroprevalence was estimated by calculating the odds ratio (OR) values (Thrusfield 2005).

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Table 1. Seroprevalence of fasciolosis in horses from the different departments of Uruguay using the FhrAPS-ELISA. The results are expressed as the prevalence (P) and the 95% confidence interval

Department	Localisation	*n*	Positive to the FhrAPS-ELISA	
			P (%)	95% CI
Rocha	Southeast	25	72	54–90
Colonia	Southwest	7	71	38–100
Lavalleja	Southeast	27	63	45–81
Tacuarembó	North	28	61	43–79
Paysandú	Northwest	80	61	51–72
Artigas	Northwest	59	51	38–64
Cerro Largo	Northeast	56	46	33–59
Montevideo	South	53	45	32–59
Rivera	North	11	45	16–75
Canelones	South	22	41	20–61
Total		368	54	49–59

RESULTS

Seasonal dynamics of IgG antibodies against *F. hepatica*

After slight variations, the IgG values increased significantly from June and peaked in August (Figure 2). Antibodies increased again from October to December (end of spring-beginning of summer), when the highest counts were recorded, and then decreased in March.

Seroprevalence

The percentage of horses positive by FhrAPS-ELISA was 54% (95% CI 49–59), with the lowest values in the northern districts and the highest values in the southern districts (Table 1); however, these differences were not significant (χ^2 = 12.178, P = 0.203). The percentage of seropositivity varied from 47% in the foals to 63% in horses older than six year ($P < 0.05$) (Table 2). The oldest equines reached an OR value of 1.7. Based on the Pearson's test, the seroprevalence and age were significantly correlated (r = 0.243, P = 0.007). For breed, the Anglo-Arabs reached significantly higher seroprevalence values (Table 2), whereas the lowest seropositivity percentage values were recorded for the Creoles ($P < 0.05$). The OR values were 5.4 for the Anglo-Arabs and 1.4 for the Arabians. Although the mares showed higher percentages of seroprevalence than the stallions, significant differences were not observed ($P > 0.05$). No significant differences based on housing were observed ($P > 0.05$). The seroprevalence rapidly increased between November and December, when the highest values were achieved. A slow increase during winter (June-August) was observed, and the percentages of seropositivity peaked again. These monthly variations were significant (χ^2 = 51.033, P = 0.001).

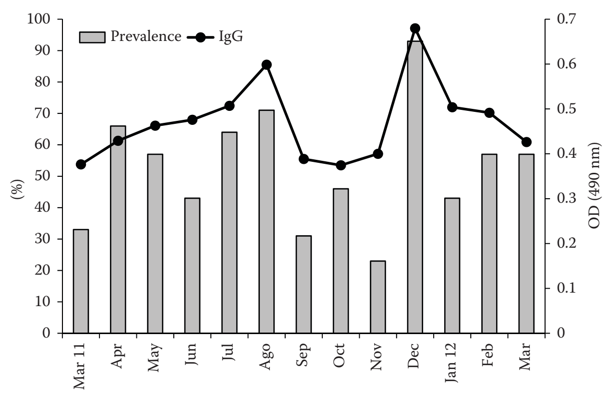

Figure 2. Monthly variations in the seroprevalence of fasciolosis and IgG antibodies against the FhrAPS recombinant surface protein in horses from Uruguay (n= 368).

DISCUSSION

A serological survey in horses from 10 out of the 19 departments of Uruguay was here performed in order to gain information regarding equine fasciolosis in this country. By analyzing the presence of serum IgG antibodies against the FhrAPS re-

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Table 2. Seroprevalence of fasciolosis in horses from the different departments of Uruguay using the FhrAPS-ELISA according to intrinsic factors. The results are expressed as the prevalence (P), 95% confidence interval (CI), and odds ratio (OR)

		n	Seroprevalence		OR	
			P (%)	95% CI	value	95% CI
Age (year)	< 3	88	47	42–52	0.7	0.4–1.1
	3– 6	163	52	47–57	0.9	0.6–1.3
	> 6	117	63	58–68	1.7	1.1–2.7
			Statistics		$\chi^2 = 6.188$, $P = 0.045$	
Breed	Anglo-Arab	21	86	82–89	5.4	1.6–18.8
	Arabian Pure Breed	45	62	57–67	1.4	0.8–2.7
	Uruguay Creole	111	50	45–55	0.8	0.5–1.2
	English Pure Breed	191	52	47–57	0.8	0.5–1.2
			Statistics		$\chi^2 = 10.969$, $P = 0.012$	
Sex	mares	196	55	50–60		
	stallions	172	53	48–58		
			Statistics		$\chi^2 = 0.096$, $P = 0.757$	

combinant surface protein, exposure to *F. hepatica* was demonstrated in more than half of the sampled horses. Although the seroprevalence of fasciolosis increased from north to south, significant differences were not observed, which may partially be due to the absence of geographical barriers that allow for the development of the liver trematode (Nari et al. 1986).

The presence of antibodies against *F. hepatica* was age-related, with the lowest values in the youngest horses and the highest values in the oldest animals. These data are consistent with previous results suggesting a cumulative effect of infection throughout the life of the horse (Arias et al. 2012a; Getachew et al. 2010). Previous investigations in Uruguay indicate that calves exhibit the highest seroprevalence against *F. hepatica* crude antigens (Sanchis et al. 2011).

One unexpected result was the elevated percentages of positivity in the FhrAPS-ELISA among the Anglo-Arabs, because the owners stated that these horses remained stabled throughout the majority of the day and therefore have no or limited access to grass. Likewise, the highest seroprevalences were obtained among the English Pure Breed, Arab and Anglo-Arab equines from Spain (85%, 75% and 60%, respectively). However, significant differences were not observed in the seropositivity regarding the equine breed (Arias et al. 2012a). It is possible that the horses are allowed access to paddocks for exercise, which may increase the risk of exposure to the metacercariae. By contrast, the lowest seropositivity values were found in the Uruguay Creoles, which are horses that receive less care and attention, in theory at least. In a recent survey, the lowest seroprevalence in Crossbreds from Uruguay has been associated with cattle that pasture on nutrient-poor grasslands, which become dry during summer, thereby reducing the risk of infection (Sanchis et al. 2014).

Because it is difficult for *Fasciola* to reach the adult stage in horses, the excretion of *F. hepatica* eggs in the faeces occurs only occasionally; therefore, the role of horses as potential trematode reservoirs has been underestimated (Arias et al. 2012a). However, the delay in mature trematode development allows the immune system to stay in contact with the trematodes and antigens, thereby eliciting a strong humoral immune response (Phiri et al. 2006). Accordingly, the analysis of horse serum samples provides useful information of the risk of *F. hepatica* infection.

The analysis of the seasonal pattern of IgG antibodies against the FhrAPS recombinant surface protein showed increased incrementally in the autumn and then significantly in the winter. Exposure to *F. hepatica* requires the ingestion of forage and/or water containing metacercariae, and the presence of these infective stages depends on their release by the snails acting as intermediate hosts, as well as on their capability to survive under adverse climate conditions. Because of the decrease

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in temperature during the autumn (March-June), development of the *Fasciola* stages inside the snails surviving the summer is delayed; therefore, the cercariae are slowly released by the intermediate hosts, which may take up to four months (Nari et al. 1986; Acosta 1993).

Temperature values of less than 10 °C together with a reduction of rainfall and relative humidity decrease the activity of the snails; therefore, the lowest IgG counts were observed in September. The temperature rises in the spring (September-December), which increases the activity of *Lymnaea* intermediate hosts (Castro et al. 2001), and thus the risk to horses that ingest the metacercariae with herbage. This may explain why the highest IgG values were observed in the horses at the end of the spring (December).

Fasciolosis is endemic in cattle from Uruguay, as shown by 46% (26–67%) liver seizure for *Fasciola* in 2007 (INIA-INAC 2009). An overall seroprevalence of 67% has been reported (Sanchis et al. 2014), whereas the percentage of cattle passing eggs in the faeces reached 15% in some areas. In this study, the presence of antibodies against *F. hepatica* in 54% of horses from Uruguay was detected. These results are consistent with those obtained in NW Spain, an endemic area where the seroprevalence of fasciolosis is 71% in cattle, 68% in sheep and 60% in horses, as demonstrated by FhrAPS-ELISA (Arias et al. 2007; Paz-Silva et al. 2007; Arias et al. 2012a).

In conclusion, analysis of horse sera for the presence of antibodies against *F. hepatica* is useful for determining the distribution of infection. Two primary risk periods for *F. hepatica* liver fluke infection in Uruguay were identified. The first period is the end of the spring, and the second period is in the autumn. Exposure to the liver fluke should be taken into account among those horses feeding on pastures also grazed by ruminants. The FhrAPS-ELISA gives useful information about the risk of liver fluke infection for horses. This assay minimises the impact of specific regional effects, and in this way, the results obtained in different laboratories can be compared.

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