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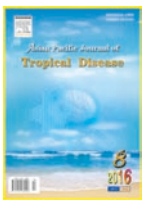

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Development and validation of a meat juice ELISA for the diagnosis of *Fasciola hepatica* in cattle in Cuba

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ABSTRACT

Objective: To establish and validate a home-made ELISA for determination of antibodies against excretory-secretory proteins of *Fasciola hepatica* in bovine meat juice samples.
Methods: The validity criteria of the assay were defined based on standards of International Organization for Standardization. The following parameters were evaluated: excretion/secretion antigen concentrations for coating, anti-bovine immunoglobulin G dilution, linearity, accuracy and precision.
Results: The assay was validated on 126 meat juice samples with known infection status. Using the receiver operating characteristic (n = 126) the optimal cut-off for the ELISA assay was 0.78, above this value the probability for an animal to have fasciolosis was 11 times. And the specificity and sensitivity were 100% and 90.91% respectively. The repeatability of the intra- and inter-assay tests had coefficients of variation lower than 10% and 20% respectively.
Conclusions: The ELISA is a suitable test for further use in studies towards the epidemiology of *Fasciola hepatica* in Cuba.

1. Introduction

Fasciolosis is a parasitic disease of great economic impact that affects mostly domestic ruminants and is caused by two species: *Fasciola hepatica* (*F. hepatica*) and *Fasciola gigantica* (class Trematoda). The disease is responsible for considerable economic losses in the cattle industry, mainly through mortality, reduced production of meat, milk and wool, and expenditure for the anthelmintic treatment. Global losses in animal productivity due to fasciolosis are estimated at US $3 billion per year with a total of 600 million animals infested in agricultural rural communities and on commercial farms reviewed by Dar *et al*.[1].

In animals, the post mortem diagnosis of fasciolosis is based on the presence of immature flukes in the liver parenchyma and adult flukes in the bile ducts. Routine diagnosis *in vivo* is done by microscopical examination of faecal samples for the presence of parasite eggs. These methods have the advantage that they have a high specificity and can be performed in laboratories with limited equipment and resources. However, their low sensitivity is an issue for epidemiological studies[2]. These tests can yield false negative results when the disease is in the acute phase, where the parasite migrates to the liver parenchyma without reaching sexual maturity[1].

Different immunodiagnostic tests for the presence of antibodies against *F. hepatica* in serum, milk and meat juice have been marketed[3]. Availability and simplicity of these tests allow extensive epidemiological studies. However, their cost and the import procedure constrain their implementation in Cuba. Hence the need to develop in-house diagnostics can be performed with the available resources.

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Extensive epidemiological studies are currently lacking in Cuba. The only available data come from pathological routine inspections of slaughterhouses in the western and central provinces[4-6]. These studies indicated prevalence between 20%–50%. It is necessary to extend the studies to other regions with important ruminant production in Cuba. Camagüey Province in the east of the center region of Cuba is the main dairy region of the country and handles 25% of the national meat and milk production.

The use of meat juices as sample matrix has several advantages compared to the use of sera. Because it is not necessary to make visits to farms and there is no need for specialized materials for the extraction and processing of blood[7]. Previously, good correlations had been described between antibodies to *F. hepatica* in meat juice and serum samples[8]. The aim of the present study was to establish and validate an ELISA for the determination of bovine fasciolosis in Camagüey Province, Cuba. Firstly, it was necessary to collect excretion/secretion (E/S) antigens from adult flukes and to develop an anti-bovine immunoglobulin G (IgG), in sheep. The ELISA was established evaluating the E/S antigens, anti-bovine IgG, anti-sheep IgG horseradish peroxidase and meat juice dilutions. It was validated considering the standards of International Organization for Standardization[9]. The optimal cut-off for the ELISA was determined using the receiver operating characteristic (ROC) curves non-parametric test.

2. Materials and methods

2.1. E/S of F. hepatica antigens

Bovine livers infected with *F. hepatica* were obtained from the slaughterhouse in Cesar Escalante (21°24'1.1" N, 77°57'48.1" W) in Camagüey Province. In the laboratory, liver cross sections were performed and the bile canaliculi were slightly pressed to extract trematodes. The adult parasites were carried to a flask containing 300 mL of phosphate buffered saline (PBS) 1× (KH_2PO_4 0.17 mmol/L; Na_2HPO_4 8.45 mmol/L; KCl 2.61 mmol/L; NaCl 137.00 mmol/L, pH 7.2) and were washed several times with this solution until they were clean and all traces of blood and bile were removed. Ten centrifuge tubes each with 45 mL of PBS were used to collect E/S antigens. In each tube 15 *F. hepatica* adults were placed and incubated at 37 ℃ for 4 h. The tubes were centrifuged at 5 000 g for 10 min at 4 ℃ and the supernatants with the E/S antigens were pooled to achieve homogeneity.

2.2. Protein quantification

Protein concentration was determined by the method of Bradford[10]. Briefly, 250 µL of Bradford reagent (1.17 mol/L Coomassie Blue G-250 (AppliChem, Darmstadt, Germany), 1.47 mol/L phosphoric acid and ethanol 5% v/v) was added to the sample of IgG or E/S antigens. It was incubated for 10 min at 25 ℃ in the dark and then the absorbance was measured at 595 nm using a Multiskan MCC/340 microplate reader (Fisher Scientific, Pittsburg, USA). Concentrations were estimated by a standard curve made with bovine serum albumin.

2.3. Obtaining anti-bovine IgG

To detect antibodies from cattle that recognize *F. hepatica*, it was necessary to develop a polyclonal anti-bovine IgG in sheep. Briefly, 100 µL of sepharose CL 4B (GE Healthcare, Invitrogen, Carlsbad, USA) coupled with protein G were incubated with 10 mL of bovine serum in immunoprecipitation buffer (Nonidet P40 1% v/v, 150 mmol/L NaCl, 50 mmol/L NaH_2PO_4-Na_2HPO_4, pH 7.4) for 2 h at 20 ℃. The sepharose CL 4B was washed three times with 1 mL of immunoprecipitation buffer. Total bovine IgG was eluted with 100 µL of glycine 50 mmol/L, pH 3 and neutralized with 100 µL of Tris-HCl, pH 8.0.

Then, a sheep of 30 kg live weight was injected three times intramuscularly with 50 µg of bovine IgG each, using adjuvant Montanide ISA 25 VG (SEPPIC, Paris, France) according to the manufacturer's recommendations. Injections were performed once a week during three consecutive weeks. Seven days after the last immunization, 15 mL blood collected via the jugular vein and serum was obtained by centrifugation at 3 000 g for 15 min.

2.4. Meat juice samples and worm counts

Meat juice samples were obtained from the diaphragm of 126 cattle from 15 herds of Vertientes and Jimaguayú Municipalities. Worms were recovered by liver necropsy. Briefly, the gall bladder was removed, the major bile duct was opened with blunt scissors and any visible flukes were removed with blunt forceps. Finally, the liver was cut into 1–2 cm slices and reviewed carefully for any stages of the *F. hepatica*. The liver was classified as positive or negative (according to their infection or not with *F. hepatica*). About 50 g of the diaphragm of each animal was frozen overnight. One milliliter of the transudate was centrifuged for 5 min at 14 000 g for removal of particulate matter and the supernatant was collected and stored at –20 ℃ until used.

2.5. Establishment of immunosorbent assay (ELISA)

The 96-well microtiter plates (PolySorp, Nunc, Roskilde, Denmark) were coated with 5 µg/mL (100 µL/well) E/S antigens diluted in coating buffer (carbonate-bicarbonate sodium, 0.1 mmol/L pH 9.6) and incubated at 4 ℃ overnight. The wells were washed four times with Tween-20 solution (Merck, Schuchardt, Munich, Germany) 0.05% v/v. Blocking was performed with skim milk 5% w/v for 1 h at 20 ℃. The wells were washed four times with Tween-20 solution

0.05% v/v. Meat juice samples (with dilution according to the assay) and controls were applied (100 µL/well) diluted in PBS 1× pH 7.2, always in duplicate, excepted in the precision assay. After incubating plates for 1 h at 20 ℃, wells were washed four times with Tween-20 solution 0.05% v/v and the sheep anti-bovine IgG 1:2 000 in PBS 1× was added. The wells were washed four times with Tween-20 solution 0.05% v/v and anti-sheep IgG horseradish peroxidase 1:10 000 (Sigma A3415) was added. The wells were washed four times with Tween-20 solution 0.05% v/v and 2,2′-azino-di[3-ethylbenzothiazoline sulfonate buffer was added and incubated in the dark at 20 ℃ for 30 min. The optical density (OD) was determined on a microplate reader (Multiskan MCC/340) at a wavelength of 405 nm.

2.6. Validation parameters

2.6.1. Precision

Precision was estimated by the coefficient of variation using the meat juice from three animals infected with more than 10 adults of *F. hepatica* and three with no *F. hepatica* found in the liver. The intra-assay variation was obtained from 14 replicates of these three animals on the same plate. The inter-assay variation was obtained from 14 replicates of these three animals running on five different days.

2.6.2. Recovery

Three samples of meat juice from animals infected with *F. hepatica* were diluted 1:2 with meat juice from an uninfected animal. Eight repetitions per sample were performed. The expected value was estimated by linear regression. Recovery was expressed as the percentage of the value and obtained to the expected value [(value obtained/expected value) × 100%].

2.6.3. Linearity and parallelism

Three samples of meat juice from animals with a high level of infection with *F. hepatica* were diluted (1:10, 1:20, 1:40, 1:60, 1:80, 1:100) with meat juice from an uninfected animal and pipetted into wells in triplicate. Linear regression was performed for each sample.

2.6.4. Sensitivity and specificity

To determine the sensitivity and specificity, 126 samples of meat juice (42 negative, *i.e.* no flukes found in the liver and 84 with at least one fluke found) were used. The ELISA was performed and the values obtained from both groups were used in the non-parametric ROC analysis (Graphpad Prism 5.0). The cut-off was obtained based on the value giving the highest sum of sensitivity and specificity and the highest likelihood ratio for a positive test result.

3. Results

The concentration of lots of E/S antigens was 264 µg/mL and 5 µg/mL was selected as the coating E/S concentration (Figure 1). The optimal sheep anti-bovine IgG dilution was 1:2 000 (Figure 2).

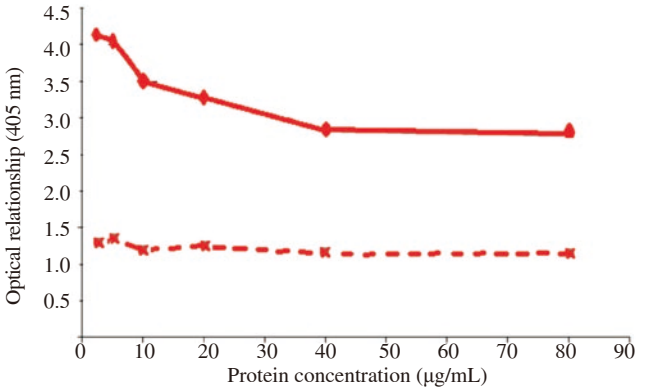

Figure 1. Effect of coating the ELISA plate with different concentrations of E/S protein of *F. hepatica* in the optical relationship (OD of the sample/OD of the PBS, at 405 nm).
---x---: Non-infected animal meat juice; ---•---: Infected animal meat juice).

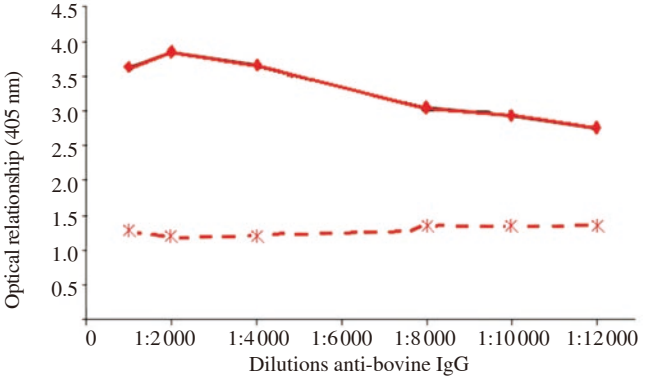

Figure 2. Effect of dilution of anti-bovine IgG in optical relationship (OD of the sample/OD of the PBS, at 405 nm).
----*---: Animal meat juice without infection of *F. hepatica*; ---•---: Animal meat juice with infection of *F. hepatica*).

The parallelism test between the lines was generated by plotting OD and the meat juice dilutions indicated a high accuracy of our assay. A meat juice dilution of 1:20 was selected to ensure the lineal rank. No difference between the slopes of the different curves (P = 0.77) was found (Figure 3) and the range of the coefficient of determination (r^2) was 0.958 and 0.974.

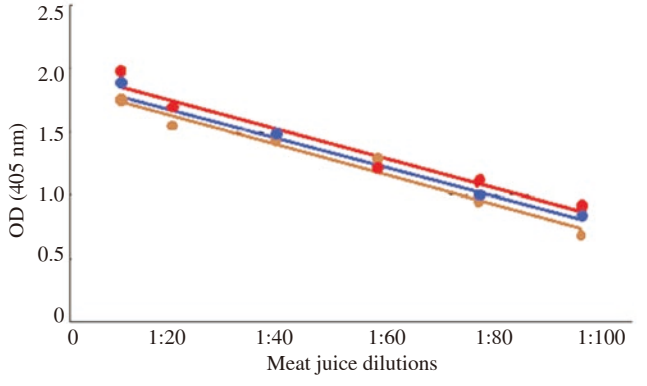

Figure 3. Parallelism test for the ELISA. OD at 405 nm of three samples of animals infected with *F. hepatica*.

Table 1 shows the repeatability of the positive and negative intra-assay and inter-assay controls. The intra-assay coefficient of variation

was less than 10% in both controls, and the inter-assay coefficient of variation was less than 15%. Table 2 shows the accuracy, where the recoveries ranged between 90% and 100%.

Table 1
Comparison of the intra- and inter-assay variations.

Sample	*F. hepatica* infection	Intra-assay (n = 14)		Inter-assay (n = 5)	
		OD 405 nm	CV (%)	OD 405 nm	CV (%)
1	Yes	1.698 ± 0.109	6.44	1.488 ± 0.152	10.23
2	Yes	1.854 ± 0.157	8.47	1.978 ± 0.254	12.86
3	Yes	1.957 ± 0.187	9.56	2.134 ± 0.252	11.79
4	No flukes	0.561 ± 0.046	4.61	0.503 ± 0.075	14.98
5	No flukes	0.457 ± 0.024	5.42	0.493 ± 0.068	13.78
6	No flukes	0.524 ± 0.015	2.86	0.554 ± 0.061	10.94
Buffer*	PBS	0.414 ± 0.023	8.05	0.386 ± 0.036	9.26

Data were expressed as mean ± SD. *: Inter-assay (n = 12); CV: Coefficient of variation.

Table 2
Recovery in the accuracy test (n = 8).

Samples	Rank	Recovery (%)
1	92.3–99.6	96.4 ± 4.2
2	90.7–94.5	92.1 ± 2.3
3	91.5–100.0	97.3 ± 8.7

Recovery data were expressed as mean ± SD.

ROC curve analysis for samples of the positive and negative animals showed that OD cut-off for the ELISA was 0.776 (Table 3) showing 100.00% specificity and 90.91% sensitivity. The likelihood ratio indicated that the absorbance values greater than the cut-off point were 11 times and more likely to have a *F. hepatica* infection. The gray area was found in the narrow range of 0.597 to 0.776.

The area under the curve (0.959) showed the ability of our ELISA to discern *F. hepatica* infected from non-infected animals.

Table 3
Analysis of ROC curves. Sensitivity, specificity and likelihood of a positive test result at different cut-offs.

Cut-off DO 405 nm	Specificity	Sensitivity	Likelihood
< 0.498	11.11	100.00	-
< 0.547	33.33	100.00	-
< 0.597	44.44	100.00	-
< 0.663	55.56	90.91	6.11
< 0.686	66.67	90.91	7.33
< 0.726	77.78	90.91	8.56
< 0.757	88.89	90.91	9.78
< 0.776	100.00	90.91	11.00
< 0.808	100.00	81.82	-
< 0.845	100.00	72.73	-
< 0.921	100.00	54.55	-
< 1.007	100.00	36.36	-

The shadowed lines represent the "gray zone". The darkness of the line represents the selected cut-off for this assay. -: Non detected.

4. Discussion

The concentration of lots of E/S antigens was higher than 7.293 μg/mL of the E/S products of *F. hepatica* obtained by Farahnak *et al.*[11] to detected superoxide dismutase activity. However, it was lower than 1 005 μg/mL of E/S obtained to establish an ELISA for the diagnosis of human fasciolosis[12] and may be due to the high density of *F. hepatica* (2 flukes/mL) used by these authors.

The effect of coating concentration of E/S antigens was evaluated using the optical relationship at 405 nm from samples and PBS. It was observed that as E/S concentration increased, the optical relationship values from infected animal decreased and mainly due to the increase of the OD background in PBS wells. The coating E/S concentration selected in the present work was similar to 1–10 μg/mL *F. hepatica* antigens that have also been used previously for coating immunoassay plates[13,14]. However, only 0.5 μg/mL was used with E/S in an epidemiological study of *F. hepatica* in dairy cattle and sheep[15,16].

It was observed that when the dilution of the sheep anti-bovine IgG increased, the difference between optical relationship at 405 nm from samples (infected and uninfected animals) and PBS decreased, mainly as a consequence of the decrease of the signal from the sample of the meat juice from the animal infected with *F. hepatica*. ELISA dilution of anti-IgG to detect bovine fasciolosis was generally between 1:1 000 and 1:10 000[13-17].

The present results were similar to the coefficient of variation of intra-assay and inter-assay for an ELISA for haptoglobin protein in pigs, which were 1.70% and 5.10% to 5.97% respectively[15]. Low coefficients of variation in intra- and inter-assay values guarantee repeatability and reproducibility of the ELISA[9].

The recoveries obtained in the present work are similar with the value recommended by International Organization for Standardization 5725[9]. A recovery between 80% and 120% is generally accepted as a good indication that the assay is suitable for use with the tested sample[16]. This also indicates that the present ELISA was robust enough to cope with meat juice. The high recovery values ensure the accuracy of the enzyme immunoassays[18-22].

Diagnostic ELISAs by E/S antigen to detect antibodies against *F. hepatica* in cattle have been reported with different sensitivity-specificity [(90% to 100%)–99.3%[12]; 98%–96%[13]; 100%–98%[23]; 100%–96%[24]]. The assay properties may vary with the population in which it is used[25], but observations in this study are in line with these previous reports. ROC analysis is more accurate to determine the cut-off point than the SD approach[26] and may have further contributed in optimizing the specificity and sensitivity of the ELISA.

The validated ELISA differentiated the immune responses developed by *F. hepatica*-infected animals from animals not affected by the parasite. The results supported the use of ELISA on bovine meat juice samples. It is concluded that the ELISA is a suitable test for further use in studies towards the epidemiology of *F. hepatica* in Cuba.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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