Evaluation of Coprological and Serological Techniques For Diagnosis of Bovine Fasciolosis

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ABSTRACT

This study was carried out to determine the prevalence bovine fasciolosis in Erzurum Province in Turkey and to evaluate sensitivity and specificity of the coproscopy by sedimentation and antibody-ELISA tests considering the copro-ELISA test as the gold standard. A total of 282 cattle (230 female and 52 male; Holstein, n=6; Simmental, n=6; Brown Swiss, n=159; and crossbreed cattle, n=111), at an average of 3.55±2.49 [mean±SD, (range 1.00-15.00)] years old, from local farms were monitored for fasciolosis between April 2011 and June 2011. Animals were grazed and watered on communal areas during days and housed in barns at nights. Blood and fecal samples were collected for coproscopy and serology for *F. hepatica*. Data were subjected to Chi-square analysis, analysis of variance, and receiver operating characteristics curve development. The prevalence rate was 35.46, 61.70, and 34.04% when assessed by the copro-ELISA, antibody-ELISA, and sedimentation tests, respectively ($P < 0.0001$). Cattle that were female, purebred, and in advanced ages (≥6 years) had greater fasciolosis prevalence than those were male, crossbred, and in younger ages (2≤ years) (40.4 *vs.* 3.6%, $P < 0.0002$ for sex; 22.8 *vs.* 55.0%, $P < 0.0001$ for breed; and 57.8 *vs.* 20.0%, $P < 0.0001$ for age) as attained by the copro-ELISA test. Sensitivity and specificity were 100.0% and 59.3% for the antibody-ELISA technique and 96.0% and 100% for the sedimentation technique, considering copro-ELISA technique gold standard. These data suggest that fasciolosis prevalence can greatly vary by the diagnostic methods and should be cautiously interpreted as they reflect disease status at different stages.

Keywords: Diagnostic method; Fasciolosis; Prevalence; Sensitivity; Specificity.

INTRODUCTION

Fasciolosis caused mainly by *Fasciola hepatica* as well as *F. gigantica* is a widespread parasitic disease of ruminants. In adult cattle, the infection usually follows a chronic course, with no obvious clinical signs. Even when asymptomatic, fasciolosis may cause economic losses in the cattle industry (1) due to compromised weight gain (2), milk yield, and fertility (3). In endemic areas grazing animals have the same susceptibility of fasciolosis as confined animals, only the risk for infection is lower (4).

The diagnosis of fasciolosis is usually based on the detection of *F. hepatica* eggs in feces or *F. hepatica* specific antibodies in serum or milk. The enzyme-linked immunosorbent assay (ELISA) often relies on excretory-secretory (ES) products from the liver fluke (3, 5). Recently, a method based on detection of a *F. hepatica* specific coproantigen has been developed and commercialized (4). The ELISA method developed for determination of *Fasciola* coproantigens in feces appears to be an alternative to coprological examination (6, 7).

Nevertheless, the diagnosis of fasciolosis is complicated due to the liver fluke's biological cycle within the definitive host. After ingestion of metacercariae, juvenile worms migrate through the intestinal wall to the peritoneal cavity, penetrate the liver parenchyma through which they migrate, and pass into the biliary tract, where they ultimately reach maturity and start oviposition. That is, eggs become present in feces several weeks after the ingestion of metacercariae (8). Moreover, ingested parasites, depending on immune status of the host, may not always maturate in the liver. In less severe infestation, eggs can be observed in only a serial fecal collection because egg counts exhibit diurnal variation. In contrast to Charlier *et al.* (9) who found that egg enumeration, depending on the method used, may be used to identify the heavily infected animals, and thus can guide treatment decisions (4, 5).

Alternative immuno-serological methods have been developed for early diagnosis of fasciolosis. The ELISA test is easy to perform for herd monitoring at early stage of fasciolosis (5, 10). Especially, the copro-ELISA test, based on determination of ES antigens in feces, has high specificity as confirmed by necropsy and high sensitivity as confirmed by lacking cross-reaction with antigens from other helminthes. It also shows positivity during the first 1-5 weeks after parasites reaching the biliary tracts (8). The objectives of this study were to determine prevalence of bovine fasciolosis, with associated risk factors in Erzurum Province in Turkey and to compare sensitivity and specificity of the coprology by sedimentation and antibody-ELISA tests considering the copro-ELISA test as the gold standard in diagnosis of fasciolosis.

MATERIALS AND METHODS

Study area and animals

The study was conducted in Erzurum (39°52' N, 41° 17' E, 1853 m above sea level) province, located in the eastern part of Turkey. The region receives 453 mm rainfall annually with a temperature ranging from -35 to 35°C. The animal production is mainly based on intensive grazing.

The requied sample size was estimated to be 323, using the following formula: n = $(Z_{1-a}^2$ x $[P$ x $(1-P) / D^2])$, where P = prevalence, Z = confidence interval at 95%, P = absolute precision, % (11). A total of 282 cattle (230 female and 52 male in terms of sex / Holstein, n=6; Simmental, n=6; Brown

Swiss, n=159; and crossbreed cattle, n=111 in terms of breed), an average of 3.55±2.49 (mean±SD, range 1.00-15.00) years old, from local farms were monitored for fasciolosis between April 2011 and June 2011. Animals were grazed and watered on communal areas during days and housed in barns at nights.

Blood and fecal samples

Ten milliliters of whole blood was drawn from the jugular vein into additive-free vacutainers. Sera were harvested following centrifugation of clotted blood at 1,500 rpm at 20°C and aliquots were stored at -20°C until ELISA analysis. Fecal samples (50-100 g) were collected per rectum using gloved fingers for coprological tests.

The study protocol was approved by the Animal Care and Use Committee at Atatürk University (20.10.2009-2009/105 decision number).

Coprological examination

Coproscopy was performed by Benedect sedimentation. Briefly, 6 g fecal samples were suspended in tap water, sieved through a grid (mesh size 250 μm) into a beaker in a volume of 250 ml. After 3 min the solution was decanted and refilled with water. This process was repeated twice. Then, the sediment was stained with 2 drops of methylene blue, decanted into a Petri dish and scanned for *Fasciola* eggs using a binocular with 100 *X* magnification (12).

Fecal samples were also subjected to determination of *F. hepatica* ES antibody using the ELISA test (BIO K 201, *Fasciola hepatica* Antigenic ELISA Kit, BIO-X Diagnostics, Jemelle, Belgium). The optical density (OD) was measured at 450 UV wavelength by a fully automatic ELISA reader (μQuant, Bio-Tek Instruments, Inc., Winooski, VT, United States) equipped with a fully automatic microplate washer (ELx50 microplate washer, Bio-Tek Instruments, Inc., Winooski, VT, United States). Blank reading was subtracted from each sample reading. As recommended by the manufacturer, the cut-off OD value was > 0.150 and the positive control OD value was > 1.533.

Serological examination

The odd colums (1,3,5,7,9,11) of microplates were coated with *Fasciola hepatica* ES antigen captured by the monoclonal antibody (BIO K 211, Bio-X Diagnostics, Jemelle, Belgium). Whereas, the even columns (2,4,6,8,10,12) contained only

the monoclonal antibody (BIO K 211, Bio-X Diagnostics, Jemelle, Belgium) serving as a negative controls to distinguish specific anti *F. hepatica* antibodies from non-specific ones.

The test blood sera were diluted 1:100 in the dilution buffer and each serum sample was applied to a coated cell and an uncoated well. After washing at the end of incubation period plates were added with the conjugate (a peroxidase-labelled anti-bovine IgG1 monoclonal antibody (Bio-X Diagnostics, Jemelle, Belgium)). The plates were incubated at room temperature and washed again. Then, the enzyme's substrate (hydrogen peroxide) and the chromogen tetra-methylbenzidine were added. The intensity of the resulting blue color was proportional to the titer of specific antibody in the sample. The OD's in the microwells were evaluated spectrophotometrically (μQuant, Bio-Tek Instruments, Inc., Winooski, VT, United States) using a 450 nm filter and the absorbance of the uncoated well was subtracted from the absorbance of the coated well. The corrected absorbance values were divided by the corresponding positive control serum OD value. The results were categorized as negative and three positive antibody levels; -, +, ++, and +++, respectively . For titer levels the results were characterized as < 15, 15-45, 45-75, and > 75, respectively.

Statistical analysis

Due to small sample size for Holsteins and Simmentals, animals were categorized as purebred and crossbred.

Animals were also categorized by age (< 2, 2-3, and > 6 years). Cross-tables were generated using the PROC FREQ procedure to determine association of animal factors (sex, age, and breed) with fasciolosis in the Chi-square analysis (29). Antibody titers were analyzed using the PROC MIXED procedure and mean differences by the degree of antibody-ELISA score were assessed using the PDIFF option.

The receiver operating characteristics (ROC) curves were developed to compare sensitivity (ability to detect fasciolosis), specificity (ability to avoid misclassifying healthy animals as animals with fasciolosis), positive likelihood ratio (low specificity or how much the odds of the disease increases when a test is positive), and negative likelihood ratio (low sensitivity or how much the odds of the disease decreases when a test is negative) for the antibody-ELISA and sedimentation methods under consideration that the copro-ELISA method is a gold standard (MedCalc version 9.6.2.0, MedCalc Software, Mariakerke, Belgium). These tests were compared based on their under areas of curves using z-test. Statistical significance was declared at $P < 0.05$.

RESULTS

Prevalence and risk factors

The overall prevalence of fasciolosis was 35.46, 61.70, and 34.04% as assessed by the copro-ELISA, antibody-ELISA, and sedimentation methods, respectively (Table 1). Females

Table 1: Risk factors for bovine fascioliasis

Risk Factor	Diagnostic Test					
	Copro-ELISA		Antibody-ELISA		Coproscopy by Sedimentation	
	- (n=182; 64.54%)	+ (n=100; 35.46%)	- (n=108; 38.30%)	+ (n=174; 61.70%)	- (n=186; 65.96%)	+ (n=96; 34.04%)
Sex						
Female (n=230; 81.56%)	137	93	73	157	140	90
Male (n=52; 18.44%)	45	7	35	17	46	6
	X^2 = 13.48; $P < 0.0002$		X^2 = 22.71; $P < 0.0001$		X^2 = 14.38; $P < 0.0001$	
Age (yr)						
2≤ (n=130; 46.10%)	104	26	72	58	107	23
3-5 (n=107; 37.94%)	59	48	25	82	60	47
≥6 (n=45; 15.96)	19	26	11	34	19	26
	X^2 = 27.50; $P < 0.0001$		X^2 = 29.81; $P < 0.0001$		X^2 = 31.42; $P < 0.0001$	
Breed						
Purebred (n=171; 60.64%)	132	39	87	84	134	37
Crossbred (n=111; 39.36%)	50	61	21	90	52	59
	X^2 = 30.40; $P <.0001$		X^2 = 29.09; $P < 0.0001$		X^2 = 29.77; $P < 0.0001$	

were affected by fasciolosis at a greater incidence than males as diagnosed by the copro-ELISA (40.4 *vs.* 3.6%; $P < 0.0002$), antibody-ELISA (68.3 *vs.* 8.8%; $P < 0.0001$), and sedimentation (39.1 *vs.* 2.6%; $P < 0.0001$) methods (Table 1).

Bovine fasciolosis was more common in advanced ages when determined by the copro-ELISA (57.8%) and sedimentation (57.8%) techniques (Table 1). Fasciolosis was the most frequent in mid-age group when the assessment was made by the antibody-ELISA method (76.6%). The frequency of affected animal younger than 2 years old was 20.0, 44.6, and 17.7% when diagnosis was made by the copro-ELISA, antibody-ELISA, and sedimentation techniques, respectively.

The frequency of crossbred animals with fasciolosis was 1.36, 1.65, and 2.46-fold greater than purebreds when diagnosis was made by the copro-ELISA, antibody-ELISA, and sedimentation methods ($P < 0.0001$ for all; Table 1).

Seropositivity assessed by the antibody-ELISA technique increased in females, advanced ages, and purebreds, whereas it decreased in males, young animals, and hybrids ($P < 0.0001$ for all; Table 2). The frequency of animals with fasciolosis detected by the copro-ELISA and sedimentation techniques increased with the degree of seropositivity ELISA score ($P < 0.0001$ for both; Table 2).

ROC curves for diagnostic methods

The antibody-ELISA method had high sensitivity (100.0%) and low specificity (59.3%), whereas the coproscopy by sedimentation method had both high sensitivity (96.0%) and specificity (100.0%) when the copro-ELISA technique was

considered gold standard (Table 3; Figure 1). The ROC curves for the antibody-ELISA and sedimentation methods were different ($P < 0.001$; Table 3; Figure 1). The results of diagnostic techniques varied by the method principles that are related to life cycle of *F. hepatica* and reflect pathobiology of fasciolosis (Figure 2).
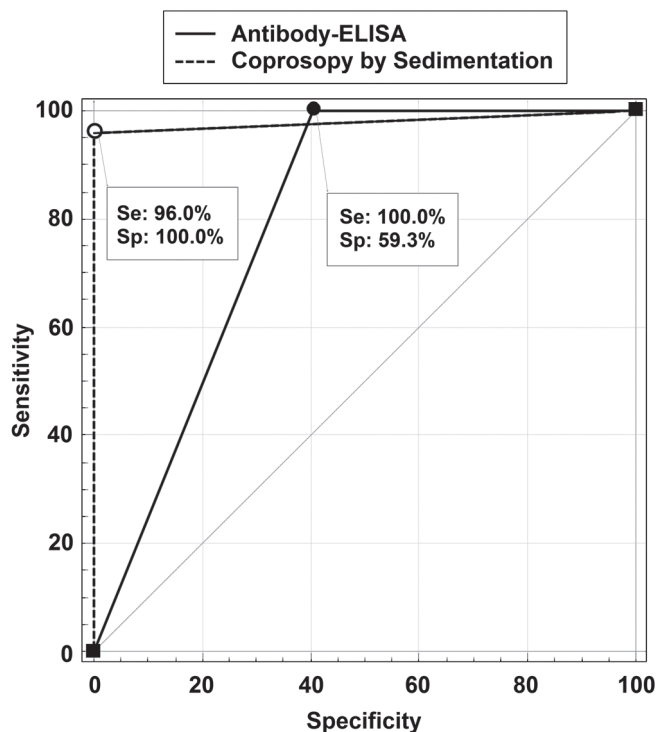

Figure 1: Comparison of specificity and sensitivity of the antibody-ELISA and sedimentation techniques when the copro-ELISA technique was considered gold standard.

Table 2: Distributions of cattle and diagnostic test results by antibody levels for *F. hepatica* infection

| | | | Degree and Titer[1] | | | | Significance |
			- (n=108; 38.30%) 2.6 ± 0.3^d	+ (n=34; 12.06%) 27.6 ± 1.4^c	++ (n=44; 15.60%) 58.4 ± 1.5^b	+++ (n=99; 34.04%) 122.1 ± 3.4^a	$P < 0.0001$
Animal Factors	Sex	Female (n=230)	73	32	38	87	$X^2 = 23.49$; $P < 0.0001$
		Male (n=52)	35	2	6	9	
	Age (yr)	2≤ (n=130)	72	15	9	34	$X^2 = 42.47$; $P < 0.0001$
		3-5 (n=107)	25	17	20	45	
		≥6 (n=45)	11	2	15	17	
	Breed	Purebred (n=171)	87	19	24	41	$X^2 = 31.89$; $P < 0.0001$
		Crossbred (n=111)	21	15	20	55	
Diagnostic Method	Copro-ELISA	- (n=182)	108	29	19	26	$X^2 = 133.36$; $P < 0.0001$
		+ (n=100)	0	5	25	70	
	Sedimentation	- (n=186)	108	30	20	28	$X^2 = 129.37$; $P < 0.0001$
		+ (n=96)	0	4	24	68	

[1] Data are presented as LS means ± SE. Different superscripts among the columns differ ($P < 0.05$)
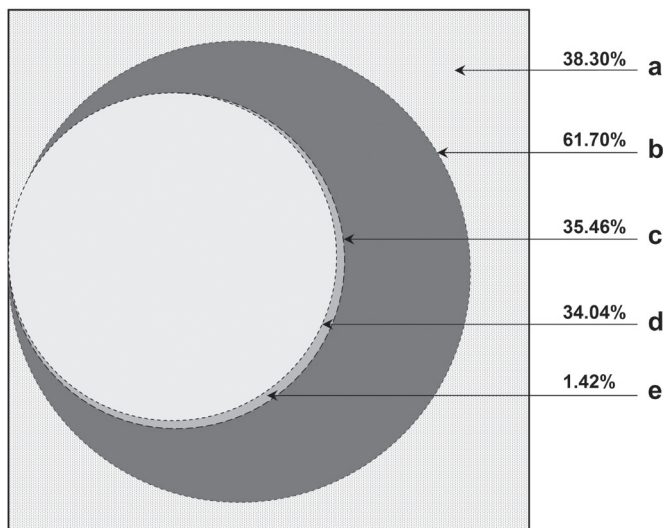
Figure 2: Interpretation of the fasciolosis detection rate by diagnostic tests. a: Surely healthy animals based on all tests; b: Animals with seropositivity based on the antibody-ELISA technique, reflecting animals with current fasciolosis, infected with other trematodes, recovered from past infection, or all; c: Animals with positivity based on the copro-ELISA technique, reflecting a patent infection. d: Animals with positivity based on the coproscopy by sedimentation, indicating a chronic infection; e: Animals with positivity based on the copro-ELISA technique and negativity based on the coproscopy by sedimentation method, indicating a prepatent infection.

DISCUSSION

The coproscopy by sedimentation, antibody-ELISA, and copro-ELISA (gold standard) techniques were utilized to attain the prevalence of bovine fasciolosis in Erzurum Province, considering associated risk factors as well as their diagnostic sensitivity and specificity. In Turkey, epidemiology studies on fasciolosis are mostly based on fecal examination or inspection at slaughterhouse, and few researchers have employed immuno-serological methods. Recent surveys employing the

antibody-ELISA technique by Yildirim et al. (13) and the copro-ELISA technique by Sen et al. (14) reported that prevalence of bovine fasciolosis in the Cappadocia region was 65.2 and 3.03%, respectively. In the present study, bovine fasciolosis prevalence in the Erzurum Province was 34.04, 35.46, and 61.70% as determined by the coproscopy by sedimentation, copro-ELISA, and antibody-ELISA techniques, respectively (Table 1). Prevalence reports based on the anitibody-ELISA test were greater than those based on the copro-ELISA test. This could be related to development of antibodies much earlier than presence of eggs in feces during the course of fasciolosis (10, 15, 16).

The literature coping with gender association with fasciolosis prevalence is inconsistent. Studies reporting no sex effect on fasciolosis are available (17, 18). In agreement with the present study (Table 1), dairy cattle were shown to be more vulnerable to fasciolosis than beef cattle (13, 19). It appears that this is not a direct effect of sex, but of the animal production system (19). The facts in the region are that males have shorter life-spans than females and that females are grazed whereas males are confined. These (age and grazing vs. confined) increase predisposition to fasciolosis.

Cattle in advanced ages (> 2-3 years) are more prone to fasciolosis than those in younger ages (< 2 years) (13, 17, 19, 20, 21). In this study, cattle older than 2 years were affected by fasciolosis in all techniques at a greater frequency than those younger than 2 years (P < 0.0001; Table 1). This could be a result of decreased immune-potency as age advances (17). It could also be probable that older cattle have prolonged host-parasite association and they are exposed to intermediate hosts in longer periods (21).

Table 3: Sensitivity and specificity of diagnostic tests for fascioliasis*

Test	Sensitivity Mean	95% CI	Specificity Mean	95% CI	+LR	-LR	AUC Mean	SE	95% CI	z	P <
Antibody-ELISA	100.0	96.4-100.0	59.34	51.8-66.5	2.46	0	0.797	0.0183	0.745-0.842	16.253	0.0001
Coproscopy by Sedimentation	96.0	90.1-98.9	100.00	98.0-100	-	0.04	0.980	0.00985	0.956-0.993	48.744	0.0001
							Antibody-ELISA vs. Coproscopy by Sedimentation				
							Difference	SE	95% CI	z	P <
							0183	0.0207	0.143-0.224	8.837	0.001

*The copro-ELISA test considered golden standard.

In disagreement with the present study, several researchers reported lacking breed effect on fasciolosis (13, 14, 21). In all three methods, fasciolosis was more common in crossbred cattle than purebred cattle (1.36-2.46 folds; $P < 0.0001$; Table 1). This is opposed to expectations that local breeds and crossbreeds are more resistant to bacterial and parasitic infections than purebred breeds due to their adaptation to habitat. Thus, this finding could also be a consequence of production system in the region. The fact is that purebred breeds are mostly raised in confined system or fenced grasslands, whereas local breeds and crossbreeds are grazed on communal areas with poor vegetation. Indeed, metacercariae (infective form of the parasite) are colonized in parts of grasses close to soil and/or water (22), which could contribute greater prevalence of cattle grazing at a late stage of the vegetation period.

The diagnosis of fasciolosis is confirmed by the observation of parasite eggs in the feces of infected animals, but due to the long pre-patent period in cattle, coprological methods are only sensitive 8-9 weeks after infection (23). Thus, more accurate diagnostic methods for the early detection of fasciolosis are invaluable (24). Because antibodies are present approximately 1-5 weeks before eggs evident in feces or infection matures, the antibody-ELISA technique is more sensitive than the coproscopy by sedimentation technique (10, 15, 16). This suggests that cattle with mature infections may not excrete detectable numbers of eggs in feces. Indeed, 4 and 78 cattle with negativity based on the coproscopy by sedimentation technique yielded positivity based on the copro- and antibody-ELISA techniques, respectively (Table 1). One disadvantage of the antibody-ELISA is that positive results only imply exposure to the trematode at some time, but not necessarily current infection (25). In addition antibody levels in most animals persist above the positive threshold of the antibody-ELISA for about 12 weeks after treatment (26), suggesting that prevalence data based on the antibody-ELISA may not be reliable about current infection status. Cattle with increased antibody levels reflected positivity at a higher percentage in both the coproscopy by sedimentation and copro-ELISA techniques (Table 2), which could be related to the fact that currently infected animals may have higher level of antibodies than those recovered from the past infection(s).

The copro-ELISA method was developed for determination of *F. hepatica* and *F. gigantica* coproantigens in feces (6, 7). The copro-ELISA technique is considered gold standard to diagnose fasciolosis in the prepatent phase of infection when fecal examination remains negative (8, 27, 28). This test is highly sensitive (confirmed by necropsy) and specific (no cross reaction was observed with antigens from other helminthes), and enables detection of *Fasciola* infections 1-5 weeks before patency (27).

The copro-ELISA technique is reported to have high sensitivity (94%) and specificity (100%) (27). Coproantigen detection was carried out using copro-ELISA test (Bio-X Diagnostics, Jemelle, Belgium), with a specificity of 100% (24). Salimi-Bejestani *et al.* (5) reported 98% sensitivity and 96% specificity for the antibody-ELISA technique. Similar to the present survey, Charlier *et al.* (9) conducted an experiment under field conditions to compare sensitivity and specificity of diagnostic tests. They (9) reported that overall, sensitivity and specificity of, 64% (53-74%) and 93% (87-97%) for the coproscopy by sedimentation (10 g), 87% (78-93%) and 90% (83-95%) for the antibody-ELISA, and 94% (87-98%) and 93% (86-97%) for the copro-ELISA, respectively (9). In this field survey, the antibody-ELISA technique had high sensitivity (100%) and low specificity (59.3%), whereas the coproscopy by sedimentation method had both high sensitivity (96%) and specificity (100%) (Table 3; Figure 1).

Interpretation of prevalence rate attained by different techniques requires caution. For instance, the antibody-ELISA test result (61.70%) may reflect antibodies developed from past fasciolosis despite being treated, cross-reactions among other trematodes, current fasciolosis, or all these factors together (Figure 2, arrow b). Difference in prevalence rates between the coproscopy by sedimentation (34.04%) and copro-ELISA (35.46%) techniques was 1.42%. The coproscopy by sedimentation method is based on enumeration of eggs in feces that are produced by mature parasite in the host (Figure 2 arrow d), whereas the copro-ELISA method is based on determination of ES-antigens of immature/mature parasites residing in biliary tract, (Figure 2 arrow c). Thus, 1.42% difference may reflect parasites not mature enough to produce eggs that could be determined in feces and indicates prepatent infections (Figure 2 arrow e).

In conclusion, in this study a greater predisposition of females and crossbreds to fasciolosis than males and pure breeds could be artifacts, probably resulting from the production system practice in this region, and as a consequence the results cannot be generalized. Older cattle (> 2 years) were more prone to fasciolosis than younger cattle (< 2 years).

Different bovine fasciolosis prevalence rates obtained from the antibody-ELISA (61.70%), copro-ELISA (35.46%), and coproscopy by sedimentation (34.04%) methods could be related to principles of methods (determination of antibody in serum, ES-antibody in feces, and egg enumeration in feces, respectively), in association with different course of fasciolosis. Considering the copro-ELISA gold standard, sensitivity and specificity were 100 and 59.3% for antibody-ELISA technique and 96.0 and 100% for sedimentation technique. It is recommended that to determine active infection, the coproscopy by sedimentation method should be performed simultaneously with the copro-ELISA technique.

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