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Diagnosis of fasciolosis antibodies in Brazilian cattle through enzyme-linked immunosorbent assay (ELISA) employing both native and recombinant antigens

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The following paragraph should be rewritten. The results show whether it is in the stool or in the serum. Please rewritten clearly. It is difficult to distinguish the eggs of Fasciola species by microscopic observation. How was the diagnosis made?

Reviewer #2 (Comments for the Author):

The number of sample size is confusing, there are 500, 433 and 139. The number of samples should be the same or tallying.
2. The Number of positives should indicated and in brackets, you put the positive percentage eg 111/139 (80%) unlike only 80% or 0.80. This can be misleading to some readers.
3. The Confidence interval should be recalculated, it is misleading or misreading. This is a range of 95% CI 80% (72-85%) OR 95% CI 0.80 (0.72-0.85) OR 95% CI 0.80 (0.72; 0.85)

Diagnosis of anti-fasciolosis antibodies in Brazilian cattle through enzyme-linked immunosorbent assay (ELISA) employing both native and recombinant antigens

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Abstract

Bovine fasciolosis is a parasitic disease with global reach. Coprological based on egg detection in fecal samples and liver inspection to evaluate the presence of the parasite is currently the gold standard for diagnosing cattle fasciolosis. However, these techniques are labor-intensive and ineffective during the acute phase of the disease. Serodiagnosis using native and recombinant antigens has become an interesting alternative in efforts to identify cattle fasciolosis. In the present study, we evaluated cattle from abattoirs through liver inspection and from farms through coprological examination. Our laboratory team optimized and validated an ELISA to detect serum anti-fasciolosis antibodies in cattle. This assay utilized native antigens, somatic antigen and excretory/secretory proteins, in addition to the recombinant antigen cathepsin L-1. The native antigens were derived from parasite, the recombinant antigen was produced in the laboratory. For animals from abattoirs, both *Fh*ES and *Fh*rCL-1 presented an area under the ROC curve of 0.80, with a sensitivity of 0.80, (95% CI 0.46; 0.95) and 0.70 (95% CI 0.38; 0.90) and specificity of 0.81 (95% CI 0.73; 0.87) and 0.87 (95% CI 0.80; 0.92), respectively. *Fh*ES gave the best performance for those originating from the farm, with an AUROC of 0.84, sensitivity of 0.79 (95%CI 0.60; 0.90), and specificity of 0.86 (95%CI 0.82; 0.89). In conclusion, our study highlights the potential of serodiagnosis for accurately screening cattle fasciolosis. When comparing liver inspection and coprological examination, the promising sensitivity and specificity values of *Fh*ES highlight its importance as a tool for cattle fasciolosis diagnosis.

Keywords: Fasciolosis, cattle, native antigens (*Fh*ES and *Fh*SA), recombinant antigen (*Fh*rCL-1), ELISA.

Introduction

Fasciola hepatica, a plant-borne trematode species, is responsible for the zoonotic disease known as fasciolosis or liver fluke disease in humans and animals (1–3). The disease has traditionally been characterized as important in the veterinary context due to the substantial production and economic losses it causes in livestock (4–7). Herbivorous mammal hosts such as cattle, goats, and sheep are the most important disease transmission path to humans (8). Human fasciolosis is considered a neglected tropical disease by the World Health Organization (WHO), with estimates of 2.4 million infected individuals and 180 million people at risk of infection worldwide (9,10).

This trematode has an extensive global distribution and is found on every continent except Antarctica. Human fasciolosis poses major health problems in Europe, Cuba, Oceania, and the Americas (1,11), with a higher number of cases reported in South America (Bolivia, Peru, Chile, Ecuador, and Venezuela) than in other regions (3,12–14). In contrast, non-Andean, lowland countries in South America have reported sporadic and isolated human cases, including Uruguay (15) and Brazil (16,17). Among animals, studies in the Americas have demonstrated a wide prevalence in goats and a lower prevalence in cattle (18,19). In the Brazilian state of Santa Catarina, a prevalence of 10.8% in cattle was documented in an abattoir (20). Fasciolosis causes economic losses related to cattle production and severely impacts public health (6,20,21). Such economic losses have been quantified at a national level in Brazil, with a 5.8% reduction in carcass weight translating to a 35 USD loss per head in this country (22,23).

Fasciola hepatica is adaptable to different environmental conditions and has the ability to switch hosts (24), resulting in a broad host range (10). Its spread is also related to the geographic expansion of its original intermediate host, the Lymnaeidae snails (1). The life cycle of this disease comprises three stages, each characterized by distinct symptoms. The acute phase, initiated through ingestion of metacercariae in contaminated vegetation and water, lasts two to four months and manifests as abdominal pain, fever, urticaria, and gastrointestinal disturbances (2,25). The latent phase involves newly encysted juveniles penetrating the intestinal wall and peritoneum, migrating to the liver tissue, and reaching the bile ducts over several months (26–28). In the chronic phase, mature parasites in bile ducts produce eggs, causing severe liver and bile duct damage.

The established diagnostic method for bovine fasciolosis is the identification of eggs in feces (coprological examination), which is cost-effective and the gold standard for various parasitic diseases in humans and animals (27,29). Diagnosis throughout coprological examination often occurs during the chronic phase, when much of the liver damage has already occurred (28). However, there is a consensus that this method is not completely reliable for several reasons. A period of 8–15 weeks post-infection is required for *F. hepatica* eggs to appear in feces, when many pathological lesions have already manifested (30,31). Additionally, the method may not detect low-intensity or intermittent infections (27,32). In regions in which the disease is not endemic, infections with immature flukes are not detected. Furthermore, the eggs are released intermittently from the bile ducts, so stool samples from infected patients (humans and animals) may not contain eggs (27).

Post-mortem worm counting in the liver can be considered a valuable diagnostic method if the livers are appropriately sliced and soaked. *Fasciola hepatica* can also be

identified by inspecting cattle livers for adult worms in abattoirs. Postmortem examination of the bovine liver is a key approach to assessing the severity of *F. hepatica* infections. This entails examining livers for juvenile worms and bile ducts for adults, along with any associated pathological changes. Different rates of bovine fasciolosis have been reported in different abattoirs globally, with Brazil, for instance, documenting a 29.51% infection rate among animals (33). However, even mild or prepatent infections can evade detection, impacting the estimated sensitivity and specificity of the test.

Serological techniques, including lateral flow assays (34), and the indirect enzyme-linked immunosorbent assay (ELISA) (35–38), have been explored for detecting specific antibodies. ELISA-based detection of serum antibodies is a widely used diagnostic tool. It is highly regarded for its sensitivity and reliability in diagnosing acute infections, and it can complement fecal analysis for diagnosing latent and chronic infections (27). The antigens traditionally employed in serological tests consist of native antigens (somatic antigens and excretory/secretory antigens) of *F. hepatica* (35). To enhance diagnostic specificity, several purified *F. hepatica* antigens and recombinant antigens (36,37) have been used, most notably cathepsin L, a major protease involved in bovine fascioliasis. Serological tests have demonstrated high accuracy in diagnosing human, bovine, and ovine fasciolosis. The recombinant cathepsin L1 test utilizes recombinant pro-cathepsin L1 and targets antibodies against cathepsin, a cysteine protease, for diagnosing fasciolosis caused by *F. hepatica* (37,39), with no reported cross-reactions. Similarly, other studies observed no cross-reactions in native antigens and cathepsin-based ELISA tests, reporting strong performance (39–44). While many serological methods have been published, only a few have been commercially adopted.

In this context, the present study aimed to assess the potential of available native antigens, both somatic (*Fh*SA) and excretory/secretory (*Fh*SE), and the recombinant antigen cathepsin L (*Fhr*CL-1) for serodiagnosis of cattle fasciolosis in Brazil.

Materials and methods

The protocols and methods used were approved by the Ethics Committee of the Evandro Chagas Institute (INI/FIOCRUZ) (protocol CAAE: 10324719.6.0000.5262).

Characteristics of the cattle included in the study

Five hundred serum and fecal samples (420 from females and 80 from males) were obtained from 37 cattle farms in southern Santa Catarina. The samples were collected from cattle ranging in age from six months to 20 years. Fecal samples (6g) were used for in vivo diagnostics of cattle fasciolosis through coprological examination based on a sedimentation protocol (32). The tests were conducted in triplicate, and the entire sediments were analyzed under a stereomicroscope (32,45).

We found 405 negative and 95 positive results for eggs in the fecal samples. Of the 95 positive animals, 38 (7.6%) were positive for *F. hepatica*, 28 for *F. hepatica* eggs only, and 10 for *F. hepatica* and other parasites: seven also contained eggs of strongylid genera, two *Eimeria* eggs, and one Strongylidae and *Eimeria* eggs. The examination also showed that 44 (8.8%) cattle were positive for strongylid genera only and 13 (2.6%) for both strongylid genera and *Eimeria*. Animals positive for other parasites than *F. hepatica*

were excluded from the diagnostic performance evaluation of native and recombinant antigens.

A total of 139 serum samples were collected from a cattle abattoir located in southern Santa Catarina. The presence of cattle fasciolosis was determined through liver inspection. According to this approach, 10 (7.2%) cattle were diagnosed with *F. hepatica*, with no other parasites investigated during the veterinary inspection.

Serum samples collected from both the abattoir and farms were processed, divided into aliquots, and stored at -30°C for subsequent ELISA testing.

FhSA and FhES

The *Fh*SA and *Fh*ES preparations were carried out as follows: intact and live adult parasites were obtained from cattle livers at a local abattoir. Initially, the parasites underwent a series of 3–4 washes at room temperature using 0.01 M phosphate-buffered saline (PBS) with a pH of 7.2 to eliminate any traces of blood and bile.

For the *Fh*SA preparation, the parasites were kept in a PBS solution and transported to the laboratory. Subsequently, the parasites were macerated and divided into separate portions. The protease inhibitor trans-Epoxysuccinyl-L-leucylamido(4-guanidino) butane (E-64) (Sigma-Aldrich, US) was added to each sample at a concentration of 10 µM to minimize protein degradation. The antibiotics penicillin (100 U/mL) and streptomycin (0.25 mg/mL) were also incorporated to counteract bacterial activity.

For the *Fh*ES preparation, parasites were incubated in Roswell Park Memorial Institute (RPMI) 1640 medium at 37°C for 6 h. Within the laboratory setting, the parasites were subjected to five washing rounds with PBS containing antibiotics (penicillin and streptomycin). The first two washes used a volume of 10 mL PBS with antibiotics, while the subsequent three used a volume of 8 mL. Subsequently, the parasites were transferred using forceps into a 15 mL falcon tube containing RPMI 1640 medium preheated to 37°C. They were then cultured at a concentration of six parasites per 3 mL for 6 h at 37°C.

After incubation, the falcon tube was centrifuged at 14,000g for 30 min. The supernatant was then collected and divided into three microtubes, each containing 1 mL. E-64 was introduced to prevent protein degradation. The secretory/excretory antigens were obtained by culturing *F. hepatica* in RPMI medium and filtered using an Amicon Ultra-15 100 kDa centrifugal filter (Millipore, UK). During the antigen filtration process from the excretory/secretory systems, the RPMI medium was replaced with a saline buffer.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to analyze the protein content within *Fh*SA and *Fh*ES. Quantification of both the somatic antigen and the excretory/secretory antigens was carried out using a fluorimetric method in a Qubit® (Thermo Fischer Scientific, US) instrument. Following protein quantification, the supernatants of *Fh*SA and *Fh*ES were divided into aliquots and stored at -30°C until use.

Expression and purification of FhrCL-1

The full-length cDNA of *F. hepatica* preprocathepsin L1 (U62288.2) was obtained commercially in the pPIC9K vector from (GenScript, US). Protein expression was conducted using the multicopy system of the *Pichia pastoris* GS115 strain. The recombinant sequence featured a single amino acid substitution, replacing the active site Cys25 with Gly. This alteration resulted in the loss of functional activity while preserving the enzyme's conformation, rendering it more stable during fermentation and downstream isolation processes (39,46,47).

To generate the inactive enzyme, fermentation was performed in a liquid minimal medium containing yeast extract and glycerol (BMGY) to enhance yeast cell density. Cultivation in BMGY took place for 16 h at 30°C with agitation at 250 rpm. Once the yeast cell density reached an OD600 of 2–6, approximately 1 mL of the inoculum was transferred to a liquid minimal medium containing yeast extract and methanol (BMMY) to induce *Fh*rCL1 expression. Cultivation in BMMY lasted 92 h at 30°C under agitation at 250 rpm. During this time, the medium was supplemented with 1% methanol every 24 hours.

After completing the cultivation period, the culture was centrifuged at 10,000g for 30 min at room temperature. The resulting pellets were discarded. *Fh*rCL-1 was isolated from the supernatant using Ni-NTA affinity chromatography, following previously described methods (39,47,48).

ELISA optimization and development

FhSA, *FhES, and FhrCL-1*

To define ELISA conditions, we performed a matrix comparison using various antigen concentrations, dilutions of the primary sera, and dilutions of secondary antibodies for *Fh*SA, *Fh*ES, and *Fh*rCL-1 antigens, respectively.

Optimal antigen concentrations and serum dilutions were determined by checkerboard titrations. *Fh*SA, *Fh*ES, and *Fh*rCL-1 antigens (0.5µg/mL, 1.0µg/mL, and 1.0µg/mL, respectively) were dissolved separately in bicarbonate/carbonate coating buffer at pH 9.0 and added to each ELISA plate (Sarstedt AG & Co. KG, DE). One hundred microliters of the solution were then added to each well and incubated overnight at 4°C. The plates were washed three times with 0.05% Tween-80 in water. After coating, an additional blocking step with 100µL 1% skimmed milk in 0.05% Tween-80 was performed for 1 h at 37°C. After a further washing procedure, 100µL of sera-diluted pooled samples were added to each antigen (1:50, 1:100, and 1:50, respectively), and the plates were incubated for 1 h at 37°C. Following another wash, 100µl of peroxidase-conjugated anti-bovine antibody (Sigma-Aldrich, US) for each antigen (1:10.000, 1:10.000, and 1:30.000, respectively) was added to the wells, and the plates were incubated for 30 min at 37°C. After a final washing step, bound antibodies were detected by adding 100µL of tetramethylbenzidine (TMB) (Thermo Fischer Scientific, US). The color was developed for 10 min, and the reaction was stopped with 50µL of 0.1 M sulfuric acid. The plates were read on an ELISA reader at 450nm to determine absorbance values.

After developing and optimizing serological ELISA conditions, we tested samples from cattle collected in an abattoir and cattle farms. Negative and positive controls were used to diagnose fasciolosis in cattle by ELISA, using *Fh*SA, *FhES,* and *Fh*rCL-1 as

antigens. A pool of four samples (two negative samples for the presence of fasciolosis in the visceral inspection and two negative samples for the coprological examination) was used as a negative control on each plate. As a positive control, a pool of four samples was used on each plate (two positive samples for the presence of fasciolosis in the visceral inspection and two positive samples for the coprological examination). Positive control, negative control, and plate control were used in duplicate in all experiments.

Statistical analysis

To evaluate the diagnostic performance of native (*Fh*SA and *Fh*ES) and recombinant antigens (*Fhr*CL-1), we used liver inspection and coprological examination as the gold standard test for cattle from abattoirs and farms, respectively. Initially, the distribution of the quantitative values for the serodiagnosis tests was analyzed according to the categories (positive or negative) of the gold standard tests, aiming to explore their descriptive statistics, such as minimum, maximum, and median values, first and third quartile, mean values and standard deviation, as well as to inspect for outliers.

The optimal cutoff value for each ELISA method was based on a logistic regression model, considering as response variable the gold standard test results (positive or negative) and as predictor the quantitative values of the serodiagnosis test. Briefly, we applied a logistic regression model to adjust a classifier and a leave-one-out cross-validation (CV) technique to evaluate its diagnostic performance in data not used for its adjustment. Thus, on each CV iteration, the observations were randomly divided into training and test data; the former was used to adjust a logistic model and the latter to estimate the probability of being classified as a positive sample. After all samples were part of the training and test data, the vector of estimated probabilities was used to evaluate the diagnostic performance of the model. For this, it was necessary to choose a cutoff point for the estimated probability, aiming to classify samples as positive or negative. We chose the cutoff that maximizes the model's sensitivity and specificity and calculated the area under the receiver operating characteristic (AUCROC) curve, sensitivity (S), specificity (E), positive predictive values (PPV), and negative predictive values (NPV) and the respective 95% confidence intervals (CI) of all of these estimates. The analyses were performed on R using caret, pROC, and CompareTests packages.

Results

Table 1 shows a summary of the *Fh*ES, *Fh*SA, and *Fhr*CL-1 values according to the presence (positive group) or absence (negative group) of fascioliasis detected by liver inspection of cattle from the abattoir (n=139). In general, for the three tests, the positive group had higher values for the first and third quartiles as well for median and mean than the negative group.

Insert Table 1

The AUROC for *Fh*ES, *Fh*SA, and *Fhr*CL-1 adjusted models were 0.80 (95%CI: 0.67; 0.92), 0.74 (95%CI: 0.55; 0.93) and 0.80 (95%CI: 0.61; 0.98), respectively (Figure 1). For each test, we chose the cutoff point that maximizes the model's sensitivity and

specificity (Table 2). The three tests had a moderately accurate performance, although *Fh*ES showed higher sensitivity and NPV (0.80 and 0.98, respectively), indicating that the test is suitable for fasciolosis screening, as it had a low frequency of false negative results: two of approximately 10 cases of the disease were mistakenly classified as negative. Thus, out of 106 negative results, only two were false. Even though the specificity of the tests was high, the fact that the disease occurs rarely (7.2%) means that the tests cannot be used to confirm the presence of the disease due to the large number of false-positive results (for example, for *Fh*ES, 25 of 33 positive results were false).

Insert Table 2 and Figure 1

Table 3 shows the summary values of *Fh*ES, *Fh*SA, and *Fh*rCL-1 according to the presence (positive group) or absence (negative group) of fasciolosis detected by coprological examination in cattle from farms. The positive group had higher values for the first and third quartiles as well as for the median and mean for the three serological tests than the negative group.

Insert Table 3

The AUROCs for *Fh*ES, *Fh*SA, and *Fh*rCL-1 adjusted models were 0.84 (95%CI: 0.76; 0.93), 0.73 (95%CI: 0.61; 0.85), and 0.67 (95%CI: 0.54; 0.80), respectively (Figure 2). For each test, we chose the cutoff point that maximizes the model's sensitivity and specificity (Table 4). For this scenario, the *Fh*ES also presented better results, with a sensitivity of 0.79 and an NPV of 0.98. Of the 353 negative results, only six were false negatives when using the chosen cutoff value for the *Fh*ES. This confirms its suitability for screening fasciolosis. Despite the high specificity, the disease prevalence was only 5.6%, out of the 80 positive results, and 58 were false positive results.

Insert Table 4 and Figure 2

Aiming to investigate the impact of cross-reactions on the diagnostic performance of the three antigens (*Fh*ES, *Fh*SA, and *Fh*rCL-1), for the 95 positive samples (according to the coprological examination), we evaluated the distribution of absorbance values and the cut-off points obtained from the adjusted models that considered samples positive for fascioliasis only (Supplementary Figure 1). Overall, these results show that positive samples for fasciolosis had higher absorbance values. We also adjusted the logistic regression models by considering cattle samples from farms positive for parasitic infections other than fasciolosis (n=500). Supplementary Table 1 shows the summary values of *Fh*ES, *Fh*SA, and *Fh*rCL-1 according to the presence (positive group) or absence (negative group) of fasciolosis and the Supplementary Table 2, the results of the adjusted models. The AUROC curve for the *Fh*ES, *Fh*SA, and *Fh*rCL-1 were similar to those that considered only samples positive for fascioliasis: 0.83 (95%CI: 0.75; 0.90), 0.73 (95%CI: 0.63; 0.83), and 0.68 (95%CI: 0.58; 0.79), respectively (Supplementary Figure 2).

Discussion

Our study is the first to compare native and recombinant antigens for diagnosing cattle fasciolosis in Brazilian animals. Coprological and liver inspection were used as the gold standard diagnostic tests for both farm and abattoir animals. The *Fh*ES serological test was better able to discriminate positive and negative samples for both farm and abattoir animals, and it seems suitable for screening purposes. The economic and public health problems caused by cattle fasciolosis have been reported in different parts of the world, including Brazil (20,21,28,49). It is important to develop and establish a reliable, simple, and rapid diagnostic tool for properly diagnosing cattle fasciolosis in Brazil, especially in endemic areas. In the present study, we evaluated the performance of three ELISA tests using *Fh*ES, *Fh*SA, and *Fh*rCL-1 antigens to diagnose cattle fasciolosis based on information obtained from a meta-analysis study (50).

Liver necropsy, which diagnoses fasciolosis when bile ducts are dissected, is the only conclusive diagnostic procedure for *F. hepatica* (7,51). This is impractical as a herd or flock management tool, as it can only be carried out postmortem. Specific ELISA tests for liver flukes have been developed to meet these requirements and are now routinely used for cattle (30,52). ELISAs for *F. hepatica* are versatile tests capable of detecting specific antibodies or antigens in fecal samples as well as pooled or individual milk and sera (36,37,53). One significant drawback of relying on fecal egg counts is the inability to diagnose immature migrating stages of liver flukes within the final host. Consequently, using ELISA tests with early diagnostic potential represents a notable advantage (27,36). The most detrimental phase of this infection occurs during the migration of immature stages (36,37). The application of ELISA techniques for *F. hepatica* diagnosis has consistently exhibited enhanced sensitivity compared to coprological methods (36–38). Moreover, it offers the distinct advantage of detecting pre-patent infections.

Serological diagnosis of cattle fasciolosis based on fractions of adult worm antigens has been reported in different studies worldwide (36,43,54). To this end, we used two cattle populations with known infection status (the presence of eggs in the feces or parasites in the liver). Our first serological panel comprised more than 100 cattle samples collected in an abattoir. A small number of articles that evaluated the diagnosis of fasciolosis in cattle used samples collected in abattoirs (36). Our second serological panel consisted of 500 samples of blood and feces from cattle collected on farms. The studies that evaluated the serological diagnosis of bovine fasciolosis used small panels with up to 100 animals (43,54–56).

A critical point for evaluating a new immunodiagnostic test is to propose a cut-off point that properly discriminates between negative and positive samples. The absorbance values of *Fh*ES, *Fh*SA, and *Fh*rCL-1 antigens tested had a good ability to distinguish between positive and negative samples in abattoir samples. Only the *Fh*ES antigen performed well in differentiating positive and negative cattle fasciolosis on serum samples collected on farms. Our investigation demonstrated that the absorbance values for the *Fh*ES antigen were comparable to those reported in other studies when sera from cattle with fasciolosis were examined using coprological testing as the gold standard (55).

Our study established a cut-off value for each proposed ELISA test based on positive and negative samples using liver inspection and coprological examination as the gold standard tests. The cut-off points for *Fh*ES, *Fh*SA, and *Fh*rCL-1 were, respectively,

0.4895, 0.379, and 0.1050 for cattle from the abattoir, and 0.4105, 0.4830, and 0.1270 for those from farms. The native antigens *Fh*ES and *Fh*SA consist of a complex mixture of proteins, potentially leading to elevated absorbance values. In contrast, the recombinant antigen *Fh*rCL-1 is a single purified protein, which could account for the comparatively lower absorbance values observed. Different approaches are employed when developing ELISA tests for serological diagnosis of fasciolosis in cattle. The cut-off values reported by studies assessing one of these antigens vary, although they are often higher than those found in our analysis. Different methods based on the average absorbance value and the ROC curve are used in the ELISA tests created using native and recombinant antigens for the serological diagnosis of bovine fasciolosis (36–38,55,57).

Serology offers the advantage of earlier detection of infections in comparison to fecal egg detection. In addition, when compared to coprological methods, serological approaches, particularly the ELISA test, are very sensitive and specific. Since *F. hepatica* is the main cause of cattle fasciolosis, most of the studies related to the disease diagnosis focus on purified subunits from either *Fh*SA or *Fh*ES (native antigens) of this parasite species (36,43,58,59). The cattle in this study come from farms in southern Santa Catarina, where the prevalence of the disease is considered low (20). Despite the observed low prevalence of the disease, the antigen *Fh*ES showed good diagnostic performance for both samples collected in the abattoir and farms, with sensitivities of 80% and 79% and specificities of 81% and 86%, respectively. Other studies that also used native antigens reported sensitivity ranging from 80 to 100% and specificity from 50 to 100% for serological diagnosis of bovine fasciolosis (37,60).

Serological diagnosis for cattle fasciolosis using recombinant antigens (cathepsin and saposin) has been developed in the last years. Cathepsin is an important enzyme the parasite uses to elicit a humoral response in cattle as early as two weeks after infection (36,38). In our study, the antigen *Fh*rCL-1 presented diagnostic performance as good as those observed in *Fh*ES for abattoir cattle.

Sera samples from farm cattle infected with other parasites were used to evaluate the impact of cross-reactivity in our ELISA tests. Cross-reactivity analysis is fundamental since fasciolosis is a worldwide parasitic disease that can co-occur with other cattle parasitic diseases. Furthermore, current parasitological methods depend on the worker's expertise because *F. hepatica* eggs can be confused with eggs from other helminths. Therefore, a good diagnostic test needs to be able to distinguish between *Fasciola* and other parasitic diseases. We did not observe substantial differences between the adjusted models without and with positive samples for other parasites, which suggests that the test differentiated animals positive for fasciolosis from cattle samples with other parasites.

In our study, the cattle in the positive group had positive fecal egg counts or the presence of *F. hepatica* in the liver, indicating that each animal was currently infected. Diagnosis of this infection is usually based on coprological techniques. The intermittent nature of the eggs' evacuation through the feces was the reason for the low sensitivity of the coproscopy in detecting fasciolosis in cattle (31). Moreover, a prolonged pre-patent period of 8–15 weeks after the infection is required for the eggs to be shed in the feces (27,31). Compared to fecal egg counts, serology can detect infections 7–8 weeks earlier (36,37) and is considered a very sensitive method (61), but it does not distinguish between current and past infections. Results indicated that indirect ELISA using *Fh*ES

and *Fh*rCL-1 antigens could be an efficient and rapid diagnostic method for cattle fasciolosis compared to coprology. Therefore, using both methods together provided excellent information about the real infection situation. Of the three antigens (*Fh*SA, *Fh*ES, and *Fh*rCL-1) tested for the serological diagnosis of *F. hepatica* in cattle, the *Fh*ES presented satisfactory results in both scenarios, when compared to liver inspection in cattle from abattoir and to coprological examination in those from farms, suggesting it may be used for the development of ELISA tests for fasciolosis screening.

Conclusion

We have developed three ELISAs utilizing two native antigens and one recombinant antigen for detecting *F. hepatica* antibodies. We validated these ELISAs using cattle serum samples collected from farms and an abattoir, considering coprological examination and liver inspection as gold standard tests, respectively. The ELISA test using *Fh*ES as an antigen had good diagnostic performance in the two scenarios (abattoir and farm) evaluated as a test for screening fasciolosis. Notably, the results were promising even in the face of the relatively low prevalence of cattle fasciolosis. The proposed ELISA test has the potential to be used in situations where it is more challenging to do a coprological investigation or examine the liver of cattle. These assays constitute a vital component of the immunodiagnostic toolkit that our laboratory is developing to improve the serodiagnosis of fasciolosis in Brazilian cattle. Recognizing that positive outcomes in antibody detection tests may not necessarily indicate ongoing infections but a history of exposure, we are actively exploring alternatives, such as an antigen detection ELISA using monoclonal antibodies. As a prospect, it is important to apply the test to more positive samples and also to explore cross-infection. Furthermore, ongoing research efforts are focused on adapting our in-house ELISA methods into more streamlined and dependable formats, such as immunochromatography or dot ELISA. This adaptation aims to facilitate potential commercialization and validation within Brazilian regions where the disease is endemic.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

GD, LGM, and FBF: Study conception and design. GD: Conceptualization, methodology, manuscript writing, and drafting the manuscript. HG dos S: Data curation, meta-analysis, and manuscript reviewing. MM da GP: Manuscript reviewing. LGM: Manuscript reviewing. FBF: Manuscript writing, reviewing, and editing. LGM and FBF supervised the study. All authors contributed to the article and approved the submitted version.

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Table 1. Descriptive summary for the three tests when applied to cattle from abattoirs (n=139).

	Native antigens		**Recombinant antigen**
Summary values	***Fh*ES**	***Fh*SA**	***Fhr*CL-1**
Positive group (n= 10)			
Minimum	0.360	0.196	0.076
1st quartile	0.490	0.270	0.111
Median	0.571	0.443	0.252
Mean (SD)	0.573 (0.141)	0.439 (0.186)	0.235 (0.126)
2nd quartile	0.668	0.568	0.336
Maximum	0.815	0.716	0.436
Negative group (n= 129)			
Minimum	0.192	0.114	0.057
1st quartile	0.298	0.169	0.078
Median	0.362	0.222	0.090
Mean (SD)	0.393 (0.133)	0.263 (0.144)	0.104 (0.064)
2nd quartile	0.452	0.303	0.107
Maximum	0.806	1.247	0.638

Legend: SD= Standard Deviation.

Table 2. Diagnostic performance measures for the three tests by considering the presence of the parasite in the liver as the gold standard method (n=139).

	Native antigens		**Recombinant antigen**
Performance measures	***Fh*ES estimate (95%CI)**	***Fh*SA estimate (95%CI)**	***Fhr*CL-1 estimate (95%CI)**
Cutoff	0.4895	0.379	0.1050
Sensitivity	0.80 (0.46; 0.95)	0.70 (0.38; 0.90)	0.70 (0.38; 0.90)
Specificity	0.81 (0.73; 0.87)	0.86 (0.81; 0.92)	0.87 (0.80; 0.92)
PPV	0.24 (0.17; 0.34)	0.30 (0.19; 0.45)	0.29 (0.18; 0.43)
NPV	0.98 (0.94; 0.99)	0.97 (0.94; 0.99)	0.97 (0.94; 0.99)

Legend: CI= confidence intervals; PPV= positive predictive values; NPV= negative predictive values.

Table 3. Descriptive summary for the three tests when applied to cattle from farms (n=433).

	Native antigens		**Recombinant antigen**
Summary values	***Fh*ES**	***Fh*SA**	***Fhr*CL-1**
Positive group (n= 28)			
Minimum	0.213	0.168	0.058
1st quartile	0.412	0.440	0.102
Median	0.529	0.637	0.141
Mean (SD)	0.560 (0.213)	0.641 (0.299)	0.184 (0.116)
2nd quartile	0.776	0.927	0.248
Maximum	0.828	1.312	0.454
Negative group (n= 405)			
Minimum	0.085	0.078	0.054
1st quartile	0.202	0.266	0.088
Median	0.259	0.382	0.103
Mean (SD)	0.289 (0.122)	0.406 (0.191)	0.113 (0.044)
2nd quartile	0.353	0.510	0.126
Maximum	0.898	1.373	0.410

Legend: SD= Standard Deviation.

Table 4. Diagnostic performance measures for the three tests by considering the coprological examination as the gold standard method (n=433).

	Native antigens		**Recombinant antigen**
Performance measures	***Fh*ES estimate (95%CI)**	***Fh*SA estimate (95%CI)**	***Fhr*CL-1 estimate (95%CI)**
Cutoff	0.4105	0.4830	0.1270
Sensitivity	0.79 (0.60; 0.90)	0.71 (0.52; 0.85)	0.57 (0.39; 0.74)
Specificity	0.86 (0.82; 0.89)	0.70 (0.66; 0.75)	0.75 (0.71; 0.79)
PPV	0.28 (0.22; 0.34)	0.14 (0.11; 0.18)	0.14 (0.10; 0.19)
NPV	0.98 (0.97; 0.99)	0.97 (0.95; 0.98)	0.96 (0.94; 0.97)

Legend: CI= confidence intervals; PPV= positive predictive values; NPV= negative predictive values.

Figure 1. ROC curves for FhES, FhSA and FhrCL-1 for cattle from abattoirs (n=139)

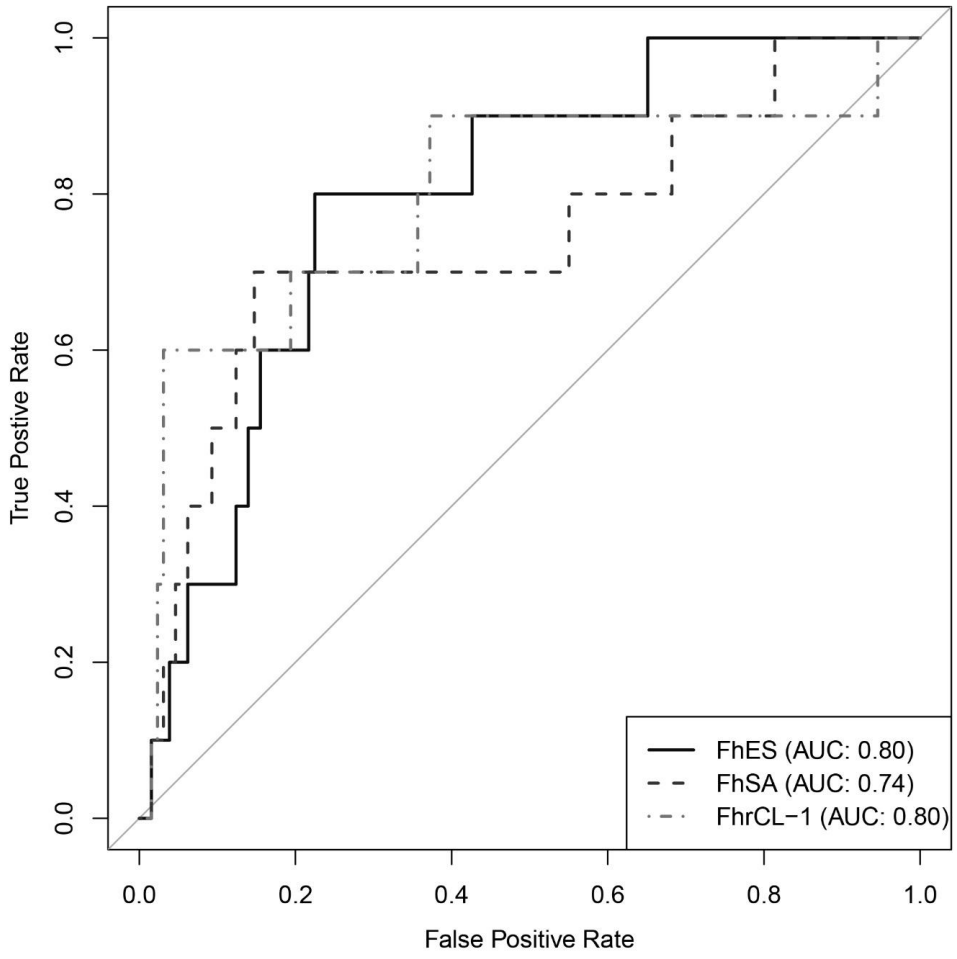
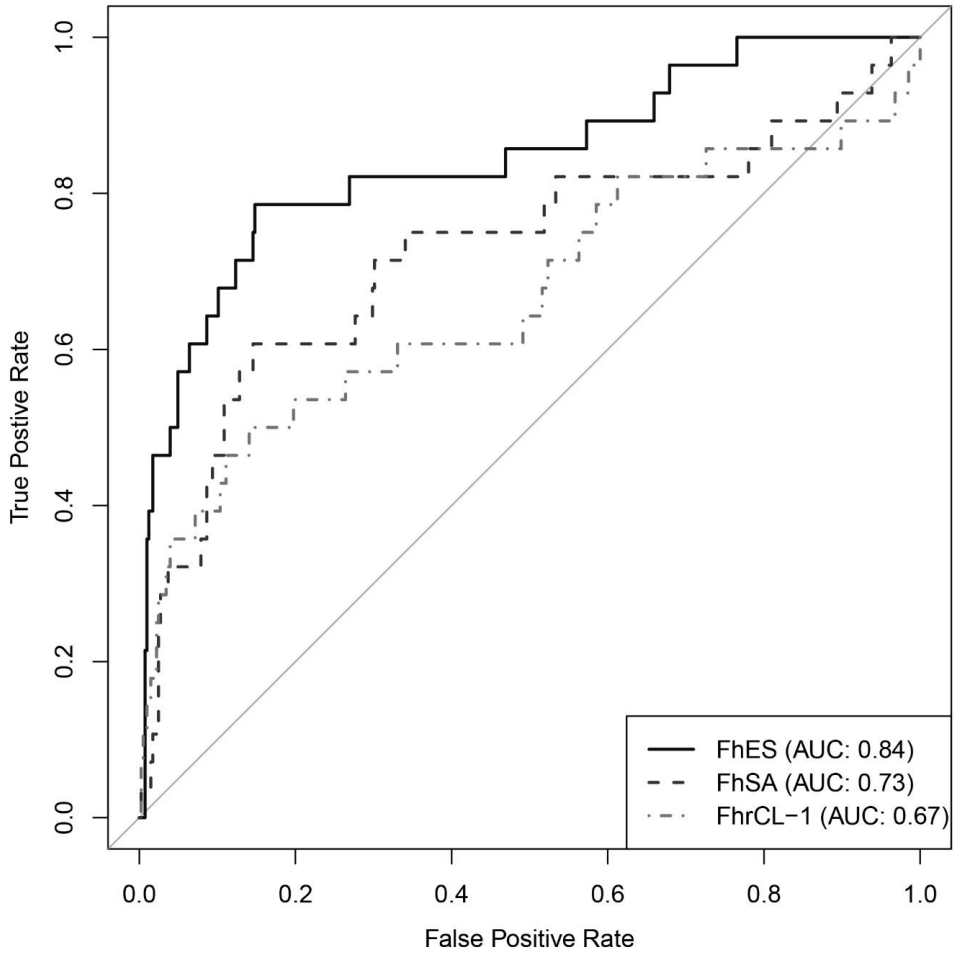


Figure 2. ROC curves for FhES, FhSA and FhrCL-1 for cattle from farms (n=433)



Reviewer's comments

	Line number	Current	Suggestion or Comments	Reasons
1	1	**Diagnosis of anti-fasciolosis antibodies in Brazilian cattle through enzyme-linked immunosorbent assay (ELISA) employing both native and recombinant antigens**	**Diagnosis of fasciolosis in Brazilian cattle through enzyme-linked immunosorbent assay (ELISA) employing both native and recombinant antigens"**	The title should change because we are detecting the disease using antibodies through ELISA **(serological technique),** hence we cannot say diagnosis of anti-fasciolosis antibodies.
2	24-25	In the present study, we evaluated cattle from abattoirs through liver inspection and from farms through coprological examination.	In the present study, we evaluated cattle from abattoirs and farms through liver inspection and coprological examination respectively	Repetition of wording
3	Abstract	ROC curve of 0.80, with a sensitivity of 0.80, (95% CI 0.46; 0.95) and 0.70 (95% CI 0.38; 0.90)	ROC curve of 0.80, with a sensitivity of 0.80, (95% CI 0.46- 0.95) and 0.70 (95% CI 0.38- 0.90) OR ROC curve of 0.80, with a sensitivity of 0.80, (95% CI 0.46, 0.95) and 0.70 (95% CI 0.38, 0.90)	The CI should be written as a range. However, check this range it's too big from 46% to 95% the other one from 38% to 90%
	66	….translating to a 35 USD loss per head in this country	…..translating to a 35 USD loss per animal in this country	Per head may be confusing to other readers.
	85	In regions in which the disease is not endemic, infections……	In regions, where the disease is not endemic, infections….	
	106	bovine fascioliasis	Bovine fasciolosis	Be consistent
	125	500 samples	How did the authors come up with this samples size? Any sample size formula?	
	127	collected from cattle ranging in age from six months to 20 years.	…collected from cattle ranging from six months to 20 years old	
	131-142	We found 405 negative and 95 positive results for eggs in the fecal samples. Of 132 the 95 positive animals, 38 (7.6%) were positive for *F. hepatica*, 28 for *F. hepatica* eggs only, and 10	Move to results section	Because this section is for methodology only and not results.

		for *F. hepatica* and other parasites: seven also contained eggs of strongylid genera, two *Eimeria* eggs, and one Strongylidae and *Eimeria* eggs. The examination also showed that 44 (8.8%) cattle were positive for strongylid genera only and 13 (2.6%) for both strongylid genera and *Eimeria*. Animals positive for other parasites than *F. hepatica* were excluded from the diagnostic performance evaluation of native and recombinant antigens. A total of 139 serum samples were collected from a cattle abattoir located in southern Santa Catarina. The presence of cattle fasciolosis was determined through liver inspection. According to this approach, 10 (7.2%) cattle were diagnosed with *F. hepatica*, with no other parasites investigated during the veterinary inspection.		
	131	38 were positive for *F. hepatica*	Were the 38 included in the 139?	
	Table 2	0.80 (0.46; 0.95), 0.70 (0.38; 0.90), 0.70 (0.38; 0.90)	Recalculate all the ranges of CI, For example 0.80 (0.72-0.85); 0.7 (0.62-0.77) Also include the positive figures unlike only percentage positivity 80% of 139 is 111/139 (80%)	They look to be too wide and appears to be out of range, considering (n=139)
	Table 4	0.79(0.60;0.90)	Recalculate all the ranges of CI, e.g (0.79 (0.75-0.82	Some of them look to be too wide and appears to be out of range, considering (n=433)
		The number of samples differ. You have 500 in initial stage but you are reporting 433	The samples collected should all be analysed. Where are other 67 samples?	
		Only 139 serum and 500 feacal samples	For serum samples you analysed only 139 why not	

			analysed serum from all 500 animals so that you compare the results for feacal examination and serology results?	
	143	Serum samples collected from both the abattoir and farms were processed, divided into aliquots, and stored at -30°C for subsequent ELISA testing.	This suggest that you had 500 serum samples why conduct ELISA on 139 only?	

Generally the research is okay but needs major revision. The number samples are confusing there 500 then 433 and 139. The number of samples should the same or tallying.

2. The Number of positive should indicated and in brackets you put the positive percentage eg 111/139 (80%) unlike only 80% or 0.80. This can be misleading to some readers.

3. The Confidence interval should be recalculated it is misleading or misreading. This is a range 95% CI 80% (72-85%) OR 95% CI 0.80 (0.72-0.85) OR 95% CI 0.80 (0.72; 0.85)

We thank the reviewer for their helpful suggestions that improved the message of our manuscript. Please find in below our point-to-point answers and commentaries on some text improvements we have made. In the revised version of our manuscript, major changes/insertions are outlined in red.

Independent Review Report, Reviewer 2

1. Line 1. ***Current:*** "Diagnosis of anti-fasciolosis antibodies in Brazilian cattle through enzyme-linked immunosorbent assay (ELISA) employing both native and recombinant antigens"/ ***Suggestion:*** "Diagnosis of fasciolosis in Brazilian cattle through enzyme-linked immunosorbent assay (ELISA) employing both native and recombinant antigens."
RESPONSE: thank you for mentioning this. We modified the title as suggested by the Reviewer.

2. Lines 24–25. ***Current:*** "In the present study, we evaluated cattle from abattoirs through liver inspection and from farms through coprological examination"/ ***Suggestion:*** "In the present study, we evaluated cattle from abattoirs and farms through liver inspection and coprological examination respectively."
RESPONSE: we appreciate this comment. The text was rewritten as suggested by the Reviewer.

3. Abstract. ***Current:*** "ROC curve of 0.80, with a sensitivity of 0.80 (95% CI 0.46; 0.95) and 0.70 (95% CI 0.38; 0.90)"/ ***Suggestion:*** "ROC curve of 0.80, with a sensitivity of 0.80, (95% CI 0.46- 0.95) and 0.70 (95% CI 0.38- 0.90)." ***Comment:*** The CI should be written as a range. However, check this range it's too big from 46% to 95% the other one from 38% to 90%.
RESPONSE: thank you for mentioning this. We modified de CI written as suggested by the Reviewer. Regarding the reviewer's observation about the size of the confidence interval (very large), we reviewed the analyzes performed and confirmed the values obtained. For the confidence interval estimation, we user CompareTests() function from CompareTests R package. In the current version of the manuscript, we include a link to an open access repository where the script used in data analysis was made available. These results are for the analyzes of abattoir data that includes 139 animals, of which 10 were positive for fasciolosis according to liver inspection. As the sample is small and the prevalence of the disease is low, we expected a wider (less precise) confidence interval, especially for the sensitivity measure. To aid in the interpretation of the performance measures presented, in the current version of our manuscript we highlight the sample size and the number of positive cases in the abstract and in the results section for each group we studied.

4. Line 66. ***Current:*** "…translating to a 35 USD loss per head in this country"/ ***Suggestion:*** "...translating to a 35 USD loss per animal in this country."
RESPONSE: thank you for pointing this out. The sentence was rewritten as suggested by the Reviewer.

5. Line 85. ***Current:*** “In regions in which the disease is not endemic, infections…”/ ***Suggestion:*** “In regions, where the disease is not endemic, infections….”
RESPONSE: thank you for pointing this out. The sentence was rewritten as suggested by the Reviewer.

6. Line 106. ***Current:*** “bovine fascioliasis”/ ***Suggestion:*** “bovine fasciolosis.”
RESPONSE: we appreciate this comment and apologize for the mistake. The sentence was rewritten according to the suggested correction.

7. Line 125. ***Current:*** “…500 samples”/ ***Comment:*** “How did the authors come up with this samples size? Any sample size formula?”
RESPONSE: thank you for mentioning this. Serum and fecal samples obtained from farms (n=500), as well as serum samples obtained from abattoir (n=139) represent convenience samples, and therefore, were not obtained from probabilistic sampling plans, as they included the voluntary participation of regional agricultural establishments in the State of Santa Catarina, located in the south of Brazil.

8. Line 127. ***Current:*** “…collected from cattle ranging in age from six months to 20 years”/***Suggestion:*** “…collected from cattle ranging from six months to 20 years old.”
RESPONSE: thank you for pointing this out. The sentence was rewritten as suggested by the Reviewer.

9. Lines 131–142. ***Current:*** “We found 405 negative and 95 positive results for eggs in the fecal samples. Of 132 the 95 positive animals, 38 (7.6%) were positive for *F. hepatica*, 28 for *F. hepatica* eggs only, and 10 for *F. hepatica* and other parasites: seven also contained eggs of strongylid genera, two *Eimeria* eggs, and one *Strongylidae* and *Eimeria* eggs. The examination also showed that 44 (8.8%) cattle were positive for strongylid genera only and 13 (2.6%) for both strongylid genera and *Eimeria*. Animals positive for other parasites than *F. hepatica* were excluded from the diagnostic performance evaluation of native and recombinant antigens. A total of 139 serum samples were collected from a cattle abattoir located in southern Santa Catarina. The presence of cattle fasciolosis was determined through liver inspection. According to this approach, 10 (7.2%) cattle were diagnosed with *F. hepatica*, with no other parasites investigated during the veterinary inspection”/ ***Comment:*** “Move to results section. Because this section is for methodology only and not results.”
RESPONSE: we appreciate this comment. Now this paragraph has been moved to the results section as suggested by the Reviewer. Furthermore, we have made some improvements to the wording of this paragraph (lines 292-299) to clarify the two independent samples we analyzed (from the farms and the abattoir).

10. Line 131. ***Current:*** “38 were positive for *F. hepatica*”/ ***Comment:*** “Were the 38 included in the 139?”
RESPONSE: we appreciate this comment and apologize for the lack of clarity in the wording of the study design, which includes two cattle samples obtained independently,

one from farms and the other from abattoir. We rewrote the method section (lines 123-143), separating the description of the samples obtained in each context into subsections to make this information clearer. In this sense, the 38 positive samples for fasciolosis correspond to samples from farms (in which, in total, 500 animals were evaluated), where the disease was investigated through coprological examination (lines 136-143 in the methods section and lines 292-299 in the results section). Among the samples from the abattoir (which totaled 139 animals), 10 were positive for fasciolosis according to liver inspection (lines 127-132 in the methods section and lines 268-272 in the results section).

11. Line 143. ***Current:*** "Serum samples collected from both the abattoir and farms were processed, divided into aliquots, and stored at -30°C for subsequent ELISA testing"/ ***Comment:*** "This suggest that you had 500 serum samples why conduct ELISA on 139 only?"

RESPONSE: thank you for mentioning this. As described in the previous item, we apologize for the lack of clarity in the description of the samples used in this research. We reiterate that improvements were made in the writing of the methods section to overcome this issue (lines 123-143). ELISA tests were conducted on both groups: on the 500 samples obtained on the farms and on the 139 obtained at abattoir. In the results section, Tables 1 and 2, and Figure 1, show the analyzes of the ELISA data for cattle at abattoir, and Tables 3 and 4, and Figure 2 correspond to the analyzes of the ELISA data for cattle on farms. These results covered 433 animals, since those that presented positive results in the coprological examination for parasites other than *F. hepatica* were excluded (n=67). Nonetheless, supplementary material shows the analyzes of all farms data (i.e., including those animals that tested positive for other parasites in the coprological examination, totaling 500 cattle) aiming to evaluate the impact of cross-infection on the diagnostic performance of serodiagnosis tests.

12. Table 2. ***Current:*** "…0.80 (0.46; 0.95), 0.70 (0.38; 0.90), 0.70 (0.38; 0.90)"/ ***Suggestion:*** "Recalculate all the ranges of CI, For example 0.80 (0.72-0.85); 0.7 (0.62-0.77). Also include the positive figures unlike only percentage positivity 80% of 139 is 111/139 (80%). They look to be too wide and appears to be out of range, considering (n=139) bovine fascioliasis."

RESPONSE: we appreciate this comment and apologize for the lack of clarity in the description of the estimated diagnostic measures, presented on Table 2 for cattle from abattoir and on Table 4 and on Supplementary Table 2 for those from farms. We added on these Tables the numerator and denominator correspondent to the estimate of each diagnostic measure. For example, in Table 2, the estimated sensitivity of 0.80 for *Fh*ES is derived from the ratio of 8 true positive cases identified by the chosen cutoff point for *Fh*ES optical density, divided by the 10 positive cases identified by the gold standard method (liver inspection). Also, we checked all the estimated diagnostic measures we presented on Tables 2 and 4 and Supplementary Table 2, as well as their confidence interval estimation. As mentioned on item 3, the wider confidence interval for sensitivity measure is expected due to the rare disease occurrence on the studied samples. Also,

we include a link to an open access repository on the current version of the manuscript where the script used in the data analysis was made available.

13. Table 4. ***Current:*** "…0.79(0.60;0.90)" / ***Suggestion:*** "Recalculate all the ranges of CI, e.g (0.79 (0.75–0.82)."
RESPONSE: thank you for mentioning this. We apologize for the lack of clarity in the description of the estimated diagnostic measures, presented on Table 4 for cattle from farms. We correct the CI presentation as suggested by the Reviewer and added information regarding numerator and denominator of the estimated diagnostic measures. Please, see the answer presented in the previous item (item 12).

14. General comment. ***Current:*** "The number of samples differ. You have 500 in initial stage but you are reporting 433"/ ***Comment:*** "The samples collected should all be analyzed. Where are other 67 samples?"
RESPONSE: thank you for mentioning this. We apologize for the lack of clarity in the description of the samples used in this research. We reiterate that improvements were made in the writing of the methods section to overcome this issue (lines 123-143). Please, see the answer presented in the items 9, 10 and 11.

15. General comment. ***Current:*** "Only 139 serum and 500 feacal samples"/ ***Comment:*** "For serum samples you analysed only 139 why not analysed serum from all 500 animals so that you compare the results for feacal examination and serology results?"
RESPONSE: thank you for mentioning this. We apologize for the lack of clarity in the description of the samples used in this research. We reiterate that improvements were made in the writing of the methods section to overcome this issue (lines 123-143). Please, see the answer presented in the items 9, 10 and 11.

Generally, the research is okay but needs major revision.

1.The number samples are confusing there 500 then 433 and 139. The number of samples should the same or tallying.
RESPONSE: thank you for mentioning this. We apologize for the lack of clarity in the description of the samples used in this research. In the method section, the subsection entitled "Characteristics of the cattle included in the study" has been revised and restructured (lines 123-143) aiming to separately describe the two studied cattle groups, the one from farms and the other from abattoir.

2. The Number of positive should indicated and in brackets you put the positive percentage eg 111/139 (80%) unlike only 80% or 0.80. This can be misleading to some readers.
RESPONSE: thank you for mentioning this. We revised Tables 2 and 4 and Supplementary Table 2 aiming to add the numerator and denominator of the estimated diagnostic measures. Please, see the answer presented in the item 12.

3. The Confidence interval should be recalculated it is misleading or misreading. This is a range 95% CI 80% (72-85%) OR 95% CI 0.80 (0.72-0.85) OR 95% CI 0.80 (0.72; 0.85)

RESPONSE: thank you for mentioning this. We correct the CI presentation as suggested by the Reviewer. Also, we checked all the estimated diagnostic measures we presented on Tables 2, 4 and Supplementary Table 2, as well as their confidence interval estimation. Please, see the answer presented in the item 12.

We thank the reviewer for their helpful suggestions that improved the message of our manuscript. Please find in below our point-to-point answers and commentaries on some text improvements we have made. In the revised version of our manuscript, major changes/insertions are outlined in red.

Independent Review Report, Reviewer 1

1. Line 21. ***Current:*** "cattle fasciolosis"/ ***Suggestion:*** "chronic fasciolosis".
RESPONSE: thank you for mention this. We replaced the term as suggested by the Reviewer.

2. *Fasciola gigantica* is more prevalent in Kettle, which species of *Fasciola* is used in this article? How is the *Fasciola* species confirmed?
RESPONSE: thank you for pointing this out. *Fasciola hepatica* is most prevalent in America. In our study, we employed both native and recombinant *F. hepatica* antigens.

For the native antigens, *F. hepatica* specimens was collected from a local cattle abattoir. The classification of these parasites as *F. hepatica* was based on morphological characteristics and parasite size during the veterinary inspection (lines 127-132).

In contrast, for the recombinant antigen, a genetic sequence encoding the cathepsin of *F. hepatica* retrieved from a genetic sequence bank was utilized (lines 181-187).

3. Lines 131–144. How did you differentiate the *Fasciola* species in Cattle? *Fasciola gigantica* is more common in cattle. How did you make a definitive diagnosis of *Fasciola hepatica*?
RESPONSE: thank you for bringing this to our attention. *F. hepatica* occurs more frequently in America. For the native antigens, the identification of *F. hepatica* was based on the morphological characteristics of the parasite. For the recombinant antigen, a genetic sequence coding for the *F. hepatica* antigen was used. The genetic sequence is described in the materials and methods of the manuscript (lines 181–187).

4. Line 223. In which temperature and condition? For TMB the time is 15-20 min. For your research, the color will be dim and therefore light absorption will be low.
RESPONSE: thank you for pointing this out. This sentence was rewritten (lines 220–223). After incubation at room temperature in the dark for 10- 20 min, the reaction was stopped with 50µL of 0.1 M sulfuric acid.

The sentence has been revised to reflect adjustments made in accordance with the ELISA protocol utilized. In light of TMB's photosensitivity, rigorous measures were instituted to ensure the accurate execution of the ELISA protocol. Detailed procedures have been outlined in the methods section of the manuscript.

Regarding the absorbance values highlighted by the reviewer, our hypothesis posits a plausible correlation between antigen composition and the observed variations in absorbance values. The heterogeneous protein composition of native antigens suggests a propensity for higher absorbance values. Conversely, the singular nature of the recombinant antigen may contribute to comparatively lower absorbance values.

5. Line 263. The purification of *Fh*SA, *Fh*ES, and *Fh*rCL-1 proteins was shown on SDS-PAGE.
RESPONSE: thank you for bringing this to our attention. We conducted an analysis of the purification of both native and recombinant antigens using SDS-PAGE; data are not shown. We followed the purification methodology described in the methods section, lines 172-177 and 196-199 for the native and recombinant antigens, respectively.

6. Line 281. … positive results were false.
RESPONSE: thank you for pointing this out. This sentence was rewritten (lines 282 - 285). Since the fasciolosis prevalence is low (10 positive cases in 139 cattle), we observed a large number of false positives and consequently a low PPV: only 8 of the 33 positive results were true positive, suggesting the serological tests cannot be used to confirm the presence of the disease.

7. Line 308. Overall, these results….
RESPONSE: thank you for pointing this out. This sentence was rewritten. Overall, these results show that samples positive for fasciolosis presented higher absorbance values (lines 329 - 330).

What sera were used for check the cross reaction? The sera of patients with hydatidosis, taeniasis, toxocariasis, etc. have a cross-reaction with fascioliasis. Especially in sheep and cattle.
RESPONSE: we appreciate this comment. During the cattle screening process on farms, coprological examination was used to screen the fecal samples for the presence of parasites eggs. The results of the coprological examination revealed the presence of *F. hepatica* eggs and other parasites, with some samples demonstrating concomitant infections. For the analysis presented in the result section, conducted in order to determine the cutoff points and diagnostic performance measures (sensitivity, specificity, positive and negative predictive values) of the serological tests (Tables 3 and 4 and Figure 2), samples positive for other parasites than *F. hepatica* were excluded (n=67).

Subsequently, these samples were used to explore their absorbance values in relation to the cutoff points established for the serological tests. Furthermore, sensitivity analysis was carried out by adjusting models that considered all samples (those only diagnosed with other parasites were classified in the negative group and those also diagnosed with *F. hepatica* in the positive group), with the aim of evaluating possible changes in the tests performance measures (Supplementary Tables 1 and 2 and Supplementary Figure 1). The description of the positive samples (for fasciolosis and/or other parasites) is now described in detail in lines 292-299.

The reviewer raised a pertinent issue concerning the assessment of cross-infection with other parasites. The utilization of serum samples from animals harboring other parasites is deemed ideal for evaluating cross-infection. Our group is just starting research into diagnostic tools for cattle fasciolosis, and we don't have serum samples from cattle that are positive for other parasites. In addition, COVID-19 has impacted the development of our research. The expectation within our laboratory is to obtain bovine

serum samples demonstrating positivity for other parasites and incorporate them into the sample panel for the development of a lateral flow assays.

8. Table 1. Add OD.
RESPONSE: thank you for mention this. We added the OD term in Table 1 as suggested by the Reviewer.

9. Table 2. Add OD.
RESPONSE: thank you for mention this. We added the OD term in Table 2, in the line correspondent to the cutoff value of each evaluated serodiagnosis test. The other information are diagnostic performance measures.

10. Table 3. Add OD.
RESPONSE: thank you for mention this. We added the OD term in Table 1 as suggested by the Reviewer.

11. Table 4. Add OD.
RESPONSE: thank you for mention this. We added the OD term in Table 4, in the line correspondent to the cutoff value of each evaluated serodiagnosis test. The other information are diagnostic performance measures.

General comment.
The following paragraph should be rewritten. The results show whether it is in the stool or in the serum. Please rewritten clearly. It is difficult to distinguish the eggs of Fasciola species by microscopic observation. How was the diagnosis made?
“We found 405 negative and 95 positive results for eggs in the fecal samples. Of 132 the 95 positive animals, 38 (7.6%) were positive for *F. hepatica*, 28 for *F. hepatica* eggs only, and 10 for *F. hepatica* and other parasites: seven also contained eggs of strongylid genera, two *Eimeria* eggs, and one *Strongylidae* and *Eimeria* eggs. The examination also showed that 44 (8.8%) cattle were positive for strongylid genera only and 13 (2.6%) for both strongylid genera and *Eimeria*. Animals positive for other parasites than *F. hepatica* were excluded from the diagnostic performance evaluation of native and recombinant antigens. A total of 139 serum samples were collected from a cattle abattoir located in southern Santa Catarina. The presence of cattle fasciolosis was determined through liver inspection. According to this approach, 10 (7.2%) cattle were diagnosed with *F. hepatica*, with no other parasites investigated during the veterinary inspection.”
RESPONSE: we appreciate this comment and apologize for the for the lack of clarity in the description of the samples used in this research. We rewritten this paragraph, as following: “The coprological examination resulted in 405/500 (81%) negative and 95/500 (19%) positive results. Of the 95 positive results, 28/500 (5.6%) were positive only for *F. hepatica* eggs, and 10/500 (2%) for *F. hepatica* and other parasites: 7/500 (1.4%) also contained *Strongylidae* eggs, 2/500 (0.4%) *Eimeria* eggs, and 1/500 (0.2%) *Strongylidae* and *Eimeria* eggs. The examination also showed that 44/500 (8.8%) cattle were positive only for *Strongylidae* eggs and 13/500 (2.6%) for both *Strongylidae* and

Eimeria eggs. Animals positive for other parasites than *F. hepatica* (n = 67) were excluded from the diagnostic performance evaluation of native and recombinant antigens described below" (lines 292-299 in the results section).

The detection of *F. hepatica* eggs in fecal samples was conducted by proficient laboratory technicians employing the sedimentation protocol. Each sample underwent diagnostic assessment in triplicate, as outlined in lines 136-143 of the methods section.

The section of the manuscript titled "Characteristics of the cattle included in the study" has been revised and restructured. Subtitles have been added to elucidate the number of samples and cattle groups we studied as well as the methodology employed to conduct the comparison with gold standard methods for the diagnostic performance estimation of the ELISA tests.

Re: Spectrum00095-24R1 (Diagnosis of fasciolosis antibodies in Brazilian cattle through enzyme-linked immunosorbent assay (ELISA) employing both native and recombinant antigens)

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