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Immunodiagnosis Assay and Cytokine Response in Naturally Fasciola-Infected Cattle in the Western Highland of Cameroon

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Abstract

FASCIOLIASIS caused by *Fasciola* is commonly reported in cattle. Various studies have evaluated the use of somatic antigen for immunodiagnosis of fascioliasis, but the results on the performance remain not clear. So, the present study was conducted to evaluate the performance of IgG-Enzyme-linked Immunosorbent Assay (ELISA) using total somatic antigens of *Fasciola* to diagnose fascioliasis and the effect of regulatory cytokines in cattle. A total of 428 serum samples from *Fasciola* spp infected cattle following the postmortem examination (coprology and liver incision) were tested for the performance of IgG-ELISA. For cytokines analysis, 150 serum samples (87 seropositive and 63 seronegative) were examined. Out of 428 samples, only 87 were diagnosed positive using the IgG-ELISA, given a performance of 20.32%. Season, sex and age did not show significant influence on the seropositivity. However a high IgG level was observed in rainy season than dry season ($p < 0.0009$). Seronegative *Fasciola* infected cattle had highly elevated serum transforming growth factor-beta (TGF-β) ($p<0.0001$), low serum Interleukin (IL)-10 (IL-10) ($p < 0.0001$), but no difference in serum interleukin (IL)-4 ($p = 0.62$) level. Furthermore, there was significant ($p<0.0001$) high level ratio of TGF-β/IL-4 in seronegative. The ratio TGF-β/IL-4 was negatively correlated with IgG level in seropositive *Fasciola* spp. infected cattle. It was concluded that overall somatic antigen did not show a convincing diagnosis of fascioliasis and this low performance may be associated with changes in TGF-β and IL-10 levels.

Keywords: ELISA, Cattle, Cytokines, Fasciola, Seroprevalence.

Introduction

Fascioliasis is an important helminth infection which is caused by *Fasciola hepatica (F. hepatica)* and *Fasciola gigantica (F. gigantica)* [9]. It is a parasite of domestic animals, wild animals and human but is most important in cattle and sheep [5]. Fascioliasis is one of the most prevalent helminth diseases throughout the world [10]. The number of grazing animals at the risk of fascioliasis exceeds 700 million worldwide [10]. *F. hepatica* and *F. gigantica* are two commonly reported liver flukes that cause fascioliasis in ruminants and human. *Fasciola* spp. infections are usually asymptomatic and disease can appear anywhere from a few days to several years after infection thereby making the diagnosis challenging [4]. Due to numerous factors, an interest in the development of an immunological method to control the disease has risen during the last few years. Despite efforts, the search for an effective diagnosis to control the disease has remained slow. Among some of the reasons of this, there may be the variation of antigen and the different mechanisms by which the parasite modulates the host immune response and affects the production of antibodies.

Like for other helminthes, studies have demonstrated that *Fasciola* parasites are able to down regulate the Th1 immune response and upregulate the Th2 response [12, 3, 6]. This may be through regulatory cytokines and/or suppress inflammatory responses. The modulation of cytokines such as IL-10 and TGF-β are common strategy used by parasites to evade immune response. Because of of this regulation, the production of antibodies is decreased and become insignificant. A lot of surveys have been conducted in this regard,

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but the results remain controversial [14, 1] and did not provide standard results on its sensitivity. It is therefore of crucial importance to search for serodiagnosis and study the host's immune mechanisms that may reduce the sensitivity. The aim of this study was to evaluate an IgG-ELISA performance using somatic of *Fasciola* parasites and the role of serum TGF-β, IL-10 and IL-4 levels in *Fasciola* infected cattle.

Material and Methods

Study area and study period

The study was carried in the Western Highlands of Cameroon agro-ecological zone that covers the entire territory of the Northwest and West Regions of Cameroon. Animals was selected at Bamenda abattoir in Bamenda the Regional capital city of the North West Region (latitudes 5˚ 45" and 9˚ 9"N; longitudes 9 ˚13" and 11˚ 13"E), and Bafoussam abattoir in Bafoussam, the metropolitan city of the West Region (latitudes 5°26' and 5°30' North and longitudes 10°20' and 10°30' East). Data were collected from 255 cattle in March to October (during the rainy season) and 173in November to February (dry season).

Blood sample collection

This study was carried on 428 Fasciola spp. infected cattle **(**195 male or 233 female) following the analysis of coprological analysis (Faecal examination using the formol-ether concentration technique) and detection of flukes after incision. Serum was obtained from blood samples after keeping the blood for 24 hr at the room temperature. Collected serum samples were used for the detection of IgG antibodies and cytokines namely TGF-β, IL-10 and IL-4.

Preparation of crude somatic proteins

Flukes collected were used to prepare the somatic proteins as described by Dar et al.[7]. Briefly, worms obtained by incision of liver of naturally infected cattle from abattoir in Bamenda were washed. Five (5) flukes were macerated in 5 ml of ice cold lysis buffer (0.01 M phosphate buffer saline (PBS), pH 7.2, containing 10 mM Tris–HCl, 150 mM NaCl, 0.5 % Triton X100, 10 mM EDTA, 1 mM PMSF and 100 µL of cocktail of protease inhibitors) using tissue homogenizer. The homogenate was centrifuged at 16000 × g for 30 minutes. Homogenization was carried out for 10 minutes at 1300 rpm taking pause of 2 minutes after every one minute of homogenization. The supernatant was collected as crude FhTA antigen and stored at -20^{o}C in 1 mL aliquots.

Detection of IgG antibody

All serum samples were analysed by IgG-ELISA for total antibody responses against somatic antigen of Fasciola spp. The assay was carried out using microplates titer (NUNC). In details, after optimization by checkerboard titration, 96-well flat-bottom polystyrene ELISA plates (Costar) were sensitized with 10 µg/mL of *Fasciola* crude antigen (100 µL/well) in 0.1 M carbonate buffer (pH 9.6) at 4°C overnight. The plates were washed 3 times with PBS containing 0.05% Tween 20 (PBST) and non-specific binding sites were blocked by addition of 100 mL of 1% BSA to each well for 1 hr at 37°C. After the blocking solution was removed by washing (four times), the plates were incubated for 1 hr at 37°C with serum (100 µL/well). After a new cycle of washing with PBST, 100 µL of peroxidase-conjugated anti-bovine IgG diluted 1:5000 in blocking solution was added to each well and then incubated for 1 hr at 37°C. The plates were then washed with PBST and then incubated in a chromogen-substrate solution of O-phenylenediamine dihydrochloride (OPD) (100 µL/well) for 30 min at room temperature (RT) in the dark. The reaction was stopped with 12.5% HCl (50 µL/well), and the absorbance was measured at 492 nm using a microplate ELISA reader after 30 min incubation at room temperature [4].

From the absorbance values of the serum samples from negative control, means and standard deviation (SD) were calculated. OD cut-off was calculated by addition of 2SD to mean of OD values of the calf fetal serum. The negative control serum samples were parasite free samples (fetal serum). Values above this cut-off were recorded as positive and below and/or equal as negative. Samples were tested in duplicate.

Quantification of some serum cytokines profile

Serum levels of IL-4, IL-10, and TGF-β were determined using reagents obtained from Ebioscience (USA) were used for the assay. For each cytokine, a microplate (NUNC) was coated overnight with the specific antibody. The following day, 10 µL of samples or control were added to well, sealed and incubated for 2 hr at RT. Plates were washed by filling each well with 400 µL of wash Buffer and decanted by inverting the plates and blotting against clean paper towels. Thereafter, 200 µL of peroxidase-conjugated antibody (detection antibody) and incubated at RT for 2 hr. Microplates were washed again as previously described and 200 µL of substrate (chromogen-substrate solution of O-phenylenediamine dihydrochloride (OPD)) was added to each well and incubated at RT for 20 min. The reaction was stopped by adding 50 µL of stop solution to each well and the optical density was read 450 nm after 30 min [11].

Statistical analysis

Statistical package for social sciences (SPSS) version 23 was used to determine the differences in proportions using Pearson's Chi-Square and to determine the odds ratio. Quantatitive data (OD and cytokine level) were expressed into means ± error and the difference between the groups was determined using by t test using Graph path Prism 7.0 version. XLSTAT 2016 was used to determine the correlation (r) between cytokine and absorbance and regression (R^2). P-value' < 0.05 is considered statistically significant.

Results

Prevalence of IgG seropositivity antibody in Fasciola spp. infected cattle.

The results of ELISA carried out with somatic antigen for detection of IgG in serum from the cattle with flukes or eggs following the seasons are summarized in Table 1. Using somatic antigens for detection of IgG antibody in serum of negative animals, a cut-off OD value obtained for the ELISA was 0.00015. Out of 428 cattle, 20.3% (87/428) were detected positive for IgG antibodies. The seropositivity was 26.01% (45/173) in dry season compared to 16.47% (42/255) in rainy season and did not show significant difference (X^2 = 22.56; p = 0.657).

The results of ELISA for detection of anti-Fasciola IgG antibody carried out with somatic antigen following the phases of the infection as classified based on the presence of eggs and season. In animals with chronic infection, 20.8% (39/404) sera were positive for the antibodies test, while 12.5% (3/24) of animals with acute infection were positive. It was observed that there is no significant difference in seropositivity among animal with chronic infection in dry and rainy seasons (p =0.815).

Influence of season, sex and age on IgG seropositivity in Fasciola spp. infected cattle.

Table 2 summarized the results of IgG seropositivity of serum in Fasciola spp infected cattle with respect to the sex. The ELISA using somatic antigen demonstrated high positivity of 34.5% in male compared to 21.1% in female in dry season, while during the rainy season high positivity was found positivity in female (19.3%) than in male (13.7%). In addition, the results of the tests for detection of antibody by ELISA using somatic antigens are correlated with the age of animals positive following the parasitology diagnosis. A higher frequency of obtaining a positive IgG-antibody titer in the ELISA using somatic antigen could be observed among animals of 8 to 10 years in dry season (32.9%) as well as in rainy season (21.8%).

Serum antibody level in relation to the season and sex in Fasciola spp. infected cattle

The relative OD492 values, which are proportion to the quantity of antibody in the sera were considered for demonstration of antibodies level. The scatter plots (Fig. 1) depict the relative OD492 values of the ELISA for demonstration of antibodies in serum. In relation to the season (Fig. 1A), the ELISA using somatic antigen detected antibodies in sera from animals with eggs or flukes in the liver in dry season and rainy season with different means, even do the mean OD value was close to the cut-off. The mean OD value was distinctly lower in dry season compared to that in the rainy season (P = 0.0009).

Independent to the season (Fig. 1B), there was no difference between the mean OD value of the assay using somatic antigen in infected females and males (t=0.156; p = 0.8757). Also, there is no difference between the OD492 values of the ELISA females and males (t=0.019; p = 0.98; Fig. 1C) and in the dry season (t=0.25; p = 0.79; Fig. 1D).

Serum antibody level in relation with age in Fasciola spp. infected cattle

The scatter plots (Fig. 2) present the relative OD492 values of the ELISA for demonstration of antibodies in positive sera in relation to the age of the animals and season of collection. The results showed that the mean OD value of the assay using somatic antigen in infected animals with different ages showed independent to the season that the mean of OD value is lower in animals of 8 to 10 years compared to those of 5 to 7 years (Fig. 2A), but there is no difference between the different groups of age (F (2, 84) = 1.608; p = 0.20). In the rainy season, there is significant difference between the groups of age (t = 2.231; p = 0.031). The mean of OD value is close to the cut-off and lower in animals of 8 to 10 years compared to those of 5 to 7 years (Fig. 2B). While, in the dry season, the results showed that the mean of OD value is lower in animals of 5 to 7 years compared to those of 8 to 10 years (Fig. 2C), but there is no significant difference between the groups of age (F (2, 42) = 1.472; P = 0.241).

Serum IL-10, TGF-β and IL-4 levels in Fasciola spp. infected cattle

Serum levels for IL-10, TGF-β and IL-4 are shown in Fig. 3. The seropositive group showed higher IL-10 level than the seronegative group. This increase showed a significant difference (P< 0.0001) between the groups (Fig. 3A). Serum level for TGF-β was higher in seronegative group compared to the seropositive (Fig. 3B), with a significant difference

($P < 0.0001$). In contrast, there were no differences between serum level of IL-4 in seropositive group and the seronegative group ($p > 0.62$; Fig. 3C). It is well known that Tregs produce not only TGF-βs but also IL-10, and the balance between the production of TGF-βs and IL-10 is important for the regulation of humoral immune homeostasis. The results showed the TGF-β/IL-10 was higher in seronegative group than in seropositive group ($P< 0.0001$; Fig. 3D). Besides, the results indicated the seronegative group showed higher ratios IL-4/IL-10 than seropositive ($P< 0.0001$; Fig. 3E) while the ratio IL-4/TGF-β was higher seropositive group than in seronegative group ($P< 0.0001$; Fig. 3F).

There was a positive correlation between the absorbance characterizing the antibody level and serum IL-10 ($r = 0.35$), TGF-β ($r = 0.079$) and IL-4 ($r = 0.009$) level for the seropositive group, while the correlation found between OD and the Ratio TGF/IL-10 ($r = -0.219$), Ratio IL4/IL-10 ($r = -0.22$) and Ratio IL4/TGF-β ($r = -0.095$) showed a negative correlation for seropositive group. However, the results did not show significant relation between the absorbance and serum IL-10, TGF-β and IL-4 group, as well as between OD and the ratio TGF-β/IL-10, Ratio IL4/IL-10 and Ratio IL4/TGF-β (Fig. 4).

Discussion

In Cameroon, during the last century, the western highlands part of the country was a hyperendemic area for *Fasciola* infection in cattle. The actual diagnosis is only based on coprology and detection of flukes in liver. However, this diagnosis is not systematic, notably incision is a postmortem test to evaluate the quality of meat. Thus, the serological tests that can be applied to living are another technique to prevent chronic infection. Antibodies' detection therefore will permit systematical investigation in a herd of cattle. Additionally, cytokines analysis emphasizes on how the infestation of *Fasciola spp*. affects the quality of meat and meat products or how the infection affects the immune response. Immunodiagnosis of *Fasciola,* IgG-ELISA, has been evaluated by different investigators [14, 1] using crude excretory protein as antigen to indicate the possibilities of immunodiagnosis. In the present study the sera of *Fasciola* infected cattle were tested using the somatic antigens. The results showed a seroreactivity of 20.32% (87) out of 428 *Fasciola* infected cattle. In contrast to the results obtained by Drescher et al. [8], the results here presented risk of an underestimation of prevalence using somatic antigens (low sensitivity, less specific and little reliable). But, this can be justified by species of *Fasciola* used to prepare the antigen, as only species of *Fasciola* from Bamenda was used. Moreover these results demonstrate that parasite may change the somatic proteins during its development. Furthermore, the state of infection can be one of the reasons to justify the low sensitivity of the test. It has demonstrated that the cytokine profile of infected individuals depends on *Fasciola* infection state (acute, subchronic or chronic) [13]. High production of some cytokine, in synergy or not, can inhibit the production of antibodies and reduce the sensitivity of an immmunodiagnosis [9].

TGF-βs and IL-10, potent inhibitory cytokines, have been investigated for their regulatory potential in humoral response [11]. Cattle naturally infected with *Fasciola* and seropositive showed high level of IL-10 in serum compared to seronegative infected cattle, suggesting a role of this cytokine in modulating the host response facilitating the parasite survival in the host. The results of the present study revealed that the role of increases in IL-10 modulating the host response is relevant in the infection. The result of this study is consistent with that reported by Pacheco et *al*. [13], who demonstrated a significant increase in the gene expression of IL-10 in liver from infected sheep during early stages of *F. hepatica* infection. Moreover, the results of the present study revealed that compared with the seronegative samples, the seropositive cattle infected with *Fasciola* showed a significantly high TGF-β level in the serum. This result agrees with that reported by Nasreldin and Zaki [11], who showed high IL-10 and TGF-β in cattle with a liver fluke. IL-10 and TGF-β are T-regulatory cytokines that are important in regulating the immune response, and they are also important in minimizing the pathology and boosting tissue repair during helminth infections. Also TGF-β has described in promotion of collagen production and fibrosis, by this means permit the encapsulation of the liver flukes, thereby limiting the evasion of parasites into the hepatic parenchyma and increase host resistance to Fasciola spp. TGF-β is also known to induce SMAD signaling, which plays an important role in fibrosis progression. In sheep, researchers have revealed increases in the expression of several genes (SMAD3, SMAD4, COL1A1) associated to TGF-β and linked with evidence of fibrosis-associated cell types [13]. The concentration of serum level of IL-4 did not show significant difference, while a significant low ratio TGF-F/IL-10 and ratio IL-4/IL-10 were noted here in seronegative cattle infected with *Fasciola spp.* This low ratio TGF-F/IL-10 and ratio IL-4/IL-10 may be associated with the seronegativity of infected cattle. TGF-β alone and particularly in synergy with IL-10 is also linked with low antibody production, and this could be in consistence with the results of Komai et al. [9], who demonstrated that TGF-β and IL-10 inhibited the proliferation and antibody production of LPS-

stimulated B cells. Consistently, studies reported that total IgG production under stimulation with IL-4 was suppressed by TGF-β1 or TGF-β3. Furthermore, it is demonstrated that TGF-β1 or TGF-β3 suppressed the differentiation of B220+CD138+ plasmablasts in anti-CD40- and anti-IgM-stimulated B cells [3]. Therefore, the high level of TGF-β could take as one the causes of seronegativity of serum from *Fasciola* infected cattle. The analysis of the correlation showed a negative correlation between the absorbance and ratio TGF-β/IL-10, demonstrating the negative of TGF-β. However, this relationship is not significant suggesting that there are other factors that may contribute to the reduction of IgG production. Therefore, the high production of TGF-β in seronegative *Fasciola* infected cattle suggests that *Fasciola* spp. induces a modulation of the host response in certain stages of infection to facilitate the parasite survival and inhibiting the production of antibodies.

Conclusion

Our study suggested that somatic components of fasciola can be potential candidate for a test to detect fasciola infection in cattle. Age, sex and season may not have influence on the sensitivity of the IgG-ELISA test using somatic antigens in cattle. However, identify of the specific component that will be used and that can differentiate acute from chronic infection may be more suitable for the evaluation of serodiagnosis as a target for anti-fasciola antibody detection. Besides, the high level of TGF-β in seronegative animals suggests that fasciola parasite induces a modulation of the host response which may reduce the sensitivity. We therefore recommend the consideration of the somatic components for search and/or evaluation of serological diagnostic methods for facioliasis. Further studies to identify, purify, and produce the immunoreactive somatic protein are highly recommended. Besides, the fact that our study did not include in the evaluation serum samples from non-infected individuals represents one limitation of this study.

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Declaration of conflict of interest

Authors of this manuscript declare that they have no conflicts of interest.

Funding statement

Not applicable

Ethical approval

This study was carried out with the authorizations obtained from the Ministry of Livestock Fisheries and Animal Industries (MINEPIA/DREPIA/SRAG/NW/40/86 of 31/03/2022). An authorization was also obtained from the head of the abattoir (No: 026/22/AS/MINEPIA/SG/DREPIA-O/SRAG of 13th July 2022).

TABLE 1. Prevalence of IgG seropositivity in *Fasciola spp.* infected cattle following the parasitological diagnosis in dry and rainy seasons

Parasitological Diagnosis	Season	Number of subjects	ELISA positive N (%)	P value
Total	Dry season	173	45 (26.01)	P= 0.657
	Rainy season	255	42 (16.47)	
	Total	428	87 (20.32)	
Coprology positive	Dry season	173	45 (26.01)	$p = 0.815$
	Rainy season	231	39 (16.9)	
	Total	404	84 (20.8)	
Liver incision positive only	Dry season	0	0 (0.0)	
	Rainy season	24	3 (12.5)	NA
	Total	24	3 (12.5)	

Coprology positive: animals with eggs and flukes in liver. Liver incision positive only: animals with only flukes in liver. Dry season: November to February; Rainy season: May to August; P value calculated using Ficher exact test (2-tailed). NA: p value is not able to be computed using Ficher exact test (2-tailed).

TABLE 2. Age and sex-related prevalence of IgG seropositivity in Fasciola spp. infected cattle in relation to parasitological diagnosis

	Parasitological Diagnosis	Rainy season		Dry season	
		Number of subjects	ELISA positive N (%)	Number of subjects	ELISA positive N (%)
Sex					
Female	Total	124	24 (19.3)	109	23 (21.1)
	Coprology positive	116	23 (19.8)	109	23 (21.1)
	Incision positive	8	1 (12.5)	0	0 (0.0)
	P value		NA		NA
Male	Total	131	18 (13.7)	64	22 (34.4)
	Coprology positive	115	16 (13.9)	64	22 (34.4)
	Incision positive	16	2 (12.5)	0	0 (0.0)
	P value		NA		NA
Age					
0 – 4 years	Coprology positive	2	0 (0.0)	2	1 (50.0)
	Incision positive	3	0 (0.0)	0	0 (0.0)
	Total	5	0 (0.0)	2	1 (50.0)
			NA		NA
5 – 7 years	Coprology positive	114	14(12.3)	95	18 (18.9)
	Incision positive	17	2 (14.3)	0	0 (0.0)
	Total	131	16 (12.2)	95	18 (18.9)
			NA		NA
8–10 years	Coprology positive	115	25 (21.7)	76	26 (34.2)
	Incision positive	4	1 (25.0)	0	0 (0.0)
	Total	119	26 (21.8)	76	26 (32.9)
			NA		NA

Coprology positive: animals with eggs and flukes in liver. Liver incision positive only: animals with only flukes in liver. NA: p value is not able to be computed using Ficher exact test (2-tailed).

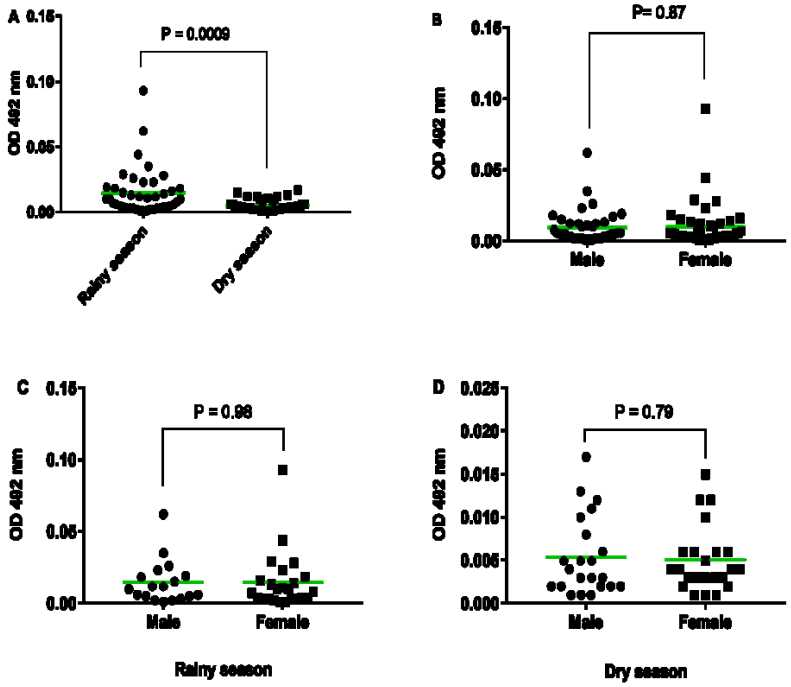

Fig. 1. Scatter plots showing OD492 values for ELISA results of antibodies in serum from Fasciola-infected cattle following the sex and season (A: season-related variation; B: sex-related variation during the 2 seasons; C: sex-related in rainy season; D: sex-related in dry season) . Bars on the plot show the mean values.

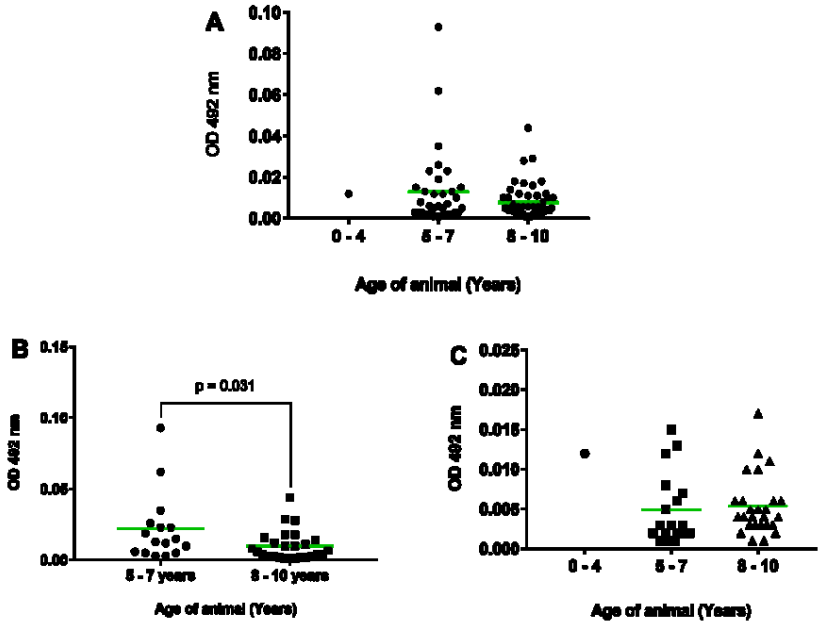

Fig. 2. Scatter plots showing OD_{492} values for ELISA results of antibodies in serum from Fasciola-infected cattle with respect to age and season(A: variation by age group during the 2 seasons; B: variation by age in the rainy season; C: variation by age in the dry season). Bars on the plot show the mean values.

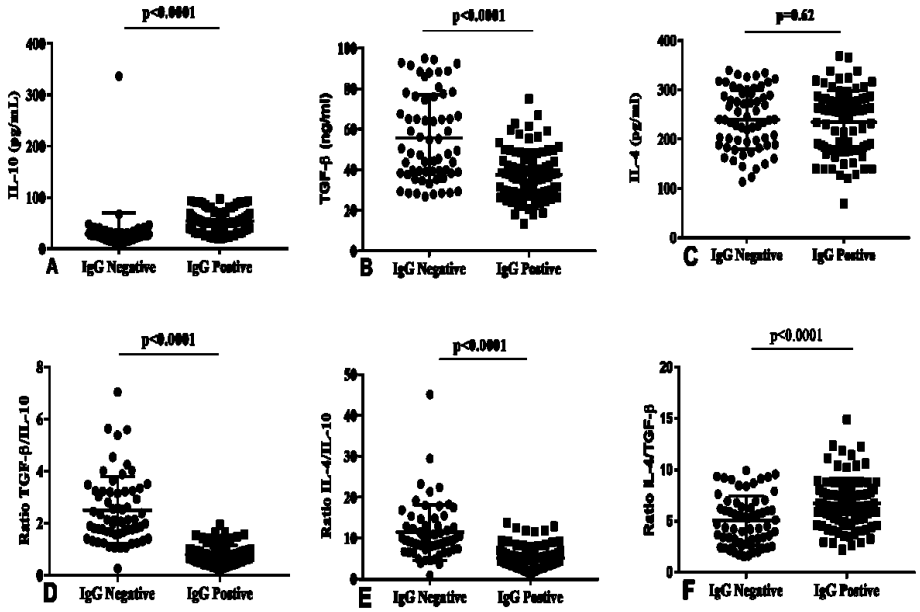

Fig. 3. Scatter plots outlining ELISA results of cytokine profiles (Mean ± S.E) for seronegative group (IgG negative) and seropositive group (IgG positive) in Fasciola -infected cattle (A. for IL-10; B. for TGF-β; C. for IL-4; D. for Ratio TGF-β/Il-10; E. for ratio IL-4/IL-10; F. for IL-4/TGF-β).

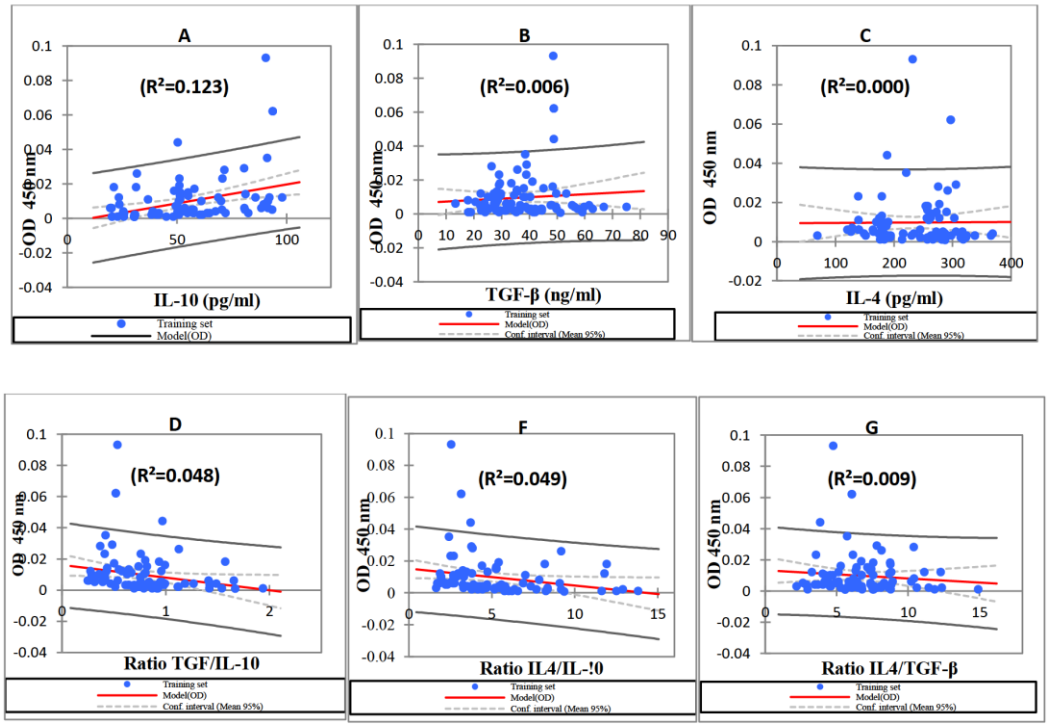

Fig. 4. Regression of OD_{492} value by IL-10 (A), TGF-β (B) IL-4 (C) levels, ratio TGF-β/Il-10 (D), ratio IL-4/IL-10 (E) and ratio IL-4/TGF-β (F) in seropositive *Fasciola*-infected cattle. OD = Optical density (absorbance), TGF-β = Transforming growth factor beta, IL- = Interleukin.

References

1. Abdolahi, K.S. and Sarkari, B. Detection of Fasciola hepatica and Fasciola gigantica common and uncommon antigens, using rabbit hyper immune serum raised against their excretory–secretory and somatic antigens. *Journal of Parasitic Diseases,* **40**, 1552–1557 (2016). DOI 10.1007/s12639-015-0726-5.

2. Ayele, M. and Hiko, A. Review on the Biology of Fasciola Parasites and the Epidemiology on Small Ruminants. *Journal of Biology, Agriculture and Healthcare,* **6**, 69 – 71 (2016).

3. Chung, J. Y., Bae, Y. A., Yun, D. H., Yang, H. J. and Kong, Y. Experimental murine fascioliasis derives early immune suppression with increased levels of TGFβ and IL–4. *Korean Journal Parasitology,* **50,** 301–308 (2012).

4. Gonzales, S. B., Dalton, J. P., Vasquez, C. F., Parkinson, M. and Ndao, M. The diagnosis of human fascioliasis by enzyme-linked immunosorbent assay (ELISA) using recombinant cathepsin L protease. *PLoS Neglected Tropical Disease,* **7**(9), e2414 (2013). doi.org/10.1371/journal.pntd.0002414.

5. Inegbenosun, C. U., Isaac, C., Anika, F.U. and Aihebholoria, O.P. Prevalence of intestinal parasites in animal hosts and potential implications to animal and human health in Edo, Nigeria. *Journal of Veterinary Science,* ***24***(1), e8 (2023). doi.org/10.4142/JVS.21211.

6. Ingale, S. L., Singh, P., Raina, O. K., Mehra, U. R., Verma, A. K., Gupta. S. C. and Mulik, S. V. Interferon-gamma and interleukin-4 expression during *Fasciola gigantica* primary infection in crossbred bovine calves as determined by real–time PCR. *Veterinary Parasitology,* **152**, 158–161 (2008).

7. Dar, J. S., Ganai, B. A., Shahardar, R. A. and Zargar, U. R. Molecular Characterization and Immunodiagnostic Potential of Various Antigenic Proteins of *Fasciola Gigantica* Species Isolated from Sheep of North West Himalayan Region. *Helminthologia*, ***56***, 93–107 (2019). doi.org/10.2478/helm-2019-0003.

8. Drescher, G., Vasconcelos, T. C. B., Belo, V. S., Pinto, M. M. d G., Rosa, J d. O., Morello, L. G. and Figueiredo, F. B. Serological diagnosis of fasciolosis (Fasciola hepatica) in humans, cattle, and sheep: a meta-analysis. *Frontiers in Veterinary Science,* **10**, 1252454 (2023). doi: 10.3389/fvets.2023.1252454.

9. Komai, T., Inoue, M., Okamura, T., Morita, K., Iwasaki, Y., Sumitomo, S., Shoda, H., Yamamoto, K. and Fujio, K. Transforming Growth Factor-β and Interleukin-10 Synergistically Regulate Humoral Immunity via Modulating Metabolic Signals. *Frontiers in Immunology,* **9**, 1364 (2018). doi: 10.3389/fimmu.2018.01364.

10. Mas-Coma, S., Bargues, M. D. and Valero, M. A. Fascioliasis and Other Plant-borne Trematode Zoonoses. *International Journal for Parasitology,* **35**, 1255- 1278 (2005).

11. Nasreldin, N. and Zaki, R. S. Biochemical and immunological investigation of fascioliasis in cattle in Egypt. *Veterinary World,* **13**, 923-930 (2020). doi.org/10.14202/vetworld.2020.

12. Pacheco, I. L., Abril, N., Morales‐Prieto, N., Bautista, M. J., Zafra, R., Escamilla, A., Ruiz, M. T., Martínez‐Moreno, A. and Pérez, J. Th1/Th2 balance in the liver and hepatic lymph nodes of vaccinated and unvaccinated sheep during acute stages of infection with Fasciola hepatica. *Veterinary Parasitology,* **238**, 61–65 (2017).

13. Pacheco, I. L., Abril, N., Zafra, R., Molina‐Hernández, V., Morales‐Prieto, N., Bautista, M. J., Rui z‐Campillo, M. T., Pérez‐Caballero, R., Martínez ‐Moreno, A. and Pérez, J. Fasciola hepatica induces Foxp3 T cell, proinflammatory and regulatory cytokine overexpression in liver from infected sheep during early stages of infection. *Veterinary Research,* **49**, 56 (2018). doi.org/10.1186/s13567-018-0550-x

14. Pfister, K. Serodiagnosis of fasciolosis in ruminants. *Revue Scientifique et Technique/ Office International des Épizooties,* **9**, 511-518 (1990).