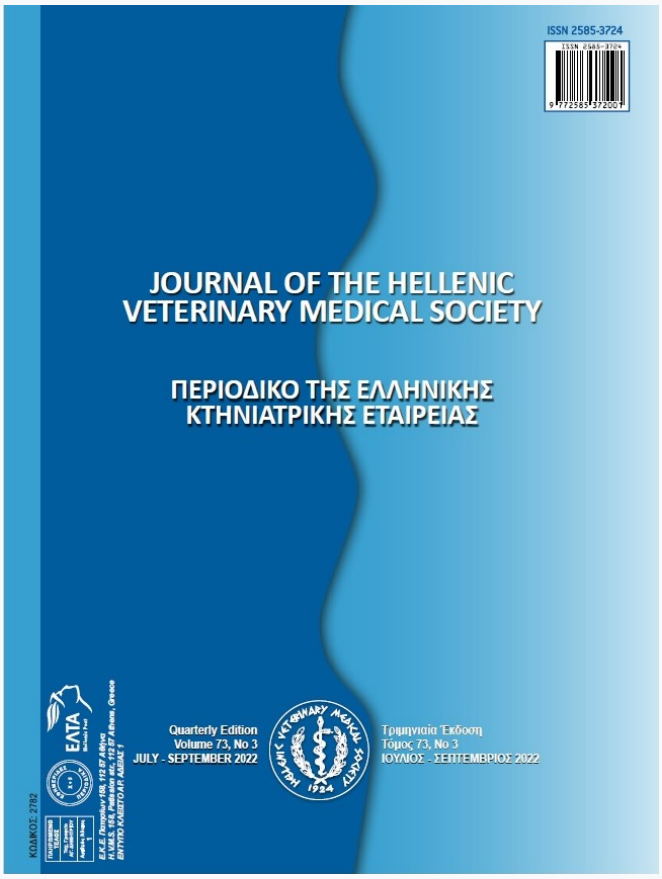
Journal of the Hellenic Veterinary Medical Society

Vol 73, No 3 (2022)



Seroprevalence of Fasciola-infection using Enzyme-linked immunosorbent assay in large ruminants from Pakistan

M Komal, K Afshan, S Zafar, S Firasat, M Qayyum

doi: 10.12681/jhvms.26436

To cite this article:

Komal, M., Afshan, K., Zafar, S., Firasat, S., & Qayyum, M. (2022). Seroprevalence of Fasciola-infection using Enzyme-linked immunosorbent assay in large ruminants from Pakistan. *Journal of the Hellenic Veterinary Medical Society*, *73*(3), 4309–4316. https://doi.org/10.12681/jhvms.26436

J HELLENIC VET MED SOC 2022, 73 (3): 4309-4316
ΠΕΚΕ 2022, 73 (3): 4309-4316

Research article
Ερευνητικό άρθρο

Seroprevalence of *Fasciola*-infection using Enzyme-linked immunosorbent assay in large ruminants from Pakistan

M. Komal[1], K. Afshan[1*], S. Zafar[1], S. Firasat [1], M. Qayyum[2]

[1] *Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan*

[2] *Department of Zoology and Biology, Faculty of Sciences, PMAS-Arid Agriculture University, Rawalpindi, Pakistan.*

ABSTRACT: Fasciolosis is a re-emerging neglected tropical zoonotic disease associated with endemic and epidemic outbreaks. Accurate diagnosis is crucial and antibody detection ELISAs based on native fluke excretory/secretory (ES), or somatic (SA) products are preferred due to their high sensitivity and specificity. The present study aimed to assess the validity of SA and ES antigens-based ELISAs for the detection of animal fascioliasis in the field. Adult flukes were collected and subjected to ES and SA antigen extraction. The polyclonal antibodies were produced by immunization of rabbits with SA and ES antigens of *Fasciola*. The results showed that the diagnostic accuracy of SA-based ELISA was 90.57%, with 95.45% sensitivity and 87.10% specificity values. The ES-based ELISA diagnostic accuracy was 92.13% with 100% sensitivity and 84.09% specificity values. The area under curve (AUC) value in the present study was 0.96 (95% CI: 0.912-1) and 0.869 (95% CI: 0.778-0.961) in SA and ES antigens, respectively. Fasciolosis was shown to be more common in buffaloes than cattle. Seropositive cases were found to be less in males as compared to females. The higher positive animals were detected in 8-10-year age groups compared to young animals. In conclusion, the present study established native SA and ES *Fasciola* antigen ELISA assay which makes them efficient screening tests in epidemiological surveys.

Keywords: Fasciolosis; Indirect ELISA; Pakistan.

Corresponding Author:
K.Afshan, Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan
E-mail address: kafshan@qau.edu.pk

Date of initial submission: 19-03-2021
Date of acceptance: 07-04-2022

INTRODUCTION

Fasciolosis is a neglected parasitic disease in domestic animals and humans with worldwide distribution (Kamel et al., 2015). *Fasciola* spp. is a parasite of great agricultural and economic importance not only in Pakistan but also in other tropic and sub-tropic regions (Shafiei et al., 2013; 2014). The number of human fasciolosis has been increasing in the last decades in a few countries, including Europe, Africa, America, Asia, and Oceania (Shafiei et al., 2015). *Fasciola*-infection incurs huge economic losses in animals that are about USD 3.2 billion worldwide (Shafiei et al., 2013; 2014). Immunological approaches have been considered as suitable methods for the early detection of fasciolosis (Khan et al., 2017). There are common and uncommon antigens in both their somatic antigens (SA) and excretory-secretory (ES) metabolites in *Fasciola* spp. Different antigenic fractions of *Fasciola* have been used for serological diagnosis of human fasciolosis (Kamel et al., 2013; Shafiei et al., 2013; 2014; 2015).

Serological and traditional parasitological detection methods of parasites have been evaluated, and circulating antigens and excretory-secretory products (ES) are considered the most useful serological tools (Khan et al., 2017). Enzyme-Linked Immunosorbent Assay (ELISA) has been used to detect serum antibodies to *Fasciola* antigens (Charlier et al., 2014). However, the sensitivity and the specificity of serological tests are affected by the circulating *Fasciola* crude and ES antigen (Attallah et al., 2013). Therefore, the present study was aimed to assess the validity of somatic and excretory-secretory antigens-based sero-ELISA for the detection of animal fascioliasis.

MATERIALS AND METHODS

Helminths and Blood Sampling

Animals (n=89) slaughtered at the Sihala abattoir were inspected for the presence or absence of liver flukes. The adult flukes were washed with 0.85% NaCl and divided into two groups for extraction of ES and SA antigens. The study was conducted by following the ethical guideline approved by the Ethical Committee of Quaid-i-Azam University Islamabad, Pakistan. Blood samples were obtained from control positive (n=45), and negative (n=44) animals confirmed with fecal/postmortem examination and from naturally infected cattle (n=177) and buffaloes (n=323) for field implementation of ELISA. The blood samples were centrifuged, sera were separated and kept at −20 °C until used for antibodies detection.

Preparation of whole worm antigens and generation of hyperimmune sera

Whole worm antigens from flukes were prepared by homogenizing them in 0.01 M phosphate-buffered saline, pH 7.2 (PBS) in a tissue blender followed by centrifugation at 4°C for 1 h at 10000 g. The PBS was supplemented with 10 mM leupeptin to inhibit proteases. Excretory-secretory antigens were prepared by incubating the worms (one worm per 5 ml) in leupeptin-supplemented PBS at 37°C for 3 h. The protein concentrations of the antigens were determined by the dye-binding procedure described by Bradford (1976) which is based on the use of Coomassie Blue. The supernatant was stored at -70 °C. Production of polyclonal antibodies to the ES and SA antigens of flukes was done as described previously by Afshan et al. (2021).

ELISA

ELISA was performed according to the method described by Murthy and Souza (2015) with some modifications. 100µl of SA and ES eluted antigens were mixed with 0.06 M carbonate buffer (pH 9.6) and coated in an ELISA plate overnight at 4°C. Washed the plates with 0.05% PBS-Tween 20, and blocked the unreacted binding sites with 0.5% skimmed milk in 0.5% PBS-Tween 20 for 2 hr. at 37°C. Following the incubation, the plates were washed and 100µl of sera obtained from naturally infected cattle and buffaloes were incubated per well at 37°C for 1 hr. Plates were washed and 100µl of goat anti-bovine IgG secondary antibodies (1:10,000), conjugated with alkaline phosphatase was added and incubated for 1 hr. at 37°C. The substrate used was pNPP (para-Nitrophenyle Phosphate) and the reaction was stopped by adding 50 µL of 2M NaOH. Absorbance values were read spectrophotometrically at 450 nm.

Statistical Analysis

The diagnostic sensitivity, specificity, accuracy, and predictive values were calculated by using Online Statistical Software MedCalc (https://www.medcalc.org/calc/diagnostic_test.php). Pearson's chi-square test was performed to compare prevalence among sex, age, and host categories. Statistical comparisons were carried out using SPSS 22.0 statistical software. Significance was defined as $P < 0.05$. The **receiver operating characteristic (**ROC) curve values were computed by using GraphPad Prism (version 9).

RESULTS

ELISA optimization and validation

The diagnostic performance of indirect ELISA on SA and ES antigens is provided in Table 1. The SA antigen ELISA results detected 4 sera as false positive (with 96.43% negative predictive value). The area under curve (AUC) values were 0.96 (95% CI: 0.912-1.00; P<0.0001) and an inverse relationship between sensitivity and specificity was observed (Fig. 1A). The diagnostic accuracy of the test was 90.57%, with 95.45% sensitivity and 87.10% specificity values. In ES antigen ELISA, 7 sera were detected false positive (100% negative predictive value) with AUC values of 0.869 (95% CI: 0.778-0.961; P<0.0001) shown in Fig. 1B. The ES-based ELISA diagnostic accuracy was 92.13% with 100% sensitivity and 84.09% specificity values.

Absorbance values were plotted on frequency histograms to visualize the separation between true positives and negative populations. The SA antigen ELISA results showed that 4 of the negative samples did not belong to the distribution and fell outside the cut-off point 0.4 (Fig 2A). Similarly, in ES antigen ELISA 7 of the negative samples fall outside the cut-off point (Fig 2B). To group the entire *Fasciola* negative samples the cut-off limit was set just below the positives at 0.9 OD units. The absorbance values of positive and negative controls were visualized in scatter graphs (Fig. 3AB), where higher OD of positive control was based on the interaction between antigens of *Fasciola* and antibodies.

Field Implementation of ELISA

The antibodies titers of *Fasciola*-infection associated with host type, sex, and age are given in figure 4AB. The mean anti-*Fasciola* IgG antibodies titer with SA-ELISA was detected 1.55 in 124/500 positive buffaloes, while 75/500 cattle were found positive with the mean OD values of 1.00. Fasciolosis was shown to be more common in buffaloes than cattle. Seropositive males were found to be 81/497 with mean OD of 1.01, and 118/497 in females with mean OD of 1.57. The higher positive animals 86/500 were detected in the 8-10year age group with mean OD of 1.3, while the lowest positive animals 28/500 were reported in the 2-4year age group with OD values of 2.08 (Fig.4A). Similarly, the anti-*Fasciola* IgG antibodies titer with ES-ELISA was detected higher in buffaloes (93/500), females (92/500), and among 8-10year age group (64/512) animals shown in Fig. 4B.

Table 1: Diagnostic efficacy of in-houseestablished ELISA for ***Fasciola*** antigens with receiver operating characteristic(ROC) curve values.

	Somatic Antigen			ES Antigen		
Parameters	**Values**	**95% CI**	***P*- value**	**Values**	**95% CI**	***P*-value**
True positive	21			45		
True negative	27			37		
False positive	4			7		
False negative	1			0		
Area under the ROC curve						
Area under curve (AUC)	0.96	0.912-1.00	<0.0001	0.869	0.778-0.961	<0.0001
Std. Error						
Diagnostic test evaluation						
Sensitivity	95.45%	77.16% to 99.88%	<0.0001	100.00%	92.13% to 100.00%	<0.0001
Specificity	87.10%	70.17% to 96.37%		84.09%	69.93% to 93.36%	
Positive likelihood ratio	7.4	2.95 to 18.55		6.29	3.19 to 12.40	
Negative Likelihood Ratio	0.05	0.01 to 0.36		0		
Disease prevalence	41.51%	28.14% to 55.87%		50.56%	39.75% to 61.33%	
Positive predictive value	84.00%	67.68% to 92.94%		86.54%	76.52% to 92.69%	
Negative predictive value	96.43%	79.84% to 99.46%		100.00%		
Accuracy	90.57%	79.34% to 96.87%		92.13%	84.46% to 96.78%	

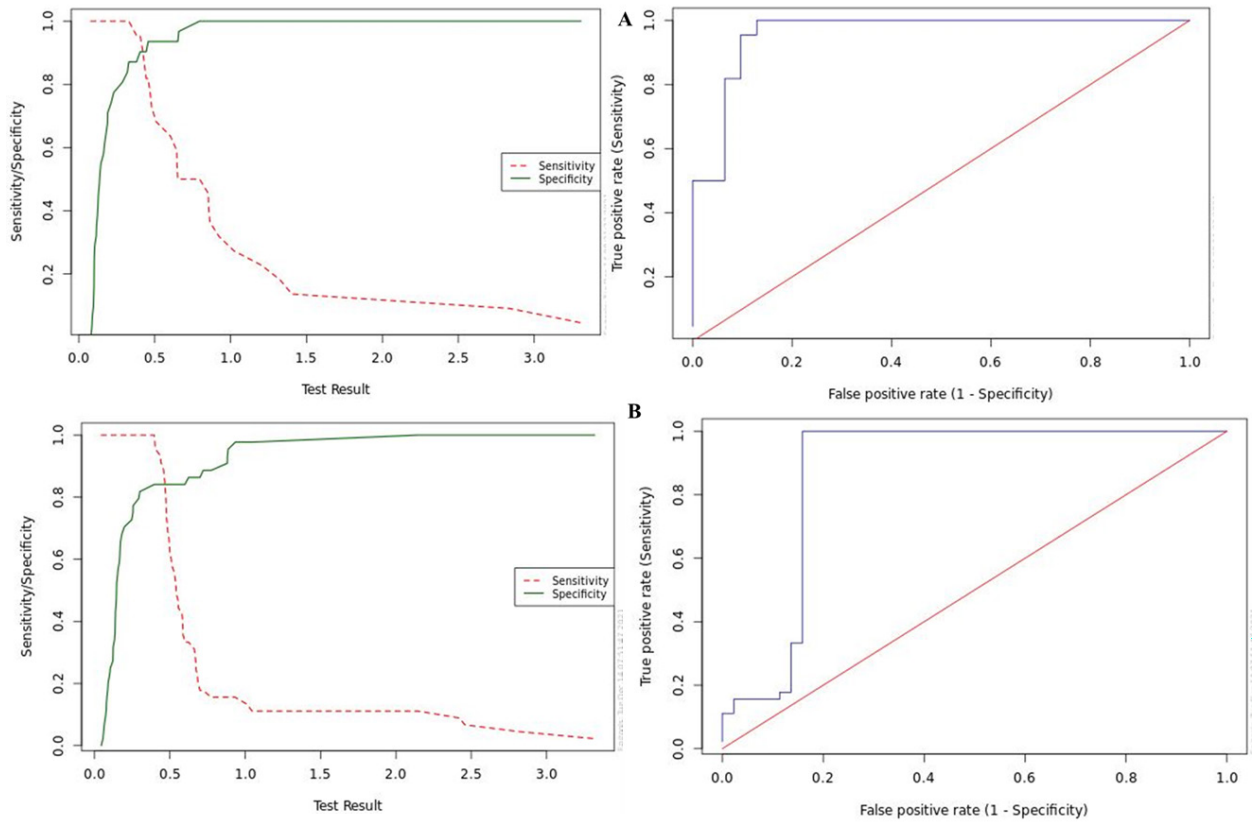


Fig. 1: Relationship between specificity and sensitivity values of Somatic antigen ELISA(A) and Excretory secretory antigen-based ELISA(B).

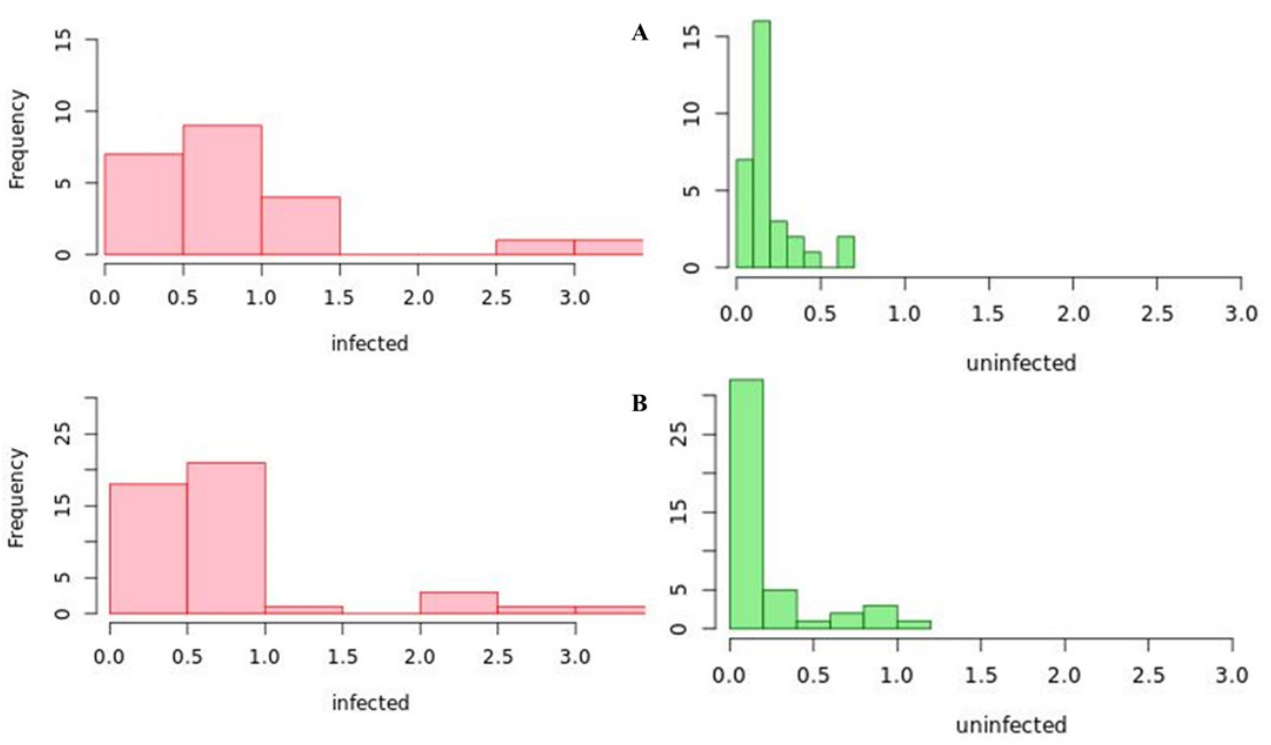


Fig. 2: The frequency and absorbance values of infected and non-infected control sera for Somatic antigen ELISA (A) and Excretory secretory antigen ELISA(B).

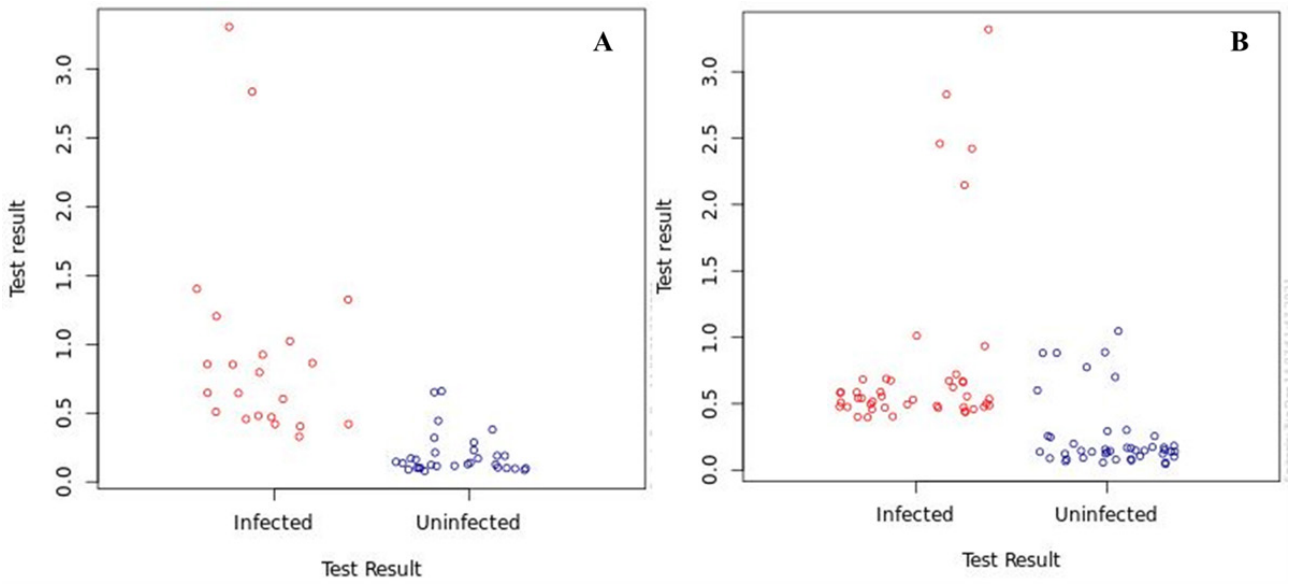


Figure 3: Scatter graph shows absorbance values of infected and non-infected controls sera for Somatic antigen ELISA (A) and Excretory secretory antigen ELISA(B).

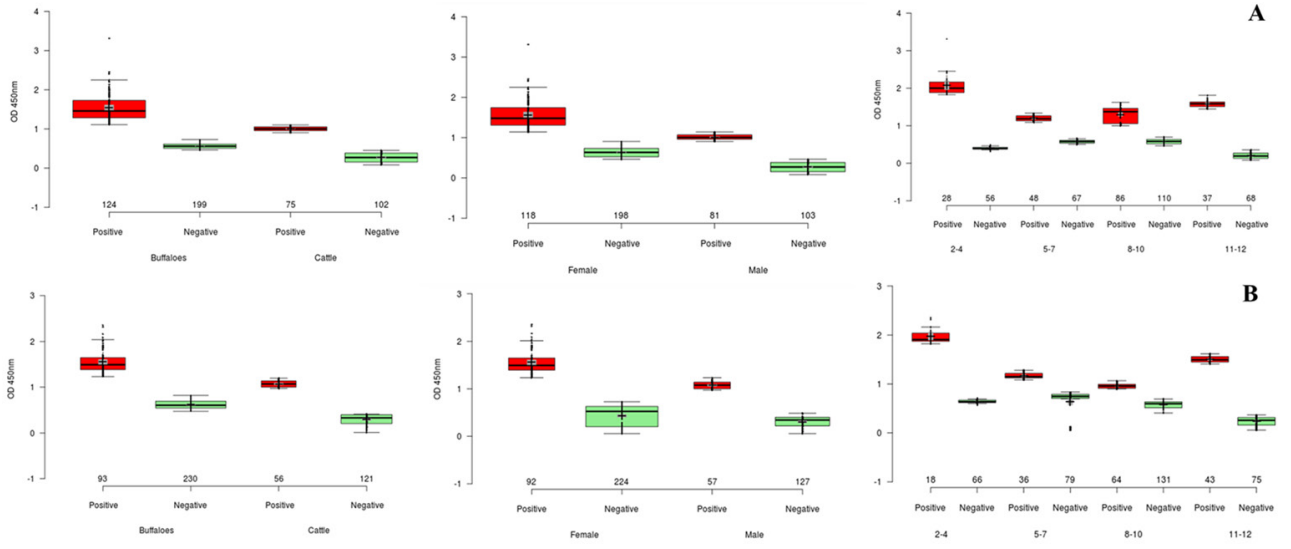


Fig. 4: The boxplot represents seroprevalence and absorbance values of fasciolosis infected animals along with associated risk factors for (A) Somatic antigen ELISA and (B) ES antigen ELISA.

DISCUSSION

An early diagnosis and disease control are very crucial considering the livestock health that is affected by the liver fluke infection not only in Pakistan but also across the world, particularly in tropical and sub-tropical countries where enormous morbidity and mortality of the livestock occurs due to fasciolosis (Anuracpreeda et al., 2013). Our results showed that diagnostic accuracy of the test was 90.57% and 92.13% for SA and ES antigen-based ELISA respectively, with higher sensitivity values. Like present work, Ardo et al. (2013) reported 100% sensitivity and 81.7% specificity using ELISA kit in the diagnosis of *Fasciola* -infection in cattle. Palmer et al. (2014) reported 100% sensitivity and 88.80% specificity in cattle, sheep, and horses in Australia. Afshan et al. (2013) also reported ELISA detection as a more sensitive technique than the conventional method in sheep and goats. Coprological examinations for detecting the parasite eggs are less sensitive due to difficulty during the patent period because eggs are expelled intermittently depending on the evacuation of the gall bladder and the biology of the *Fasciola* (Troncy, 1989).

Different antigens of *Fasciola* including somatic and ES have been used for immunodiagnosis of human and animal fasciolosis (Morales and Espino 2012;Rokni et al., 2003;Shafiei et al., 2015). The present study showed that ES and somatic antigens of *Fasciola* can be used for detecting the antibodies in the serum of immunized rabbits. Moazeni et al. (2005) also reported that somatic and ES antigens of *F. hepatica* have a strong reaction with antisera raised against both antigens. In addition, Cornelissen et al. (2001) used ELISA with somatic and ES antigens of *F. hepatica* to serodiagnosis of *Fasciola*-infection in naturally or experimentally infected sheep.

An essential characteristic of ELISA-based assays is high sensitivity. The area under curve (AUC) value in the present study was 0.96 (95% CI: 0.912-1) and 0.869 (95% CI: 0.778-0.961) for SA and ES ELISA, respectively. The AUC values represent sero-ELISA assay has good power of discrimination to the diagnosis of *Fasciola*-infection. The AUC values $0.5 < AUC \leq 0.7$ represents low accuracy, $0.7 < AUC \leq 0.9$ moderate accuracy, and $0.9 < AUC \leq 1.0$ represents high accuracy (Swets, 1988). Similarly, previous studies reported a high AUC value of 0.832 ± 0.0012 (Ducheyne et al., 2015), 0.62 (Bennema et al., 2011), and 0.76 (Kuerpick et al., 2013).

The serological assays showed the highest prevalence of *Fasciola*-infection in buffaloes as compared to cattle. Similarly, previous studies reported the high vulnerability of buffaloes towards fascioliasis as compared to cattle (Adedokun et al., 2008; Khan et al., 2011; Mehmood et al., 2017). The major contributing factor of the high susceptibility of buffaloes towards fasciolosis may include its habit to live in swampy areas (Ligda, 1998) due to an abundance of snail's intermediate hosts of *Fasciola* (Spithill et al., 1999).

In regard to our finding,female animals were more susceptible to infection than males. The reason could be the lower immunity of females due to milking a stressful physiological condition. Other studies mentioned that fasciolosis did not associate significantly between sex (Kanyari et al., 2017; Kabir et al., 2009, Khan et al., 2009), this may refer to the same management system. However, in contrast to the present study a significantly higher infection among male animals as compared to females was recorded (Umbreen and Azhar, 2012). This could be explained by fact that females are kept under healthier management and nourishing conditions than males.

The present serological assays showed higher infection in 8-10 years aged animals (cattle and buffaloes) as compared to younger ones, consistent with previous studies (Fufa et al., 2009; Kassaye et al., 2012; Isah, 2019; Zwede et al., 2019). This may be because younger animals are restricted indoors and provide a good management system, while older animals are allowed to the pasture for extended periods of grazing, which may contribute to the chances of higher exposure to parasitic stages (Zewde et al., 2019).

CONCLUSION

In conclusion, ELISA established have high sensitivity values which makes them a suitable test for screening the large number of animals infected with fasciolosis in epidemiological surveys.

ACKNOWLEDGEMENTS

The research work presented in this article is under funding provided by Higher Education Commission of Pakistan under the project grant No:7402/Federal/NRPU/R& D/HEC/2017. The research presented here is part of PhD thesis of Ms. Maria Komal.

CONFLICT OF INTEREST

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

Adedokun OA, Ayinmode AB, Fagbemi BO (2008) A comparative study of three methods for detecting *Fasciola* infections in Nigerian cattle. VeterinArskIarhiv 78 (5): 411-416.

Afshan K, Ahmad I, Komal M, Firasat S, Khan IA, Qayyum M (2021) Diagnostic efficacy of Copro-ELISA for detection of fasciolosis in cattle and buffaloes in Punjab Province,Pakistan. KafkasÜniv Vet FakDerg 27(4).

Afshan K, Qayyum M, Rizvi SSR, Mukhtar M, Mushtaq M, Miller JE (2013) Serological and coprological comparison for rapid diagnosis of *Fasciola hepatica* infection in small ruminants from sub-tropical area of Pakistan. Small Rumin Res 113 (1): 267-272.

Anuracpreeda P, Chawengkirtikul R, Tinikul Y,Poljaroen J, Chotwiwatthanakun C, Sobhon P (2013) Diagnosis of *Fasciola gigantica* infection using a monoclonal antibody-based sandwich ELISA for detection of circulating cathepsin B3 protease. Acta Trop 127(1): 38-45.

Ardo MB, Aliyara YH, Lawal H (2013) Prevalence of bovine fasciolosis in major abattoirs of Adamawa State, Nigeria. Bayero J Pure Appl Sci 6(1): 12-16.

Attallah AM, Bughdadi FA, El-Shazly AM, Ismail H (2013) Immunodetection of *Fasciola gigantica* circulating antigen in sera of infected individuals for laboratory diagnosis of human fascioliasis. Clin Vaccine Immunol 20(10): 1569-1577.

Bennema SC, Ducheyne E, Vercruysse J, Claerebout E, Hendrickx G, Charlier J (2011) Relative importance of management, meteorological and environmental factors in the spatial distribution of *Fasciola hepatica* in dairy cattle in a temperate climate zone. Int J Parasitol 41(2): 225-233.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

Charlier J, Vercruysse J, Morgan E, Van Dijk J, Williams DJL (2014) Recent advances in the diagnosis, impact on production and prediction of *Fasciola hepatica* in cattle. Parasitol 141(3): 326-335.

Cornelissen JB, Gaasenbeek CP, Borgsteede FH, Holland WG, Harmsen MM, Boersma WJ (2001) Early immunodiagnosis of fasciolosis in ruminants using recombinant *Fasciola hepatica* cathepsin L-like protease. Int J Parasitol 31 (7): 728-737.

Ducheyne E, Charlier J, Vercruysse J, Rinaldi L, Biggeri A, Demeler J, Brandt C, De Waal T, Selemetas N, Höglund J, Kaba J(2015) Modelling the spatial distribution of *Fasciola hepatica* in dairy cattle in Europe. Geospat Health 9(2): 261-270.

Fufa A, Loma A, Bekele M, Alemayehu R(2009) Bovine fasciolosis: coprological, abattoir survey and its economic impact due to liver condemnation at Soddo municipal abattoir, Southern Ethiopia. Trop Anim Health Prod 42(2): 289-292.

Isah H (2019) Spatial species distribution models to predict liver fluke disease (*Fasciola gigantica*) in cattle:A case study of Sokoto State, Nigeria (Doctoral dissertation, University of Leicester).

Kabir MHB, Eliyas M, Hashem MA, Mohiuddin M, Miazi OF (2009) Prevalence of zoonotic parasitic diseases of domestic animals in different abattoir of Comilla and Brahman Baria region in Bangladesh. Univ J Zool, RajshahiUniv 28: 21-25.

Kamel HH, Saad GA, Sarhan RM (2013) Dot-blot immunoassay of *Fasciola gigantic* infection using 27 kDa and adult worm regurge antigens in Egyptian patients. Korean J Parasitol51: 177-182.

Kamel HH, Sarhan RM, Saad GA (2015) Biochemical assessment of oxidative stress versus liver enzymes in patients with chronic fascioliasis. J Parasit Dis 39: 628-633.

Kanyari PWN, Kagira JM,Mhoma RJ (2017) Prevalence of endoparasites in cattle with zoonotic potential within urban and peri-urban areas of Lake Victoria Basin, Kenya.

Kassaye A, Yehualashet N, Yifat D, Desie S(2012) Fasciolosis in Slaughtered Cattle in AddisAbabaAbattoir, Ethiopia. Glob Vet 8: 115-118.

Khan MK, Sajid MS, Khan MN, Iqbal Z, Iqbal MU (2009) Bovine fasciolosis: prevalence, effects of treatment on productivity and cost benefit analysis in five districts of Punjab, Pakistan. Res Vet Sci 87(1): 70-75.

Khan MK, Sajid MS, Khan MN, Iqbal Z, Arshad M, Hussain A (2011) Point prevalence of bovine fascioliasis and the influence of chemotherapy on the milk yield in a lactating bovine population from the district of Toba Tek Singh, Pakistan. J Helminthol 85(3): 334-338.

Khan MN, Sajid MS, Rizwan HM, Qudoos A, Abbas RZ, Riaz M, Khan MK (2017) Comparative efficacy of six anthelmintic treatments against natural infection of *Fasciola* species in sheep. Pak Vet J 37(1): 65-68.

Kuerpick B, Conraths FJ, Staubach C, Froehlich A, Schnieder T, Strube C (2013) Seroprevalence and GIS-supported risk factor analysis of *Fasciola hepatica* infections in dairy herds in Germany. Parasitol 140(8): 1051-1060.

Ligda DJ(1998) Water Buffalo Facts (on-line), Availablefrom http://animals.nationalgeographic.com/animals/mammals/water-buffalo.html on September 25, 2008.

Mehmood K, Zhang H, Sabir AJ, Abbas RZ, Ijaz M, Durrani AZ, Saleem MH, Rehman MU, Iqbal MK, Wang Y, Ahmad HI(2017) A review on epidemiology, global prevalence and economical losses of fasciolosis in ruminants. MicrobPathog 109: 253-262.

Moazeni M, Gaur SNS, Shahangian A (2005) Cross reactivity between ES and somatic antigens of *Fasciola* spp in enzyme linked immunosorbent assay. Iranian J Vet Res 6 (2): 12.

Morales A, Espino AM (2012) Evaluation and characterization of *Fasciola hepatica* tegument protein extract for serodiagnosis of human fascioliasis. Clin Vaccine Immunol 19:1870-1878.

Murthy CK,Souza PE (2015) An enzyme-linked immunosorbent assay for diagnosis of *Fasciola gigantica* infection in cattle and buffaloes. J Parasit Dis 39(4): 783-785.

Palmer DG, Lyon J, Palmer MA, Forshaw D (2014) Evaluation of a copro-antigen ELISA to detect *Fasciola hepatica* infection in sheep, cattle and horses. Aust Vet J 92(9): 357-361.

Rokni MB, Massoud J, Hanilo A (2003) Comparison of adult somatic and cysteine proteinase antigens of *Fasciola gigantica* in enzyme linked immunosorbent assay for serodiagnosis of human fasciolosis. Acta Trop 88: 69-75.

Shafiei R, Sarkari B, Moshfe A (2013) A consistent PCR-RFLP assay based on ITS-2 ribosomal DNA for differentiation of *Fasciola species*. Iranian J Basic Med Sci 16(12): 1266.

Shafiei R, Sarkari B, Sadjjadi SM (2015) Performance of a 27 kDa*Fasciola hepatica* antigen in the diagnosis of human fascioliasis. Journal Lab Physicians 7(01): 017-020.

Shafiei R, Sarkari B, Sadjjadi SM, Mowlavi GR, Moshfe A (2014) Mo-

lecular and morphological characterization of *Fasciola spp.* isolated from different host species in a newly emerging focus of human fascioliasis in Iran. Vet MedInt.https://doi.org/10.1155/2014/405740

Spithill TW, Piedrafita D, Smooker PM (1997) Immunological approaches for the control offasciolosis. Int J Parasitol 27(10): 1221-1235.

Swets JA(1988) Measuring the accuracy of diagnostic systems. Science 240:1285-1293.

Troncy PM(1989) Helminths of livestock and poultry in Tropical Africa. In: Fischer. 1989. Manual of Trop Vet Parasitol 63-73.

Umbreen JK, Azhar M (2012) Prevalence of fasciolosis in cattle under different managementalconditions in Punjab. Pak J Zool 44:1193-1196.

Zewde A, Bayu Y, Wondimu A(2019) Prevalence of bovine fasciolosis and its economic loss due to liver condemnation at Wolaita Sodo municipal Abattair, Ethiopia. Vet Med Int.https://doi.org/10.1155/2019/9572373.