

# Seroepidemiology of goat fascioliasis in district Sargodha, Punjab, Pakistan based on excretory secretory antigens of the indigenous strains of *Fasciola gigantica*

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## ABSTRACT

The aim of this study was to screen the domestic goat (*Capra hircus*) population of district Sargodha, using ELISA based on the indigenous excretory secretory antigens (ES Ag) for *Fasciola (F.)* spp. antibodies. The ES Ag from the indigenous strains of *Fasciola* were procured from adult flukes (*F. gigantica*) for the standardization and application of ELISA for sero-epidemiology in the target goat population. A total of 3504 blood samples were collected based on stratified random sampling from district Sargodha during a calendar year (2013) for isolation of sera. The separated sera were screened for anti-*Fasciola* Ab using BioRad Microplate Reader at 450 nm wavelength. Data were statistically analyzed through multivariate analysis. Of 3504 sera, 1496 (42.69%) were found positive for Ab against *Fasciola* spp. Among the study determinants, tehsils, age and sex were found having positive statistical association with the distribution of anti *Fasciola* Ab in the goat population of district Sargodha. The distribution of anti *Fasciola* Ab was higher in adults and female hosts during the winter season. Breed was not found statistically associated with the sero-surveillance of *Fasciola* spp. Among tehsils, Bhalwal, Shahpur, Kot Momin, Sahiwal, Sillanwali and Sargodha were having anti *Fasciola* Ab in descending order of abundance. ELISA based on the indigenous strains of *Fasciola* spp. proved a successful application for wide scaled sero-surveillance of the sub-clinical disease. It is recommended to apply the test on other geographically susceptible livestock population for early diagnosis and control of the disease.

**Keywords:** Epidemiology, Excretory/Secretory Antigens, Indigenous ELISA, Goat, Fascioliasis, Sargodha.

## RESUME

### Etude séroépidémiologique de la fasciolose chez la chèvre au Pakistan

Le but de cette étude était de tester la population de chèvres domestiques (*Capra hircus*) du district de Sargodha vis-à-vis d'anticorps détectés par ELISA à l'aide d'antigènes excrétés/sécrétés (ES Ag) de *Fasciola (F.)* spp. Les ES Ag obtenus à partir des souches indigènes adultes de *Fasciola (F. gigantica)* ont été utilisés pour la normalisation et l'application de ELISA pour la détection séroépidémiologique dans la population de chèvres cibles. Un total de 3504 échantillons de sang a été recueilli sur la base d'un échantillonnage aléatoire stratifié dans le district de Sargodha au cours d'une année civile (2013). Les sérums ont été testés quant à la présence d'anticorps anti-*Fasciola* en utilisant un lecteur de microplaques BioRad à la longueur d'onde de 450 nm. Les données ont été analysées statistiquement par analyse multivariée. Sur les 3504 sérums, 1496 (42,7%) se sont révélés positifs. Parmi les facteurs déterminants de l'étude, la localisation, l'âge et le sexe ont été trouvés comme ayant une association statistique positive avec la distribution d'anticorps anti-*Fasciola*. La plus forte séropositivité a été observée chez les adultes femelles pendant l'hiver. La race est apparue sans effet. Des différences entre lieux de collectes ont été observées. Cette étude suggère d'appliquer le test de détection sur d'autres animaux sensibles pour le diagnostic et le contrôle précoce de la maladie dans la région.

**Mots-clés :** épidémiologie, antigènes, ELISA, chèvre, Fasciolose, Pakistan.

## Introduction

In domestic goats (*Capra hircus*) fascioliasis is presumed to be a less important and less frequent infection than other ruminants; however, it is prevalent in different parts of the world e.g. 14% in India [57], 18% in Turkey [61], 20% in Chile [54], 20% in Morocco [32] and 72% in China [64]. Goats are found to be very sensitive for natural as well as trial infections [49]. Conventional coprological examination through optical microscopy is extensively used for routine diagnosis of helminthes in general and *Fasciola* spp. in specific [8]. However, this fails to detect milder infections or those earlier than 8 weeks of infection [45]. Mostly, fascioliasis appears as chronic disease with low mortality; however, a less frequent acute form increases the rate of mortality [38]. These losses attributable to fascioliasis can be reduced by treating the animals diagnosed at their initial stages of infection through sensitive as well as specific tests

e.g. Polymerase chain reaction (PCR) and enzyme-linked Immuno-sorbent assay (ELISA) [65].

In Pakistan, fascioliasis is prevalent in many areas e.g. 0.66% in Islamabad [19], 4% in Faisalabad [27], 5% in Ziarat [48], 5.28% in Jammu Kashmir [43], 12.5% in Dera Ismail Khan [5], 20.93% in Quetta [4], 28.75% in Multan [59], 33.18% in Toba Tek Singh [36], and 73.2% in Jhelum Valley [18]. Recently, 38.78% prevalence of caprine fascioliasis in district Sargodha has been reported through conventional coprological assay [9]. ELISA is one of the reliable technique for early sero-diagnosis of fascioliasis among the available ones. Commercially available ELISA kits are in use for detection of fascioliasis but its sensitivity and specificity varies according to geographic variation [22,50]. So the results of commercially available ELISA kits cannot be extrapolated to the field situations of Pakistan with good accuracy. The present paper reports the application of ELISA based on the

excretory secretory antigens (ES Ag) of indigenous strains of *F. gigantica* as standardized earlier by Rehman [50].

## Materials and Methods

### STUDY AREA

Study was conducted in district Sargodha which predominantly contains flat and fertile plains; although, few small hills are present on the Sargodha-Faisalabad Road. The detailed statistics of the district Sargodha has been reported elsewhere [9].

### PREPARATION OF EXCRETORY/SECRETORY ANTIGENS

The method described by Anderson *et al.* [8] and standardized by Rehman [50] was used to prepare ES Ag from adult Fasciola. Briefly, adult parasites, in batches of about 20, were put into 50 mL of phosphate buffer saline (PBS) at 37°C. Initial regurgitant, comprising of blood, bile and debris, was removed after swirling the containers containing the flukes. Fresh PBS medium was added and this process repeated at least three times over a period of 20 minutes. The flukes were incubated in fresh PBS for 6-8 hours at 37°C. After incubation, the fluid containing the ES Ag was centrifuged at 2500 rpm at 4°C for 15 minutes, passed through a 0.22 µm filter and stored at -20°C until required. The protein concentration of this solution was estimated spectrophotometrically at 280 nm.

### SAMPLING OF ANIMALS

Sampling was done from all the six tehsils (administrative divisions) of district Sargodha through stratified random sampling and proportional allocation method at 95% confidence interval using the following formula [60] as follows:

$$n = \frac{1.96^2 P_{exp} (1 - P_{exp})}{d^2}$$

Where, n = Sample size;  $P_{exp}$  = Expected prevalence;  $d^2$  = Desired precision

Taking an expected prevalence of 25.46% [34] at 5% precision level, sample size was calculated for goat as 292. A total of 3504 sera samples were collected during a calendar year 2013 (292 during each month). According to Punjab Development Statistics [46], total goat population is 588 thousands in district Sargodha. The distribution of goat in tehsils is as follow: 104500 in Sargodha, 122500 in Bhalwal, 98500 in Sahiwal, 80500 in Sillanwali, 93500 in Shahpur and 88500 in Kot Momin. Numbers of animals to be sampled from each of six tehsils (considered as stratum) of district Sargodha was calculated by following formula:

$$n_k = n \frac{N_k}{N}$$

Where, N = Population size;  $N_k$  = Population size of stratum; n = Total sample size;  $n_k$  = Sample size from each stratum.

### COLLECTION OF BLOOD SAMPLES

About 10 mL whole blood sample from each subject were collected from all the six tehsils of district Sargodha in vacutainers (gel and clot activator) without anticoagulant. The blood was allowed to clot for 30-45 minutes before centrifugation at 2500 rpm for 15 minutes. The supernatant (serum) was aspirated carefully and stored at -20°C after making aliquots of 0.5 mL till further use.

### THE ELISA

Optimum dilutions of antigen, serum and horse radish peroxidase (HRP) conjugated anti-goat antibodies (Fitzgerald Industries International, USA) were determined by checkerboard titration (CBT) as described by Crowther [14] and Rehman [50]. Mean OD value of negative group was  $0.0868 \pm 0.059$ . The cut-off point value was determined by the following formula:

$$\text{Cut-off value} = \text{Mean OD value of negative group} + 2 (0.059)$$

The ELISA test protocol of Rehman [50] was followed. Briefly, the ES Ag at dilution of 1/200 was added (50 µL/well) into all columns of microtiter plate. After overnight incubation at 4°C, the plates were washed five times with washing solution (0.05% Tween 20 in PBS). Nonspecific binding was blocked using 5% skimmed milk in PBS at 37°C for 1 hour. Subsequently, 50 µL of 1/100 dilution of serum were added to each well. After 1 hour of incubation at 37°C, 50 µL of a 1/5,000 dilution of HRP-labeled rabbit anti-goat IgG was added to each well and the plate was again incubated at 37°C for 1 hour. Between each step, plates were washed five times in rinse solution for 2 min. After the conjugation step, tetra-methyl benzidine (TMB) was added and incubation of 10 minutes was given. Reaction was stopped by adding 50 µL of 0.6 N sulphuric acid. Optical density (OD) values were measured at 450 nm through iMark Microplate Reader S/N 14309 (BIORAD, USA).

### STATISTICAL ANALYSIS

Multiple logistic regression analysis and Odd's ratio was carried out for epidemiological studies of goat fascioliasis and its associated risk factors [55]. All statistical procedures were carried out using SAS [53] software package at 95% level of confidence.

## Results

Overall prevalence of fascioliasis in district Sargodha was found to be 42.69% (1496/3504). Prevalence of fascioliasis was found to be significantly higher ( $P < 0.05$ ) in adults and females than young stocks and males, respectively. Among seasons, fascioliasis was highly significant ( $P < 0.05$ ) in the winter followed in order by autumn and spring while, summer was found to be non-significant. All breeds of goats were equally susceptible to carry fascioliasis i.e. breed was not

found statistically associated with the sero-surveillance of *Fasciola* spp. Among tehsils, Bhalwal, Shahpur, Kot Momin, Sahiwal, Sillanwali and Sargodha were having anti *Fasciola* Ab in descending order of abundance (Fig. 1).

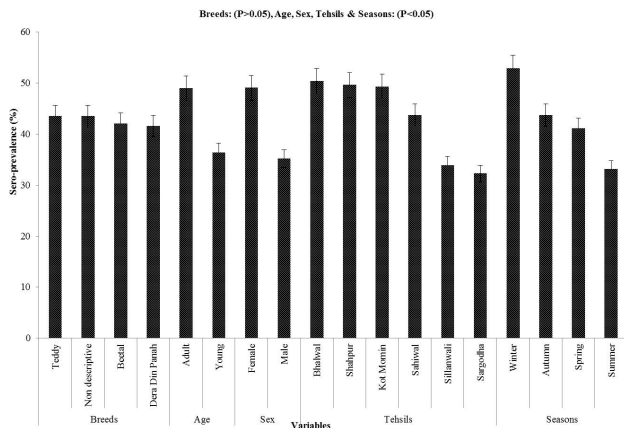


FIGURE 1: Overall prevalence of fascioliasis in goat population of district Sargodha using ELISA based on the excretory secretory antigens of the indigenous strains of *F. gigantica*.

Overall prevalence of fascioliasis was found to be highest (52.32%) in tehsil Bhalwal. Where the prevalence was significantly higher ( $P<0.05$ ) in adults while, the sex and breed was found to insignificant. In tehsil Shahpur, a highly positive association ( $P<0.05$ ) of fascioliasis was found with the sex of host being higher in female as compared to male, however, the age was found insignificant ( $P>0.05$ ) but little higher in case of adult as compared to young. Fascioliasis, in tehsil Kot Momin was found statistically associated ( $P<0.05$ ) with the age group, whereas, other determinants like sex and breed were found to be statistically non-significant. Overall prevalence of fascioliasis was 43.70% in tehsil Sahiwal. Where non-significant association was found in associated risk factors like age, sex, breed and season. In tehsil Sillanwali, a highly positive association ( $P<0.05$ ) of fascioliasis was found with the sex of host being higher in female as compared to male. The risk of disease was found to be insignificant in case of age and breed. Fascioliasis, in tehsil Sargodha was found statistically associated ( $P<0.05$ ) with the age group i.e. a significantly higher prevalence was found in adults as compared youngs. Whereas, other determinants like sex and breed were found to be statistically non-significant. Among the four seasons, fascioliasis was found to be significantly ( $P<0.05$ ) higher in winter followed in order by that of autumn, spring, and summer in all the six tehsils of Sargodha district (Fig. 2).

## Discussion

Fascioliasis is an important infectious disease of small and large ruminant population throughout the world [47] having zoonotic significance [23]. In most of the cases, fascioliasis appears as chronic disease with low mortality but acute form increases the rate of mortality [38]. In the developing countries, the diagnosis of fascioliasis in animals has been

mainly relied upon the faecal examination for detection of eggs [34]. Although this technique has been used from the last several decades, but has some drawbacks e.g. lower sensitivity which increases the probability of false negative results in earlier stages of infection, low-intensity infections [17] and/or ectopic infection [8]. However, in higher intensity infections, conventional diagnostic assays are very sensitive and have advantages over other techniques, like low cost and easier to perform [13].

Under the given limitations of the conventional copro-microscopic techniques, various other approaches such as immuno and DNA based diagnostic assays have been developed for parasitic diagnosis in general. Specifically, the use of molecular techniques have coined their worth due to their higher sensitivity, accuracy, and efficiency in early and true detection of fascioliasis [11,30,50].

The immunological response to ES, Somatic and surface Ag of *Fasciola* has been found promising; however, western blot analysis indicated higher specificity of ES Ag than those of somatic and surface Ag for sero-diagnosis of fascioliasis [10,52]. In Pakistan, an indirect ELISA based on the ES Ag of the indigenous strains of *F. gigantica* has been standardized and successfully applied in large ruminants for early diagnosis of fascioliasis with acceptable performance [50]. Our study reports the first successful implementation of this test for sero-surveillance of the goat fascioliasis.

Overall sero-prevalence of goat fascioliasis in district Sargodha was recorded higher as compared to those reported by Islam *et al.* [28], Utuk *et al.* [63], Gebeyehu *et al.* [20], Gebeyehu *et al.* [21] but lower than Hillyer *et al.* [25], Ibarra *et al.* [26], Anderson *et al.* [8], Molloy *et al.* [44], Kooshan *et al.* [37], Al-Khafajy [6], Damwesh and Ardo [16]. The prevalence and method used for the determination of fasciola infection by the above mentioned scientists is given in table I. In many districts of Pakistan, such as Faisalabad [27], Ziarat [48], Quetta [4], Multan [59], Islamabad [19], Toba Tek Singh [36], Dera Ismail Khan [5], Sargodha [2], and Jammu Kashmir [43] copro-microscopic assay has been used for detection of fascioliasis, which shows lower prevalence than this study. The presence of higher prevalence in the study area is directly correlated to the geographic location and conditions i.e. marshy and low lying area with high humidity provide suitable habitation for snail. The availability of snail, rainfall, temperature ( $>9.5$  °C), soil moisture and/or humidity propagate the development of parasite [1]. Other than this, the reason for these variations might be due to difference in agro-ecological conditions, movement of animals for grazing near to water logged areas, traditional pasture management practices, amount of rainfall, flooding during rainy season, differences in landscape e.g. agricultural irrigation practices and swampy areas [51,62]. Moreover, adaptation of unhygienic measures, use of inappropriate drugs and indiscriminating trade of animals also help in the propagation of disease [29].

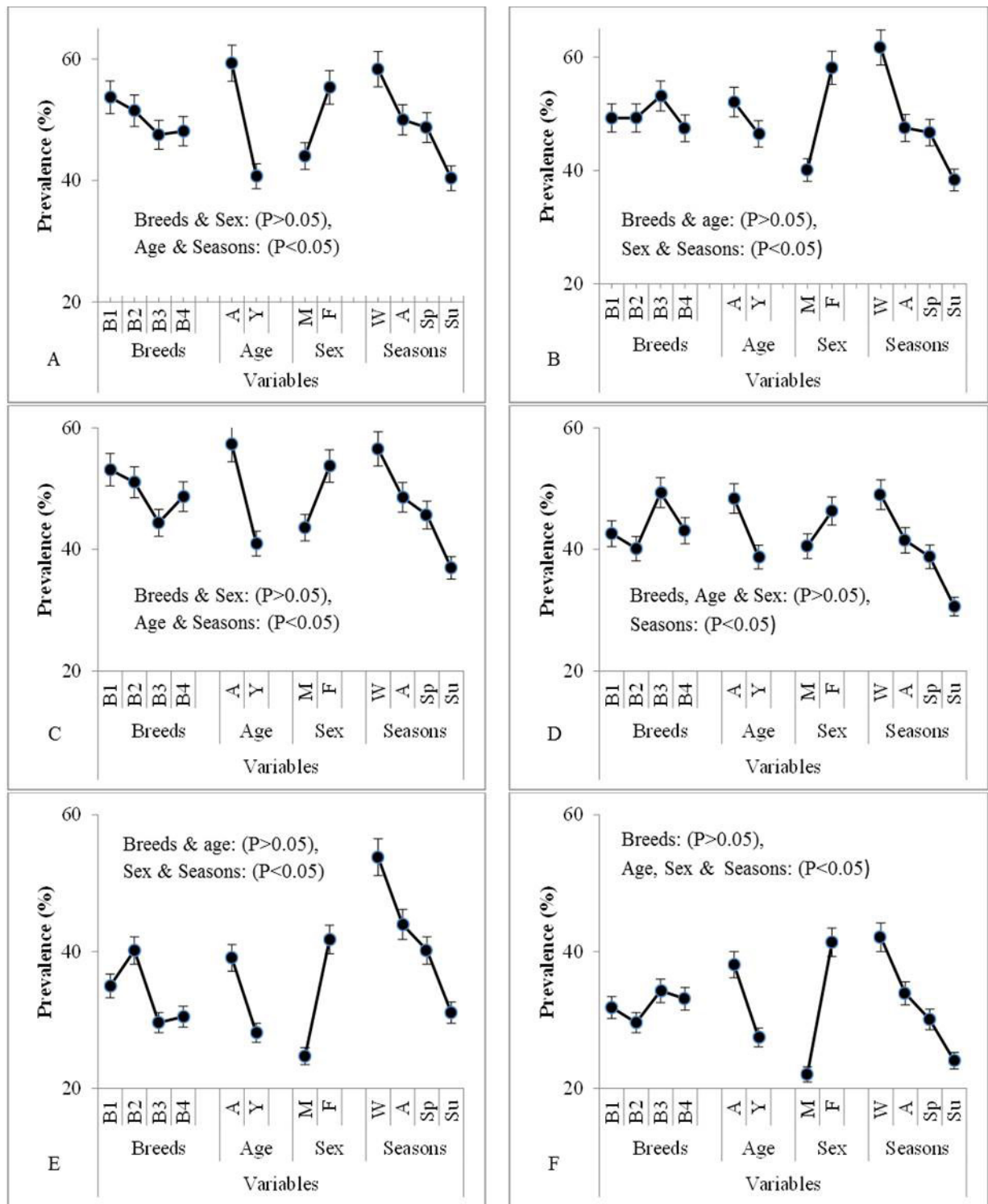


FIGURE 2: Association of determinants with the frequency distribution of goat fascioliasis in all six tehsil of district Sargodha, Punjab, Pakistan determined using ELISA based on the excretory secretory antigens of the indigenous strains of *F. gigantica*. A = Bhalwal; B = Shahpur; C = Kot Momin; D = Sahiwal; E = Sillanwali; F = Sargodha; B1 = Teddy; B2 = Non descriptive; B3 = Beetal; B4 = Dera Din Panah; A = Adult; Y = Young; M = Male; F = Female; W = Winter; A = Autumn; Sp = Spring; Su = Summer.

In most of the study, female sex was found to be highly significant for fascioliasis than male e.g. Maqbool *et al.* [40], Mazid *et al.* [41], Ahmed *et al.* [3], Talukde *et al.* [58], and Khan *et al.* [35] reported that females are more prone to fascioliasis than male. In some studies conducted by Khan *et al.* [33], Chanie and Begashaw [12] and Gebeyehu *et al.* [21] the sex was found to be a non-significant determinant for fascioliasis. The probable reasons for higher prevalence

of female goats in this country are; the females are rear for long time *i.e.* the chances of exposure to the disease increase, continuous physiological changes during productive activity such as pregnancy and lactation posed stress on females [12], lack of proper nutrition for animals especially for females during production, continuous grazing of animals on water-logged areas [56].



Prevalence (%)	Method	Country	Reference
38.00	ELISA Kit (Fasciola ELISA kit, Institute Pourquier, France)	Bangladesh	[28]
16.20	Using the commercial ELISA kit	Turkey	
15.60	Home-made ES antigen based indirect ELISA method.		[63]
01.10	ELISA test kit (IDEXX, Montpellier, France)	Korea	[20]
09.40	ELISA kit	Korea	[21]
89.00	ES antigen based ELISA	Bolivia	[25]
52.15	ES antigen based ELISA	Mexico	[26]
78.26	ES antigen based ELISA	Vietnam	[8]
60.20	ELISA Kit, Institute Pourquier (Montpellier, France)	Australia	[44]
90.00	ES antigen based ELISA	Iran	[37]
55.80	ELISA kit	Iraq	[6]
72.00	ELISA kit	North Eastern Nigeria	[16]

TABLE I: The prevalence and method used for the determination of fasciola infection in different parts of the world

Among age groups, fascioliasis has been reported higher in adults than in young's by Keyyu *et al.* [31], Tasawar *et al.* [59], Mbaya *et al.* [42], Al-Khafajy [6], Hassan *et al.* [24], Ahmad [2], Gebeyehu *et al.* [20]. The higher prevalence in adult goats might be due to their routine grazing near submerged areas, longer exposure to the infection, long latent phase in the host (about 5-6 months), and the compromised immunity of animals. However, Dagnachew *et al.* [15], Gebeyehu *et al.* [21] and Anjum *et al.* [9] recorded higher prevalence in young goats. The presence of higher prevalence in younger animals might be due to get infection immediately after birth or due to hyper endemicity of infection in those areas.

Prevalence of fascioliasis was found highest during the months of winter which is similar to the study of Lemma *et al.* [39], Selim *et al.* [56], Rehman *et al.* [50] and Anjum *et al.* [9]. This might be due to progressive increase in the percentage of animals passing fluke eggs during the end of dry season and at the start of rainy season [31]. Typical weather such as proper humidity, temperature, rainfall provide suitable conditions for growth and development of *Fasciola* spp. as well as their intermediate host snail [34].

In present study the relatively higher prevalence of goat fascioliasis was found in tehsil Bhalwal, and Shahpur. This might be due to presence of river Jhelum and river Chenab along the boundaries of these tehsils. An association between flooded areas and prevalence of fascioliasis was also determined by Alves *et al.* [7] in Brazil.

It is concluded that the ELISA, based on the ES Ag of indigenous *F. gigantica* has good diagnostic efficacy, which can be used for early detection of fascioliasis in endemic regions of the world. By using ELISA test employed in

this study, the morbidity and mortality of livestock population due to fascioliasis can be reduced by treating the animals prior to the development of liver pathology.

## Acknowledgements

Financial support of this study was provided by the Higher Education Commission (HEC), Islamabad through project No. 20-1483/R&D/09/2845 under National Research Program for Universities (NRPU). Authors would like to submit the gratitude towards the goat keepers of the study area for their cooperation during the blood sampling.

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