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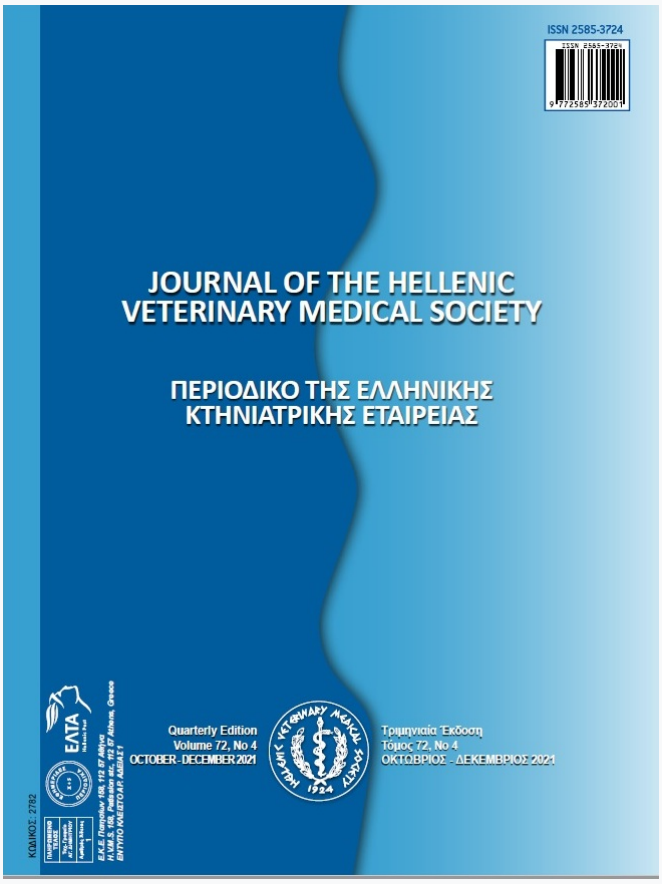

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Research article
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Epidemiology, burden and seasonal variation of fasciolosis determined through faecal examination and excretory/secretory antigens based ELISA

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ABSTRACT: The study was conducted to evaluate predominance and the related risk factors of sheep fasciolosis in Faisalabad district, Punjab, Pakistan. In addition, comparison of the coprological examinationand excretory/secretory antigens (ES Ag)-based ELISA was also performed to determine the variation in the prevalence of fasciolosis. Oft-he1200 faecal and blood samples,128 (10.67%) and 241 (20.08%) samples were found positive for *Fasciola* infection and anti-*Fasciola* antibodies respectively.The prevalence of fasciolosis was significantly ($P < 0.05$) higher in female and adultanimals. Most of the animals showed moderate (55.47%) infection which was significantly higher than mild (28.91%) and severe (15.63%) infections. The highest prevalence of fasciolosis was determined in December (26.00% through faecal examination; 42.00% through ELISA) with a mean EPG of 842.3 while lowest in May (02.00% through faecal examination; 07.00% through ELISA) with a mean EPG of 650. The frequency distribution of fasciolosis was significantly ($P < 0.05$) highest in the winter (faecal examination 20.67%; ELISA 34.67%) followed in order by the monsoon (faecal examination 09.00%; ELISA 18.67%), spring (faecal examination 08.33%; ELISA 17.33%) and summer (faecal examination 04.67%; ELISA 09.67%). The highest mean EPG of fasciolosis was determined in the spring (894) followed in order by the winter (851.67), summer (654.67) and monsoon (616.33). Although the faecal examination is the gold standard and the "best method" in the diagnosis of sheep fasciolosis; however, the serological tests should not be excluded especially the home-made ES Ag-based ELISA which might be preferred and more affordable risk assessment tool in the field epidemiology.

***Keywords*:** Coprology; ELISA; Excretory/secretory antigens; EPG; Fasciolosis;

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INTRODUCTION

Fasciolosis, an important, emerging food and water-borne parasitic zoonosis occurring in human communities, is considered among the significant public health problems in several countries (Intapan et al., 2003). Fasciolosis is a significant problem of animals and the infection is brought about by the Digenean trematodes of the genus *Fasciola*, generally called as the liver flukes. Both juvenile and adult developmental phases of the parasite are responsible for pathogeneses and production losses in the final hosts. In temperate areas without enormous occasional climatic variations, for example Ireland, the management factors unequivocally impact the spatial distribution of the *Fasciola* sp. (Munita et al., 2016). Fasciolosis causes an enormous financial loss of approximately US$ 3.2 billion around the world (Ahmad et al., 2017; Khan et al., 2017).

The conclusive analytic test for *Fasciola* sp. is the liver necropsy which gives a profoundly exact diagnosis of the fasciolosis when bile ducts have the parasite (Ahmad et al., 2017). However, this cannot be a preventive management tool for the flock and/or herd health monitoring system because we can only apply this on the post-mortem examination (Mazeri et al., 2016). Oftenly utilized ante-mortem test for the diagnosis of fasciolosis is the identification of eggs in the faeces by the sedimentation method followed by the faecal egg counts (FEC) which is reported to have a higher specificity. The later i.e. FEC is the best tool for the detection of current infection and to determine a higher precision of the burden of the parasitic infection (Rizwan et al., 2017; 2019; Ahmad et al., 2020). However, FEC does not prove to be a sensitive diagnostic tool in cases of lower magnitude of parasitism or when the juvenile (non-reproducing) stages of the flukes are in the process of internal migration towards the predilection site, i.e. the bile duct (Brockwell et al., 2013).

The technique which depends on the antibody (Ab) detection will overwhelmingly be the favorable technique for the immunodetection of *Fasciola* sp. This is due to the overall simplicity of the method and an early sero-conversion during the premature disease. Thus, most of the specialists are currently using enzyme-linked immunosorbent assay (ELISA) for the immuno-detection of *Fasciola* sp. (Rizwan et al., 2016; Acici et al., 2017; Munita et al., 2019).The excretory/secretory antigens (ES Ag)-based ELISA is explored which not only detects infection but also gives a better picture of the infection as compared to the conventional diagnostic techniques in the preventive management strategy of the *Fasciola* sp. in the livestock. The current study was performed to (a) compare the prevalence of fasciolosis through the classical coprology and ES Ag-based ELISA owing to their capabilities to detect the exposure of the sheep population to the *Fasciola* sp., (b) determine the quantitative magnitude of the parasitic load through the eggs per gram (EPG) of *Fasciola* sp., and (c) analyze an association of season as an extrinsic determinant with the prevalence and EPG of the *Fasciola* sp.

MATERIALS AND METHODS

Collection and Processing of Samples

The study was conducted in the district Faisalabad, Punjab, Pakistan which is the third largest city of the country. The faecal samples (5 g) and blood samples (5 mL) were collected from the randomly selected 100 sheep/month of the pastoral communities for one year aseptically with the owners' consent. Following criteria was used for the selection of animals: (a) clinically health, (b) normal physiological parameters, and (c) no history of the fasciolicidal treatment over the past three months. The collected faecal samples from 519 young animals, 681 adult, 713 male and, 487 female animals were processed to identify the *Fasciola* sp. infection through the standard indirect qualitative faecal examination assay (sedimentation technique) as given by Hayat and Akhtar (2000). Eggs were identified following the standard identification keys (Soulsby, 1982).

Quantitative faecal examination for the assessment of parasitic burden was performed through the "Modified McMaster Test" as given by Mazeri et al. (2016). The animals found positive for the *Fasciola* sp. were categorized as mildly, moderately and severely infected according to the FEC of 100 to 600, 700 to 1000, and over 1000 per gram of faeces, respectively. The blood samples were collected from the jugular veins into the gel-clot-activator vacutainers and indorsed to clot for 35 to 40 minutes and shipped to the Department of Parasitology Lab., following the starndard operating procedures of the transportation. The supernatant was centrifuged (2500 rpm for15 minutes), pipetted, transferred to the pre-labeled eppendorf tubes, and stored at -20°C till further use.

Preparation of *Fasciola* Excretory/Secretory Antigens

The technique depicted by Anderson et al. (1999)

was followedfor the preparation of ES Ag of the *Fasciola* sp. as described elsewhere (Rizwan et al., 2016).Briefly, flukes in groups of around 20 were placed into a phosphate buffer saline (PBS) solution. Early regurgitates containing bile, debris and blood were removed just after twirling the flasks containing parasites and the procedure was revised multiple times. After proper washing of the parasites, 50 mL fresh PBS was added and incubated at 37°C for 6 to 8 hours. Then, the contents were centrifuged (2500 rpm) for 15 minutes, filtered through the 0.22 µm filter, and kept at -20°C. Spectrophotometery was applied to determine the protein (ES Ag) concentration at A280 through the Bradford technique.

Enzyme Linked Immunosorbent Assay

The ELISA test protocol as described by Rizwan et al. (2016) was followed. Briefly, 50 µL of the ES Ag (1/200 dilution) was added into each of the wells of a micro-titer plate and incubated overnight at 4°C.The plates were washed multiple times with 0.05% Tween 20 in a phosphate buffer saline (PBS) solution. For blockage of the nonspecific binding, 5% skimmed milk in PBS was added and incubated at 37°C for 60 minutes. Then, 50 µL serum with 1/100 dilution was added into the wells and incubated at 37°C for 60 minutes. Subsequently, 50 µL HRP-labeled rabbit anti-sheep IgG with 1/5000 dilution was added to each of the wells and incubated for 60 minutes at 37°C. Between each progression, plates were washed multiple times with 0.05% Tween 20 in PBS. After this, tetra-methyl benzidine was added and incubated for 10 minutes. Then, 50 µL of 0.6 N Sulphuric acid was added to stop the reaction and the optical densities were measured through an ELISA reader at 450 nm wavelength.

Data Analyses

Differences between independent variables (breed, sex, age and season) concerning the prevalence of *Fasciola* sp. were explored using the Chi-square test (categorical variables). Data were analyzed using Minitab 17 software.

RESULTS

The overall prevalence of fasciolosis determined by the faecal examination (FE) was 10.67%, and that determined by ES Ag-based ELISA was 20.08%. The frequency distribution of fasciolosis was significantly higher in females than males and in adults than young stock (Table 1). Most of the animals showed moderate (55.47%) infection which was significantly higher than mild (28.91%) and severe (15.63) infections (Table 2). The highest prevalence of fasciolosis was determined in December (26.00% through the FE; 42.00% through ELISA) with a mean EPG of 842.3.In contrast, the lowest prevalence of fasciolosis was determined in May (02.00% through the FE; 07.00% through ELISA) with mean EPG of 650 (Fig. 1). The prevalence of fasciolosis was significantly higher in winter followed in order by the monsoon, spring and summer (Table 3).The highest mean EPG of fasciolosis was determined in spring (894.00) followed in order by winter (851.67), summer (654.67) and monsoon (616.33).

Table 1. Frequency distribution of fasciolosis in sheep population of Faisalabad district, Punjab, Pakistan determined by the faecal examination and excretory/secretory antigens-based ELISA

Variable	Level	Faecal Examination					ES Ag based ELISA				
		Examine	Positive	Prevalence	Chi-Square	P-Value	Examine	Positive	Prevalence	Chi-Square	P-Value
Breed	Kajli	497	58	11.67	1.657	0.437	497	105	21.13	1.065	0.587
	Thali	327	29	8.87			327	61	18.65		
	Lohi	376	41	10.90			376	75	19.95		
Age	Young	519	30	5.78	22.915	0.000	519	84	16.18	20.308	0.000
	Adult	681	98	14.39			681	157	23.05		
Sex	Male	713	50	7.01	24.618	0.000	713	103	14.45	28.019	0.000
	Female	487	78	16.02			487	138	28.34		

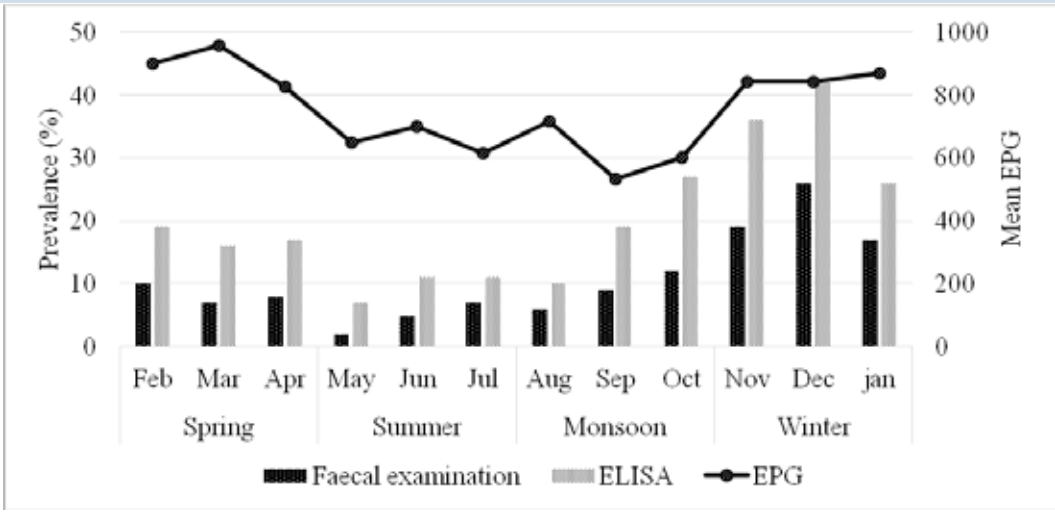
Table 2. Faecal egg count-based severity of the sheep fasciolosis in district Faisalabad, Punjab, Pakistan

Severity	Examined	Positive	Prevalence (%)	Mean Number of Eggs Per Gram (EPG)	Std. Dev.	Minimum	Maximum
Mild	128	37	28.91	364.9	170.3	100	600
Moderate	128	71	55.47	836.6	103.1	700	1000
Severe	128	20	15.63	1320	220.3	1100	1800

Table 3. Comparative prevalence of the sheep fasciolosis through the faecal examination and excretory-secretory Ag-based ELISA in Faisalabad district, Punjab, Pakistan

Diagnostic tool	Spring	Summer	Monsoon	Winter
Faecal examination (mean eggs per gram; EPG values)	8.33 (894.00)	4.67 (654.67)	9.00 (616.33)	20.67 (851.67)
Excretory-secretory Ag- based ELISA	17.33	9.67	18.67	34.67

Figure 1. Mean EPG and month-wise prevalence of fasciolosis determined by faecal examination and excretory/secretory antigens based ELISA



DISCUSSION

Fasciola sp. has an across-the-border dispersion in Africa, South America, Eastern Europe, Middle East, Eastern and South Asia (Matanovic et al., 2007). Liver fluke is a significant parasite affecting6 to 7 million ruminants around the world every year (Coma et al., 2009). The death rates due to fasciolosis rely upon the type of illness, whether chronic or acute with a high death rate being recorded in the acute form (Reddington et al., 1986). The subclinical and chronic type of fasciolosis can lead to the reduced performance and the shrouded financial losses. In chronic infections, the economic losses occur due to the delayed puberty, lowered fertility rates, milk yield, meat production, birth weight, and quantity and quality of fleece (Soulsby, 1986; Ahmad et al., 2017).

The snail acts as an intermediate host of *Fasciola* sp. which propagates in the marshy areas close to the ponds and lakes. Fasciolosis has been reported in various areas of Pakistan by many researchers in different periods (Anjum et al., 2014; Rizwan et al., 2016; Ahmad et al., 2017; Zafar et al., 2019; Rizwan et al., 2019), however, the prevalence and association of fasciolosis with various factors in sheep population have not been well-documented in the district Faisalabad.

The prevalence of fasciolosis determined by Utuk et al. (2012) was 4.9% through faecal examination, 15.6% through an ES Ag-based ELISA and 16.2% through the commercial ELISA kit. The outcomes of the serological tests were seen to coincide while faecal assessment yielded a lower efficacy rate consistent with the review of literature. Besides, 41.8% of sheep recognized negative by sedimentation were seen as positive by sandwiched ELISA test. In a study conducted by Gonenc et al. (2004)in the Central Anatolia, all the seropositive animals (through western blot analysis) presented *Fasciola* sp. eggs during the faecal examination. This was decoded by specialists that the juvenile flukes serologically gave a positive response while moving to the liver parenchyma and as they were juveniles, no egg was found in the faecal samples. In our investigation, the contrasts between the faecal assessment and serological tests can be associatedwith the primary stage of infection.

The variation in the prevalence of *Fasciola* sp. may be due to the method used for the identification, availability of snails, temperature (> 9.5°C),location, sample size, annual rainfall rate, flooding during rainy season, humidity, soil moisture and outdated pasture management practices (Coma et al., 2009).The variations in the grazing practices (grazing near the marshy areas)and agro-climatic conditions also have sound

effects on the frequency distribution of *Fasciola* sp. in animals. All these factors directly or indirectly affect the propagation of *Fasciola* sp. Besides, adjustment of unhygienic measures, irrational utilization of the medicine and indiscriminate dealing of the livestock can contribute in the development of infection (Khan et al., 2011; 2013).

Similar to the present study, Hassan et al. (2011) and Rizwan et al. (2016) found a higher incidence of fasciolosis in adult animals than in the young stock. The higher predominance in adult sheep may be because of their standard grazing close to the marshy areas, compromised immunity of the host, prolonged contact with the infectious agents and long inactive stage in the final host (around 5 to 6 months). However, Anjum et al. (2014) and Zafar et al. (2019) recorded a higher rate of infection in younger animals. The higher predominance in young sheep may be expected due to the low level of immunity development in young sheep which are not completely grown up.

The females were seen as more prone to fasciolosis than males in other studies as well (Khan et al., 2013; Rizwan et al., 2016; Zafar et al., 2019).However, in some investigations directed by Khan et al. (2011) and Gebeyehu et al. (2014),an insignificant association betweenthe sex and abundance of fasciolosis was found. The possible reasons for higher prevalence of fasciolosis in females are: rearing of females for a long time increasing the exposure of infection, consistent change in physiological parameters especially during pregnancy and lactation leading to immunosuppression, unavailability of proper nutrition especially during production and reproduction phase of life and nonstop grazing of sheep in and around the marshy areas (Najib et al., 2020).

In the present study, breed of host showed insignificant association with fasciolosis; however, Anjum et al. (2014) in Pakistan and Munita et al. (2019) in Ireland found a significant association. Anjum et al. (2014) documented a significantly higher frequency in Kajli (46.32%) breed followed in order by Lohi (37.43%), Cholistani (8.24%) and Thalli (7.90%). In Pakistan, still there is very few documented data available regarding the susceptibility of fasciolosis in different breeds of sheep. However, the insignificant association of different breeds of sheep with fasciolosis might be due to the rearing and grazing of different breeds together (Anjum et al., 2014).

The range of EPG of flock determined by Martínez - Valladares et al. (2013) was 0-154 with a mean of 17.5± 33.9. About 21% of flocks showed a medium/high level of severity with mean EPG higher than 40. A study conducted by Duthaler et al., (2010) found 46.0% of animals infected with moderate infection, 45.1% infected with heavy infection and 3.5% with low infection. An insignificant association of severity of infection was determined by Carneiro et al. (2018) i.e. about 68%, 59.36% and65.88% animals showed mild, moderate and heavy infections, respectively. This variation in the severity of infection might be due to differences in grazing patterns, contamination of grazing areas, rate of rainfall and availability of snails.

In Iran, the highest prevalence of fasciolosis was determined in spring (8.3%) while the lowest (4.0%) in summer (Khanjari et al., 2014).In Pakistan, Anjum et al. (2014) noted a significant influence of time of year on the occurrence of fasciolosis i.e. highest in winter and lowest during summer. Because, the development of intermediate host snail and hatching of eggs of parasites require suitable temperature (> 9ºC), high humidity and rainfall (Taylor, 2012). In Pakistan, such environmental condition can be found in the monsoon when numerous parasites eggs hatch, propagation of snail's increases and afterward cercariae are produced, and are discharged on wet fields before encystation onto herbage. The frequency distribution of fasciolosis in different seasons has also been determined by various scientists (Khanjari et al., 2010; Ali et al., 2011; Rizwan et al., 2016). *Fasciola* sp. Inclines towards the temperate climatic regions as its 18-30-weeksof the lifecycle requires mild temperature and high moisture both for the propagation of the intermediate hosts and the free-living stages of the parasite (Relf et al., 2011; Ducheyne et al., 2015). The necessity of explicit climatic conditions for the completion of its lifecycle leads towards the cyclic variation in the animal disease (Bloemhoff et al., 2015).

In conclusion, although faecal examination is the "best method" and the gold standard in the diagnosis of sheep fasciolosis; however, serological tests must not be excluded especially the home-made ES Ag based ELISA which might be preferred and more affordable (Utuk et al., 2012).Likewise, the propagation of the snail, rainfall, soil moisture, and temperature are the significant elements affecting the growth of *Fasciola* sp. from egg to miracidium development. Even these physiological factors are directly influencing the rate of development of the fluke inside the snails and/or the final hosts (Najib et al., 2020). Hence, local and yearly

differences in the frequency distribution of *Fasciola* ap. are related to the fundamental climatic conditions. The diagnosis of fasciolosis by home-made ES Ag-based ELISA is effective and more affordable risk assessment tool in the field epidemiology. There is need to find the specific antigens from the ES product to reduce the chances of false positive results.

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