



RESEARCH ARTICLE

Seroprevalence of *Fasciola hepatica* in Small Ruminants of District Chakwal, Punjab, Pakistan

Arsalan Zafar¹, Muhammad Kasib Khan^{1*}, Zia ud Din Sindhu¹, Rao Zahid Abbas¹, Sabiqaa Masood², Zaheer Abbas¹, Muhammad Shahid Mahmood³, Muhammad Kashif Saleemi⁴, Junaid Ali Khan⁵, Riaz Hussain⁶, Muhammad Usman Naseer¹, Zafar Iqbal¹ and Hasnain Javed⁷

¹Department of Parasitology, University of Agriculture, Faisalabad-38040, Pakistan; ²Department of Parasitology, Cholistan University of Veterinary and Animal Sciences, Bahawalpur; ³Institute of Microbiology; ⁴Department of Pathology; ⁵Institute of Physiology and Pharmacology, University of Agriculture, Faisalabad-38040, Pakistan; ⁶University College of Veterinary and Animal Sciences, The Islamia University of Bahawalpur-Pakistan; ⁷Molecular biologist Advance Diagnostic lab Punjab AIDS Control Program

*Corresponding author: mkkhan@uaf.edu.pk

ARTICLE HISTORY (18-229)

Received: June 30, 2018
Revised: February 10, 2019
Accepted: February 17, 2019
Published online: February 21, 2019

Key words:

ELISA
Epidemiology
Goat
Liver fluke
Sheep

ABSTRACT

Fascioliasis, caused by *Fasciola (F.) hepatica*, affects a large number of animals in terms of morbidity, production losses, treatment cost and high mortality rate. Economic losses of about 3.6 billion US\$ per year have been reported due to this parasite throughout the world. The present study was planned to investigate the prevalence and related risk factors of *F. hepatica* in sheep and goat population in District Chakwal, Punjab, Pakistan. To this end, a total of 384 serum samples were collected from sheep and goats in different tehsils of District and examined through enzyme-linked immunosorbent assay (ELISA) by using Excretory/Secretory antigen coated plates. A predesigned questionnaire was also used to collect the data of related risk factors. The overall prevalence of fascioliasis in the small ruminant population was 37.24%. The highest prevalence was observed in tehsil Talla Gang, followed by Chakwal, Kallar Kahar and Choa Saidu Shah. Fascioliasis was more prevalent in females as compared to males. According to age groups, the prevalence was higher in animals with 6-12 months of age. At the species level, sheep had higher prevalence as compared to goats and this prevalence was higher in animals went for grazing than those fed through stalls. Among different breeds of goats, Teddy breed had a higher prevalence of *F. hepatica* than those of Beetal and mixed breeds of goat.

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To Cite This Article: Zafar A, Khan MK, Sindhu ZUD, Abbas RZ, Masood S, Abbas Z, Mahmood MS, Saleemi MK, Khan JA, Hussain R, Naseer MU, Iqbal Z and Javed H, 2019. Seroprevalence of *Fasciola hepatica* in small ruminants of District Chakwal, Punjab, Pakistan. Pak Vet J, 39(1): 96-100. <http://dx.doi.org/10.29261/pakvetj/2019.024>

INTRODUCTION

Parasitic diseases have been great threat in livestock production systems (Abbas *et al.*, 2017a, 2017b, 2017c, 2018; Mehmood *et al.*, 2017; Naqvi *et al.*, 2017; Hanem *et al.*, 2018). Fascioliasis is an economically important disease of sheep and goat, which is caused by *Fasciola (F.) hepatica*, a flatworm belonging to class Trematoda. This disease causes annual economic losses of US\$ 3.6 billion by affecting sheep, cattle and other livestock species in the form of morbidity and mortality throughout the globe (Anjum *et al.*, 2014; Kaplan, 2001). Fascioliasis also has zoonotic importance with more than 90 million people infected all over the world (Keiser and Utzinger, 2005). The disease is more prevalent in tropical and sub-

tropical countries, particularly in Pakistan, Bangladesh, India, Turkey and Iraq. The prevalence of fasciolosis in Pakistan varies in different areas due to different climatic conditions and animals grazing habits. The highest prevalence of fascioliasis was reported in Peshawar i.e. 55% and a comparatively low prevalence was recorded in Multan and Bahawalpur Districts of south Punjab i.e. 23.97% and 17.68%, respectively. The lowest prevalence (10.48%) in Pakistan was documented in Lahore city in central Punjab region (Khan *et al.*, 2009).

Metacercariae is the infective stage for the mammalian host, which is transmitted by ingestion of infected aquatic snail i.e. *Galba truncatula* (Most common snail species in Europe, Africa, Asia and South America) and contaminated fodder. Infection mainly

occurs in two forms, first is parenchymal or acute form which involves the subsequent migration of juvenile through the liver parenchyma resulting in extensive liver damage, necrosis (liver rot), urticaria, eosinophilia, haemorrhages, anaemia and eventually death. The biliary or chronic form is the second form, which involves the obstruction of bile duct due to mature flukes resulting in biliary obstruction, cholangitis, jaundice, hyperplasia of biliary epithelium and hardened liver due to massive fibrosis. However, the mortality rate is higher in the acute form of fascioliasis. Most common clinical signs in sheep and goat include bottle jaw, anemia, emaciation and ascites, which leads to high economic losses in the form of decreased milk production, wool production, low weight gain and increased mortality (Robinson and Dalton, 2009). The severity of infection mainly depends upon the immune response, age of the host and the number of metacercariae ingested (Coma *et al.*, 2009). The excretory-secretory products of this parasite such as cathepsin and thioredoxin peroxidase cause immunosuppression within the host, which increase the severity of the disease and make the host more susceptible to other bacterial infections (Keusch, 1982; Robinson *et al.*, 2009).

Diagnosis of this parasite is mostly done by a conventional method, in which the eggs of the parasite are identified in faeces. Nevertheless, this technique may help in species identification but, this method fails in early diagnosis as major pathogenic effect occurs before the egg shedding by liver flukes. However, for the early diagnosis, serological technique is much beneficial, as the antibodies produced in response to the antigens present in the tegument of juvenile can be detected by this method, which makes this technique more sensitive (Espino *et al.*, 1987; Guobadia and Fagbemi, 1995; Itagaki *et al.*, 2010). Many serodiagnostic methods have been developed for fascioliasis, which includes indirect haemagglutination, enzyme-linked immunosorbent assay (ELISA), immunoprecipitation and indirect fluorescence antibody test. Among these techniques, ELISA is a more specific and sensitive technique for early diagnosis of fascioliasis. Moreover, ELISA kits are commercially available and are suitable for diagnosis at farm level (Awad *et al.*, 2009; Rizwan *et al.*, 2016).

In Pakistan, several studies have been conducted to evaluate the occurrence of fascioliasis in different regions of the country (Anjum *et al.*, 2014; Rizwan *et al.*, 2016). However, no study has been conducted so far on the occurrence of *F. hepatica* in District Chakwal, which indicates a dire need of surveillance in the study area so that effective policy could be made to maximize the profitability of farming community. So, keeping in view the economic importance of fascioliasis and huge population of sheep and goat in study area, this study was planned to check prevalence and associated risk factors of disease through ELISA and a questionnaire based survey.

MATERIALS AND METHODS

Study area and sample size: District Chakwal is located at 32.93° latitude towards North and 72.85 longitude towards East, at a height of 498 meters above the sea level with the semi-hilly landscape. All four season can be observed in district Chakwal namely; Spring, Summer, Autumn and

Winter. The average minimum temperature in the district is 7°C, while the average maximum temperature is 40°C. The climate of this district is mostly rainy and humid with the average humidity level of 65.8%.

During the study period (2016), district Chakwal was divided into four tehsils (administrative regions), namely Chakwal, Tala Gang, Choa Saiden Shah and Kallar Kahar. The sample size (n=384 sheep and goat) was calculated by using the following equation with 50% expected prevalence at a 95% confidence interval and 5% desired absolute precision (Thrusfield, 2007).

$$n = \frac{1.962 P_{exp} (1 - P_{exp})}{d^2}$$

Where, n = required sample size; P_{exp} = Expected prevalence, d = Desired absolute precision

Determination of associated risk factors: A questionnaire was prepared based on dichotomous type questions to check the associated risk factors. The questionnaire was refined through formal and informal testing, including interviews and personal findings. The factors like; age, sex, breed, location, feeding pattern etc. were included in the predesigned questionnaire.

Antigen preparation and ELISA: Adult *F. hepatica* was collected from the livers of infected sheep from abattoirs and washed with sterile phosphate buffer saline (PBS). The E/S antigen was prepared by following the method described by Kooshan *et al.* (2010). After extraction, E/S antigens were stored at -20°C for further use in ELISA. For seroprevalence, blood samples were collected randomly from 384 sheep and goat (203 sheep and 181 goats) from the jugular veins in 3ml serum collecting vacutainers using sterile needles (22-27gauge). Collected blood samples were centrifuged at 3000x g and 20°C for 15 min and sera were stored at -20°C until used.

The E/S antigens were diluted in 0.125, 0.25, 0.5 and 1µl/ml carbonate buffer (pH 9.5). Polystyrene microtiter plates were coated with 100µl of diluted antigen per well. After incubation for 1h, plates were sensitized overnight at 4°C. Wells were washed five times for 5 min, with 300µl PBS and 0.05% Tween-20 (PBS-T). Then, 200µl of 1% BSA in PBS-T was added to each well as blocking buffer and incubated at room temperature for 1h. Serum samples were diluted with PBS-T with the ratio of 1:10, 1:20, 1:50, 1:100 and 1:500. Then 100µl of diluted serum was added to each well and incubated at room temperature for 30 min. After further washing, 100µl anti-sheep IgG peroxidase conjugate diluted at 1:2000 and 1:5000 in PBS-T was added to each well. The plates were incubated at room temperature for 30 min and washed as described earlier. Furthermore, 100µl of substrate solution containing TMB/H₂O₂ was added to each well and the plate was incubated for 15 min in darkness at room temperature. The reaction was stopped with 1M sulfuric acid. The absorbance was measured at 492nm using a microplate ELISA reader. In the plate, 2 wells as a blank (without serum), 4 wells as a negative control (sera collected from newborn lamb) and 4 wells as a positive control (positive sera already present in the laboratory) were used (Kooshan *et al.*, 2010).

Statistical analysis: A multiple logistic regression method was used to analyze the differences among the variables. All statistical procedures were carried out using SAS software package at 95% level of confidence (Thrusfield, 2007).

RESULTS

The concentration of E/S antigens was found 30µg/ml. The cut-off value was calculated as 0.502 using the following equation (Rizwan *et al.*, 2016);

$$\text{(Cut-off Value= } 0.38 + 2(0.061)$$

Where; 0.38 was the mean value of a negative sample and 0.061 was the standard deviation. The serum samples of OD value more than 0.502 were considered positive. The overall prevalence of *F. hepatica* was found at 37.23% (143/384). A significantly higher prevalence ($P<0.05$) was observed in Tehsil Talla Gang followed by Tehsil Chakwal, Kallar Kahar and Choa Saiden Shah (Table 1). Among sheep and goat, the prevalence of 41.87% was observed in sheep, while 32.04% prevalence was observed in goats. Detailed results of sheep and goat prevalence have been presented in Table 2.

Table 1: Overall prevalence of fascioliasis in different Tehsils of district Chakwal

Tehsils	Examined	Positive	Prevalence (%)	Odd ratio	P value
Chakwal	100	37	37.00	1.39	0.2696
Talla gang	102	48	47.06	2.12	0.0117
Choa Saiden Shah	98	29	29.59	-	-
Kallar Kahar	84	29	34.52	1.27	0.4427
Overall	384	143	37.24	-	-

Table 2: Species wise prevalence of fascioliasis in different Tehsils of district Chakwal

Tehsil	Species	No. of examined	No. of positive	Prevalence (%)
Chakwal	Sheep	56	22	39.29
	Goat	44	15	34.09
Talla Gang	Sheep	62	33	53.23
	Goat	40	15	37.50
Choa Saiden Shah	Sheep	46	16	34.78
	Goat	52	13	25.00
Kallar Kahar	Sheep	39	14	35.90
	Goat	45	15	33.33
Overall	Sheep	203	85	41.87
	Goat	181	58	32.04

Host wise prevalence of fascioliasis

Prevalence of fascioliasis in sheep: Out of 203 Sheep, 85 were positive indicating the prevalence of 41.87%. Among four tehsils, which were included in the study, the highest prevalence of 47.06% was recorded in tehsil Talla Gang followed in decreasing order by Chakwal, Kallar Khar and Choa Saiden Shah. Among different associated risk factors, prevalence was higher ($P<0.05$), (64.70%) in female animals of tehsil Talla Gang, followed by Kallar Kahar (52.38%), Chakwal (50.00%) and Choa Saiden Shah (48.00%) as shown in Table 3. As far as the age of the animal is concerned, prevalence was significantly higher ($P<0.05$) in the age group of 6-12 months as compared to the other age groups in all four tehsils. Similarly, the prevalence was significantly higher

($P<0.05$) in the grazing animals as compared to stall feeders. The highest prevalence (66.66%) of fascioliasis was observed in grazers of tehsil Tala Gang followed in order by Chakwal (51.61%), Kalar kahar (50.00%) and Choa Saiden Shah (48.14%).

Prevalence of fascioliasis in goat: Out of 181 Goat 58 were found positive, indicating the 32.04% prevalence of fascioliasis in the goat. Complete results on the association of different risk factors in the prevalence of fascioliasis in goats have been presented in Table 4. As far as the age of the animal is concerned, prevalence was significantly higher ($P<0.05$) in the age group of 6-12 months as compared to the other age groups in all four tehsils. Similarly, the prevalence was significantly higher ($P<0.05$) in the grazing animals as compared to stall feeders. The highest prevalence (60.00%) of fascioliasis was observed in grazers of tehsil Tala Gang followed in order by Kalar kahar (48.00%), Chakwal (45.83%) and Choa Saiden Shah (37.03). Regarding the breed of goat population in District Chakwal, significantly higher prevalence ($P<0.05$) was found in Teddy breed as compared to Beetal and Mix breeds of all the Districts.

DISCUSSION

Liver fluke is very important parasite infecting six to seven million ruminants globally per year (Coma *et al.*, 2009). The mortality rates depend on the form of disease whether acute or chronic with high mortality rate being recorded in acute form (Reddington *et al.*, 1986). The snail acts as an intermediate host of *F. hepatica* that survives in marshy areas near lakes and ponds. Fascioliasis has been prevalent in different regions of Pakistan reported by different scientists in a different era (Sahar, 1996), however, the prevalence of fascioliasis in small ruminants has not been studied before in District Chakwal.

During present study, it was found that grazing animals have a significantly high rate of infestation as compared to stall feed. This could be due to the reason that the infective stage of parasite travel to grass blades, which can be easily transferred to grazing animals via ingestion as reported by Khan *et al.*, 2009. The stall feeders have a low prevalence of *Fasciola* spp. and even this feeding method is used to control fascioliasis in Nepal (Mahato and Harrison, 2005). The main contributing factors of high prevalence in Tehsil Talla Gang may include (i) trend of grazing practice in small ruminants (ii) presence of a high number of ponds and dams and (iii) increased number of sheep, which are assumed as deep browsers. The ponds and lakes are also very common in Tehsil Chakwal with heavy rainfall, ultimately leading to a high prevalence of fascioliasis in this tehsil (Chanie and Begashaw, 2012).

Age-wise prevalence was significantly higher in the group of sheep of less than 6-12 months of age in all four Tehsils, Chakwal, Talla Gang, Choa Saiden Shah and Kallar Kahar. Similarly, fascioliasis was more prevalent in the age group of 6-12 months of goats in all four Tehsils, Chakwal, Talla Gang, Choa Saiden Shah and Kallar Kahar.

Table 3: Determination of associated risk factors in sheep species of District Chakwal

Risk factors	Categories	Animals examined	Positive animals	Negative Animals	Prevalence %	Odd Ratio	p-value
Tehsil Chakwal							
Gander	Male	22	5	17	22.72	-	-
	Female	34	17	17	50	3.4000	0.0461
Age (months)	>12	22	6	16	27.27	0.8889	0.8859
	6-12	21	13	8	61.90	5.41	0.0340
	<6	13	3	10	23.07	-	-
Feeding Pattern	Stall feed	25	6	19	24.00	-	-
	Grazing	31	16	15	51.61	3.3778	0.0392
Tehsil Tala Gang							
Gander	Male	28	11	17	39.28	-	-
	Female	34	22	12	64.70	2.83	0.0485
Age (months)	>12	28	11	17	39.28	-	-
	6-12	27	19	8	70.37	3.6705	0.0230
	<6	7	3	4	42.85	1.1591	0.0230
Feeding Pattern	Stall feed	20	5	15	25	-	-
	Grazing	42	28	14	66.66	6.0000	0.0034
Tehsil Choa Saidu Shah							
Gander	Male	21	4	17	19.04	-	-
	Female	25	12	13	48	3.9231	0.0460
Age (months)	>12	18	3	13	16.66	-	-
	6-12	16	9	7	56.25	5.5714	0.0351
	<6	12	4	8	33.33	2.1667	0.3829
Feeding Pattern	Stall feed	19	3	16	15.78	-	-
	Grazing	27	13	14	48.14	4.9524	0.0301
Tehsil Kalar Kahar							
Gander	Male	18	3	15	16.67	-	-
	Female	21	11	10	52.38	5.5	0.0266
Age (months)	>12	16	3	13	18.75	-	-
	6-12	15	9	6	60	6.5000	0.0240
	<6	8	2	6	25	0.3333	0.3204
Feeding Pattern	Stall feed	17	3	14	17.64	-	-
	Grazing	22	11	11	50	4.6667	0.0443

Table 4: Determination of associated risk factors in goat species of District Chakwal

Risk factors	Categories	Animals examined	Positive animals	Negative Animals	Prevalence (%)	Odd Ratio	p-value
Tehsil Chakwal							
Gander	Male	19	3	16	15.78	-	-
	Female	25	12	13	48.00	0.171	0.028
Age (months)	>12	16	2	14	12.50	-	-
	6-12	18	11	7	61.11	11.0000	0.0075
	<6	10	2	8	20	1.7500	0.6089
Feeding Pattern	Stall feed	20	4	16	25.00	-	-
	Grazing	24	11	13	45.83	3.3846	0.0785
Breed	Beetal	13	2	11	15.38	-	-
	Teddy	14	10	4	71.42	13.7500	0.0069
	Mix	17	3	14	17.64	1.1786	0.8692
Tehsil Tala Gang							
Gander	Male	20	4	16	20	-	-
	Female	20	11	9	55	4.8889	0.0269
Age (months)	>12	18	4	14	22.22	-	-
	6-12	16	9	7	56.25	4.5000	0.0474
	<6	6	2	4	33.33	1.7500	0.5888
Feeding Pattern	Stall feed	20	3	17	15	-	-
	Grazing	20	12	8	60	8.5000	0.0058
Breed	Beetal	13	3	10	30.00	-	-
	Teddy	9	6	3	66.66	6.67	0.0496
	Mix	18	5	12	27.77	1.1250	0.8802
Tehsil Choa Saidu Shah							
Gander	Male	27	3	24	11.11	-	-
	Female	25	10	15	40	5.3333	0.0229
Age (months)	>12	23	3	20	13.04	-	-
	6-12	19	8	11	42.10	4.8485	0.0414
	<6	10	2	8	20	1.6667	0.6110
Feeding Pattern	Stall feed	25	3	22	12	-	-
	Grazing	27	10	17	37.03	4.3137	0.0462
Breed	Beetal	23	3	20	13.04	-	-
	Teddy	19	8	11	42.10	4.8485	0.0414
	Mix	10	2	8	20	1.6667	0.6110
Tehsil Kalar Kahar							
Gander	Male	23	4	19	17.39	-	-
	Female	22	11	11	50.00	4.75	0.0252
Age (months)	>12	16	3	13	18.75	-	-
	6-12	18	10	8	55.55	5.000	0.0445
	<6	11	2	9	18.18	0.8889	0.9075
Feeding Pattern	Stall feed	20	2	18	10	-	-
	Grazing	25	12	13	48	8.3077	0.0123
Breed	Beetal	16	3	13	18.75	-	-
	Teddy	12	7	5	58.33	6.0667	0.0378
	Mix	17	5	12	29.41	1.8056	0.4780

Most of the farmers take animals of this age group for grazing, thus, there are more chances of interaction with an intermediate host (Mor and Cardenas, 2010). Moreover, the immune system of younger animals is not fully developed, so they pass through a primary immune response that depends on the age of the animal (Anjum *et al.*, 2014). In contrary to the present study, a higher prevalence in older animals has been reported by (Hassan *et al.*, 2011).

Overall breed wise prevalence was higher in Teddy breed of goat because Teddy breed is considered to be less precious as compared to Beetal and Mixed breed of goats in the study area. Deworming schedule was followed in Beetal and Mixed breeds in the study area, which could be the possible factor of low prevalence in these breeds (Roberts and Suhardono, 1996). It was also reported that teddy breed may be less resistance to fascioliasis as compared to other breeds (Tasawar *et al.*, 2007). As in another study, Tasawar *et al.* (2011) reported a higher prevalence of toxoplasmosis in teddy breed as compared to other breeds of goats. So, it may be concluded that genetic variation and resistance to infection were the main reasons for such higher prevalence.

From the results of this study, it may be concluded that sheep and goat fascioliasis is prevalent in all areas of District Chakwal. Effective measures should be adopted by authorities for control of this economically important parasite. Farmers of teddy breed should be encouraged to adopt an effective deworming program.

Authors contribution: AZ, SM, ZuDS and HJ wrote the manuscript, while ZI and RZA analyzed the data. ZuDS, MUN, MKS, MSM, RH, JAK and MKK reviewed and edited the manuscript. ZA helped in the collection of samples from the study area.

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