The Journal of Animal & Plant Sciences, 30(3): 2020, Page: 775-779
ISSN (print): 1018-7081; ISSN (online): 2309-8694

Short Communication

IDENTIFICATION OF FASCIOLIDS AT DIFFERENT ALTITUDES OF AZAD JAMMU AND KASHMIR

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ABSTRACT

Fasciolosis is an important zoonotic disease affecting domestic animals in most parts of the world. Two important species of flukes *Fasciola (F.) gigantic* and *Fasciola hepatica* with an intermediate form was reported by many researchers from different countries of the world. For identification of species of fasciolids 167 flukes were collected from domestic animals at abattoirs from three topographic locations from three districts of Azad Jammu and Kashmir during 2012. Readings were taken using glass rulers and ocular micrometer. Characterization of *F. hepatica* revealed average length (mm) 20.66±2.27 to 23.20±5.25 and width 9.90±0.50 to 10.16±1.00 and that of *F. gigantic* 41.25±1.55 to 45.33±0.83, 9.44±0.20 to 9.71±0.37 respectively. It was noted that *F. hepatica* predominantly affecting animals at high altitudes with cooler climate and *F. gigantica* at low altitudes with warm conditions. No "intermediate form" of fasciola was found at any location or host animal. All flukes after morphometery were identified through PCR using species specific primers. Further investigations are required to investigate intermediate form from different geographic locations of Pakistan for control measures.

Keywords: *Fasciola* species; Morphometery; Altitudes; topographic locations; Azad Kashmir

https://doi.org/10.36899/JAPS.2020.3.0090 *Published online March 25, 2020*

INTRODUCTION

Fasciolosis is an important human health concern affecting domestic and wild animals in many parts of the world (Iyiola *et al.* 2018). *F. hepatica* was reported from temperate and F. *gigantica* from tropical zones, overlapped in subtropical areas (Abdulwahed and Al-Amery 2019). Human infection occurs accidentally most frequently in sheep and cattle rearing families. Proper identification based on morphology of species is necessary for understanding pathogenesis. Moreover use of molecular techniques was helpful to identify intermediate subspecies (Shahbakhsh *et al.* 2016). Both species have different epidemiological characteristics where *F. hepatica* is less pathogenic than *F. gigantic.* The reason is smaller in size instead of genetic characters. Pathogenesis may be misleading where intermediate form is present and may have significant complications in the area (Aryaeipour *et al.* 2017). Identification of species can be made on the basis of morphology or molecular analysis however, microscopic analysis is recommended. Molecular techniques have greater sensitivity but not cost effective for routine use (El-Rahimy *et al*. 2012). Characterization of fasciola species is useful for epidemiology, prevention and control of disease in endemic areas where hybridization phenomenon occurs (Akhlaghi *et al.* 2017). Such hybrids/ intermediate forms were reported from Korea (Agatsuma *et al.* 2000), Iran (Amor *et al*. 2011), Veitnam (Le *et al*. 2008), Egypt (Amer *et al*. 2011) and China (Peng *et al*. 2009). Recent studies found F. *gigantica* to be more pathogenic and widespread in Pakistan (Afshan, *et al* 2014). Accurate diagnosis of the disease needs identification of the species of the fluke involved for assessment of the risk factors and control strategy (Khan *et al.* 2009). Data on morphometric and molecular identification at species level are lacking in Pakistan which prompted this study to be carried out for morphometric measurements of the two species and conformation through PCR.

MATERIALS AND METHODS

Geographic locations and sampling: Adult liver fluke samples (n=167) were collected from affected livers of sheep, buffalo, cattle and goats. These sample were collected from three districts (Table 1&2) based on altitudes, A) Mirpur (<3000 feet), B) Neelam (>6000) and C) Poonch (3000-6000). Collected samples were preserved in physiological saline immediately after removal from bile duct, labelled and shifted to University of Health Sciences, Lahore for phenotypic and genetic identification.

Morphometric measurements: Flukes were flattened and clamped between two glass slides, fixed with formalin (10%) and kept in refrigerator. After 12

hours specimen were washed to remove debris and traces of formalin and separated from slides. Borex caramine staining was done and dehydrated for 15 minutes each in 70%, 80%, 95% and absolute alcohol. Samples were mounted in Canadabalsam as described by Periago *et al.* (2006). Measurements of specimen included body length, width, diameter of suckers and distance between oral and ventral suckers were made using eye piece micrometer and glass ruler. The average of each parameter was calculated along with standard error.

Molecular identification: Specimens collected from three topographic locations (n=50) were used for PCR. Specimens already identified on the basis of morphometery were fixed in ethanol until DNA extraction. GeneAll DNA extraction kit (GeneAll Biotechnology Co. Ltd, Korea) was used for extraction. PCR was done with specific primers reported by (Mc-Garry *et al.* 2007).

PCR Conditions: Denaturation 94ºC for 1min. Annealing 52ºC for 1 min. Extension 72ºC for 2 min. followed by 10min of final extension at 72ºC.

RESULTS

Mean worm load per liver in small and large ruminants was 11.92 with maximum load of 22. The average worm count of *F. hepatica* depicted 10.37 as compared to 14 for *F. gigantic.*

Both species were found overlapped at lower altitudes and warm climate. *F. gigantic* was not encoutered at high altitude however, *F. hepatica* found present in livers of infected animal at all topographic locations. *F. hepatica* measurements revealed body length 20.55±1.09 to 22.73±2.01mm at altitude >6000 feet, 19.81±1.35 to 22.50±2.73 mm at altitude 3000-6000 feet and 20.66±2.27 to 23.2±5.25 mm at altitude <3000 feet (Table 1). Body length of *F. gigantic* was 42.23±0.95 to 45.33±0.83 mm at altitude 3000-6000 feet and 41.25±1.55 to 41.54±1.68 mm at <3000 feet (Table 2). The body length showed a marked difference in the size of the two species. The body width of *F. hepatica* ranged from 9.90±0.50 mm to 10.16±1.00 mm as compared to 9.44±0.20 to 9.71±0.37 mm of *F. gigantica*. The diameters of suckers and distance between them were not found to be a reliable tool for differentiation however, distance between sucker (ventral) and taper end of the body was quite different in two species. This value for *F. hepatica* was 19.83±5 mm as compared to *F.gigantica*37.65±1.4 mm.

PCR product revealed 391 bp sizes for *F. hepatica* with specific primer and did not generate any product when DNA of *F. gigantica* was used. DNA of *F. gigantic* generated 235bp amplification product with specific primer and no product generated using non specific primer (Fig. 1). Hence *Fasciola* species identified on the basis of morphological parameters were confirmed with PCR.

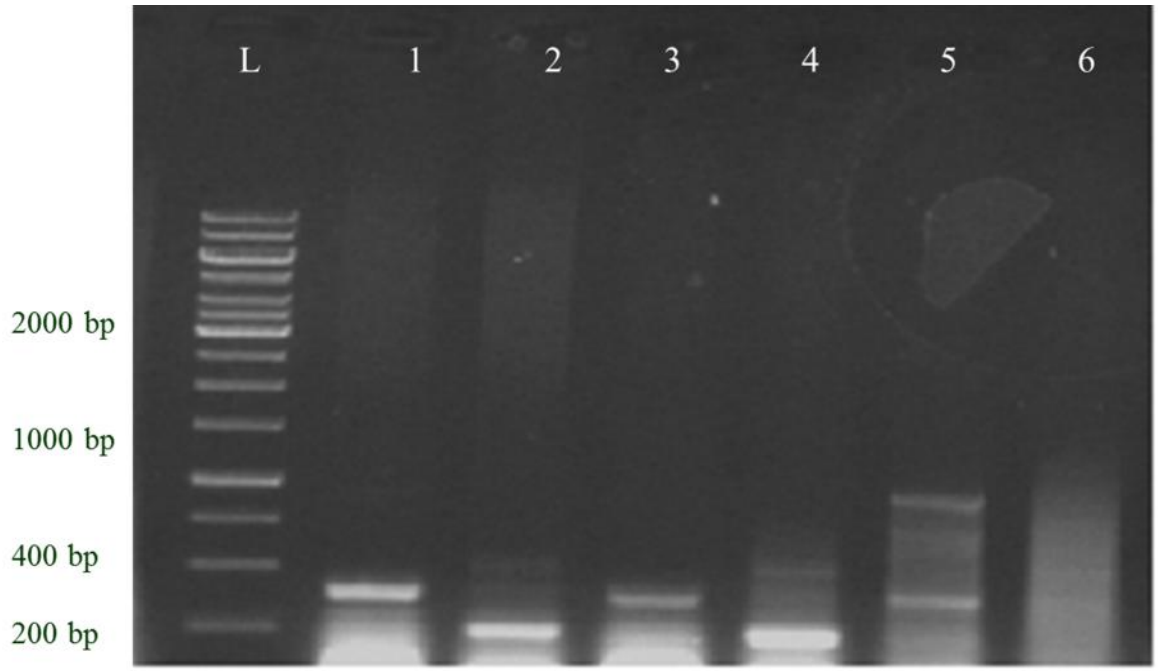

Figure 1. Amplification of DNA of fasciola species in AJK.
Lane 1. *F.hepatica* sheep Lane 2. *F.gigantica* goat Lane 3. *F.hepatica* goat Lane 4.*F.gigantica* Buffalo Lane 5. *F. hepatica* sheep Lane 6. Control -ve

Table 1. Morphometery of *Fasciola hepatica* at different geographic locations and hosts in AJK

GA	D	Alt	HS	NF	BL	BW	OS	VS	D-OS/VS	D-VS/P
					Avg±SE	Avg±SE	Avg±SE	Avg±SE	Avg±SE	Avg±SE
Sharda	Neelam	6499	Goat	22	21.32±1.14	10.05±0.24	0.76±0.008	1.27±0.02	1.40±0.06	17.93±1.16
Machal	Neelam	7350	Sheep	08	22.73±2.01	10.13±0.48	0.83±0.01	1.31±0.04	1.21±0.17	19.63±2.14
Ghamoot	Neelam	7800	Cow	17	20.55±1.09	10.15±0.11	0.73±0.01	1.29±0.02	1.43±0.09	17.53±1.25
Rawalakot	Poonch	5374	Sheep	14	19.81±1.35	10.05±0.20	0.71±0.01	1.25±0.03	1.24±0.06	16.83±1.27
Rawalakot	Poonch	5374	Cow	08	21.51±1.88	10.06±0.30	0.75±0.04	1.33±0.04	1.39±0.14	18.32±2.17
Hajeera	Poonch	3168	Goat	05	22.50±2.73	9.90±0.50	0.69±0.06	1.20±0.07	1.31±0.21	19.30±2.73
Mirpur	Mirpur	1503	Goat	03	23.20±5.25	10.16±1.00	0.74±0.08	1.31±0.11	1.47±0.43	19.83±5.21
Mirpur	Mirpur	1503	Calf	06	20.66±2.27	10.0±0.51	0.72±0.03	1.34±0.04	1.38±0.20	17.60±2.79

GA: Geographical Area; D: District; Alt: Altitude (feet); HS: Host Species; NF: Number of Flukes; BL: Body Length; BW: Body Width; OS: Oral Sucker; VS: Ventral Sucker**;3**
D-OS/VS: Distance between OS/VS, D-VS/P Distance between VS & posterior end of the body; (Measurements in mm)

Table 2. Morphometery of *Fasciola gigantica* at different geographic locations and hosts in AJK

GA	D	Alt	HS	NF	BL	BW	OS	VS	D-OS/VS	D-VS/P
					Avg±SE	Avg±SE	Avg±SE	Avg±SE	Avg±SE	Avg±SE
Mirpur	Mirpur	1503	Cow	13	41.54±1.68	9.64±0.38	0.94±0.04	1.60±0.07	1.67±0.06	38.21±1.62
Dadyal	Mirpur	1503	Goat	12	41.50±1.12	9.71±0.37	0.91±0.02	1.48±0.07	1.67±0.08	38.25.9±1.37
Rawalakot	Poonch	5374	Goat	21	42.23±0.95	9.44±0.20	0.89±0.01	1.36±0.04	1.57±0,06	38.73±0.98
Khaigala	Poonch	5500	Cow	10	43.50±1.32	9.50±0.12	0.87±0.04	1.62±0.09	1.76±0.07	39.87±1.37
Hajeera	Poonch	3168	Buffalo	19	45.33±0.83	9.66±0.25	0.85±0.02	1.71±0.07	1.94±0.06	41.73±0.91
Jatlan	Mirpur	1377	Goat	09	41.25±1.55	9.57±0.42	0.92±0.51	1.64±0.12	1.62±0.08	37.65±1.48

GA: Geographical Area; D: District; Alt: Altitude (feet); HS: Host Spescies; NF: Number of Flukes; BL: Body Length; BW: Body Width; OS: Oral Sucker; VS: Ventral Sucker; D-OS/VS: Distance between OS/VS, D-VS/P Distance between VS & posterior end of the body;(Measurements in mm)

DISCUSSION

Livestock farming is important in sustainable economic development in developing world. Fasciolosis is a serious challenge to livestock particularly sheep and goats farming worldwide because of its high occurrence. Moreover incidence of human infection is high in some countries of high animal infections (Amer *et al.* 2016). Being a zoonotic disease with limitations in drugs availability for treatment control measures are urgently needed (Knubben-Schweizer and Torgerson 2015). Data on genetic and phenotypic characteristics of Fasciola species is limited in the state of AJK. Present study elucidated such characteristics of Fasciola species infecting large and small ruminants from different localities. Morphometric features of two species were studied to identify presence of hybrid form where overlapping occurs. Parameters like specimen length, width, diameter of two suckers were considered practical criteria for differentiation between species of liver flukes (Akhlaghi *et al.* 2017). Results of present study indicated that measurements differed considerably between two species at three different topographic locations. Comparable results were reported from different host animals (Ashrafi *et al.* 2006) and (Yakhchali *et al*. 2015).

Microscopic measurements are helpful in characterization of liver flukes in areas with low occurrence. However, in countries where presence of intermediate forms such as Japan and Korea cannot be classified as *F. gigantica* and *F. hepatica* (Itagaki *et al*. 2005). Hybrids of two species were listed by different researchers (Afshan *et al* 2014; Ai *et al.* 2010; Itagaki *et al*. 2009; Ali *et al*. 2008).

In Asian countries like Pakistan overlap occurs in areas of different altitudes with movements of animals in summer and winter. Prompt control strategies are required to asses overlap scenario and to identify hybrid forms of *Fasciola* (Afshan *et al*. 2014). Present study depicted existence of overlap in two districts but no intermediate form was found at any geographic location. These findings were in agreement with (Mas-Coma *et al*. 2009), as they found *F. gigantica* and *F. hepatica* as only species of liver flukes in areas with overlapping. The present study concludes that flukes collected from high altitudes were found as *F. hepatica* and at lower altitudes *F. gigantic*. *F. hepatica* population decreased as altitude decreased and *F.gigantica* was not present in animals of district Neelam (high altitudes). However, mixed infection found in animals of district Mirpur (lower altitudes). No intermediate form of the *Fasciola* was encountered at any geographical location or host species of animals.

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