Pak. J. Agri. Sci., Vol. 52(1), 183-189; 2015
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
http://www.pakjas.com.pk

EVALUATION OF COMMERCIAL ELISA KIT FOR DIAGNOSIS OF SMALL RUMINANT FASCIOLIASIS IN PAKISTAN

Rubina Anjum[1,*], Muhammad Nisar Khan[1], Muhammad Sohail Sajid[1] and Muhammad Tariq Javed[2]

[1]Department of Parasitology, University of Agriculture, Faisalabad-38040, Pakistan;
[2]Department of Pathology, University of Agriculture, Faisalabad-38040, Pakistan
[*]Corresponding author's e-mail: *rubinadr@gmail.com*

ELISA is a serological assay claimed for higher sensitivity and specificity in diagnosing sub-clinical fascioliasis. The purpose of this investigation was to evaluate the sensitivity and specificity of an ELISA kit (DRG®, Germany) for the diagnosis of small ruminant fascioliasis in district Sargodha, Punjab, Pakistan. Microscopically, 226 animals out of 600 (37.66%) were found positive for fascioliasis. Higher prevalence (38.78%; 121/312) was found in goats as compared to sheep (36.46%; 105/288). Prevalence of *Fasciola hepatica* was significantly higher as compared to *Fasciola gigantica* ($p<0.005$). Commercial ELISA kit detected anti-fasciola antibodies in higher percentage in sheep and goat population as compared to coprological examination. Seroprevalence was 48.61 and 49.36% in sheep and goat population. Commercial ELISA kit was more specific than coprological examination when compared in Win episcope by screening of comparative diagnostics.

Keywords: Clinical diagnosis**,** ELISA, goats, helminth infection.

INTRODUCTION

Fascioliasis is the most common and economically important helminth infection of livestock in Punjab, Pakistan (Khan *et al*., 2009). Ahmad (2008) reported US$ 0.36 and 0.17 million in economic losses due to liver condemnation and weight reduction in small ruminants of the district of Sargodha. Other factors associated with economic losses are decreased wool production, decreased milk yield and reduced fertility (Rioux *et al*., 2007). It is important as it has to multi definitive host species including livestock, human and wild animals (Rondelaud *et al*., 2001). The most important species of parasite include *Fasciola (F.) hepatica* and *F. gigantica* (Soulsby, 1982).

It has already been reported that fascioliasis is prevalent in livestock of district Sargodha (Maqbool *et al*., 2002; Khan *et al*., 2009; Ahmad, 2012; Rehman, 2013; Anjum *et al*., 2014). Climate and unhygienic measures are the key factors for a higher prevalence of the disease in the study area. The agro-climatic conditions of Pakistan are presumed very favorable for the propagation of snails which act as the intermediate hosts for this parasite. The low lying and swampy areas of Punjab like Jhang, Toba Tek Singh, Lahore and Chiniot have been reported to support a high incidence of fasciolosis (Khan *et al*., 2009). A variable prevalence of fascioliasis ranging from 17.6-55.0% in different animals has been reported from Pakistan (Chaudhry and Niaz, 1984; Masud and Majid, 1984; Khan *et al*., 2009; Rehman, 2013).

Among available conventional and modern diagnostic techniques, ELISA (Enzyme Linked Immunosorbent Assay) is the most sensitive and reliable diagnostic tool for fascioliasis. However, due to antigenic diversity, there is demographic variation in sensitivity and specificity of the test (Greiner and Gardner, 2000). Many scientists have developed specific ELISAs using excretory/secretary antigens of indigenous strains of Fasciola in various countries (Hillyer *et al*., 1996; Ibarra *et al*., 1998; Anderson *et al*., 1999; Ortiz *et al*., 2000; Reichel, 2002; Charlier *et al*., 2008; Afshan *et al*., 2013; Jalali *et al*., 2011). The present investigation was planned to evaluate microscopic faecal examination and a commercial ELISA kit under local conditions.

MATERIALS AND METHODS

***Study area*:** District Sargodha consists of flat, fertile plains and small hills having six tehsils (Sargodha, Silanwali, Shahpur, Kot Momin, Sahiwal and Bhalwal). The river Jhelum flows on the western and northern sides, and the river Chenab lies on the eastern side of the city. The riverine areas are used as pasture for grazing of livestock. The soil contains alluvial deposits which results in the stagnation of water, being one of the suitable habitats of snails (Rondelaud *et al*., 2001; Khan *et al.,* 2009). According to Punjab Development Statistics (2008), total goat and sheep population is 337,941 and 99,874, respectively.

***Selection of experimental animals*:** A cross sectional study was designed to investigate the prevalence of fascioliasis involving indigenous breeds of sheep (*Ovis aries*) and goats (*Capra hircus*) of district Sargodha. Selection of

experimental animals was routed through simple random sampling and proportional allocation method. Different epidemiological aspects of fascioliasis including tehsil, host, species of parasites, age, sex, climate and husbandry practices was maintained on a pre-designed questionnaire to determine frequency distribution of the possible determinants influencing the prevalence of fascioliasis (Thrusfield, 2007) and Lauridsen (2005).

***Collection of faecal and blood samples*:** Faecal samples were collected directly from rectum of the selected animals. The samples were preserved in wide-mouthed plastic bottles containing 10% formalin as preservative and labeled properly. Faecal samples were scanned microscopically for the identification of parasitic species (Soulsby, 1982). Sera were separated from collected blood samples using standard procedures and stored at –20°C.

***Serological assay*:** The collected sera were analyzed to detect anti-fasciola antibodies through an ELISA kit (DRG®, Germany) according to the manufacturer's instructions. Briefly, all components were brought to 21±3°C before use. Dilution buffer and washing solution were prepared as instructed in the composition of kit. Positive serum and test sera were diluted @ 1/100 before distributing 100µl of these sera. Sera were added in duplicate in adjacent odd and even columns. After incubation (1 hr; 21°C) and washing, conjugate antibodies (100 µl) were dispensed to all the wells of plate. Again after incubation (1 hr; 21°C) and washing, 100 µl of chromogen solution was added to each well. Plates were then incubated for 10 minutes and 50 µl of stopping solution was added per microwell and plate was read at 450 nm using iMark Microplate Reader (Bio Rad®, USA).

***Statistics*:** ELISA results were analyzed in terms of sensitivity, specificity, positive predictive value, negative predictive value and screening comparative diagnostics by Win episcope by taking coprological examination as a standard test. Results of coprological survey were analyzed by Chi square test and multiple logistic regression analysis. Paired characteristics were analyzed by Odd's ratio. All these procedures were carried out using SAS statistical software package (SAS, 1998) at 95 % confidence level. Comparative sensitivity and specificity of microscopy and ELISA was determined as under:

$$\text{Sensitivity} = \frac{TP}{TP+FN} \times 100$$

$$\text{Specificity} = \frac{TN}{TN+FP} \times 100$$

TP is true positive, TN is true negative, FP is false positive, FN is false negative

Microscopy serves as gold standard for true positive and true negative.

RESULTS

Coprological examination revealed the presence of *Fasciola* spp. eggs in 226 animals out of 600 with a prevalence of 37.66%. Higher prevalence (38.78%, 121/312) was found in goat population as compared to sheep (36.46%, 105/288) (Table 1). Prevalence of *F. hepatica* was significantly higher (P<0.005) as compared to *F. gigantic*a.

Age and sex of hosts was statistically associated with the distribution of fasciolosis in small ruminants of the Sargodha district. Female population of both species (sheep and goat) had a higher prevalence rate than males in the population. The prevalence of fasciolosis was highest in young age group, in both sheep and goats population, after which it decreased gradually with advancement in age in descending order.

Monthly variation of fascioliasis (coprological/serological) was also observed and found to be the highest in July and lowest in December in the sheep population, and highest in July and lowest in January in the goat population. Fig. 1 shows the monthly prevalence of disease in comparison to metrological data for the study area.

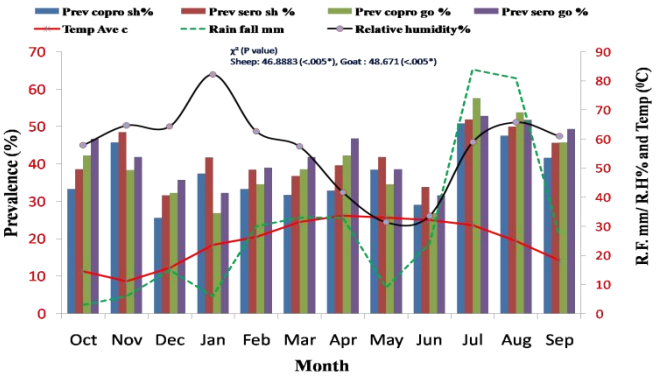

Figure 1. Comparative sero/copro prevalence of Fascioliasis with relation to the metrological data of study area

Table 1. Evaluation parameters of DRG kit ELISA using coprological examination as standard method

	Microscopic examination	ELISA Kit	Microscopic examination	ELISA Kit
	Sheep	Goat	Sheep	Goat
Prevalence (%)	36.46	48.61	38.78	49.36
Sensitivity (%)	72.92	100.00	77.55	100.00
Specificity (%)	67.78	75.83	68.25	82.87
Predictive value -	59.41	70.04	67.87	78.15
Predictive value +	100.00	100.00	100.00	100.00

A significant trend of ovine fascioliasis and non-significant trend of caprine fascioliasis prevalence in the selected six tehsils of district Sargodha has been observed.

Tables 2 and 3 summarized the association of various determinants with the frequency distribution of small ruminant fasciolosis in district Sargodha. The higher trend of prevalence was observed in grazers followed by ground and trough feeders. It was observed that prevalence of disease was higher in population provided with river water followed by pond water and tap water.

Commercial ELISA kit have detected antifasciola antibodies in higher percentage in sheep and goat population (48.61%

Table 2. Prevalence of Fascioliasis related to associated determinants by coprological/ELISA in sheep (*Ovis aries*) of Sargodha District

Associated determinants	Prevalence (%)		χ^2	95% C.I.	95% C.I.	Odds
	Copro	Sero	(P value)	Lower limit	Upper limit	ratio
Sex	47.41	48.93	23.5913	42.49	52.71	1.17
(male/female)	52.83	53.98	(<0.005)	47.58	57.89	
Age	32.59	34.69	34.4023	28.03	38.53	3.18
(young/adult)	22.05	23.92	(<0.005)	17.91	27.43	
Parasite species	29.31	30.95	70.6325	24.06	34.79	1.71
(*F. hepatica/ F. gigantica*)	7.15	8.08	(<0.005)	2.59	12.69	
Feeding sys (Grazing/ Ground/	63.95	65.62	29.9996	58.24	68.44	2.37
Trough)	33.69	34.96	(<0.005)	28.79	38.89	
	2.45	3.92		0.69	10.39	
Watering sys (River water/ Pond	63.41	66.01	28.2102	58.43	68.63	2.07
water/ Tap water)	36.59	37.79	(<0.005)	30.89	40.16	
	4.95	5.02		0.87	10.12	
Tehsil (Bhalwal/ Sargodha/	39.62	41.03	34.7484	34.79	44.82	1.83
Silanwali/ Sahiwal / Shahpur/ Kot	34.09	37.93	(<0.005)	29.19	39.81	
momin)	54.05	56.23		48.11	58.97	
	32.56	34.73		27.75	37.89	
	27.91	28.99		22.79	32.54	
	33.82	35.06		28.68	38.34	

Sheep screened for Fascioliasis (N=288)
Infested animals (n/N) = (105/288); Prevalence (%) = n/N×100 = 36.46%

Table 3. Prevalence of Fascioliasis related to associated determinants by coprological/ELISA in goat (*Capra hircus*) of Sargodha District

Associated determinants	Prevalence (%)		χ^2	95% C.I.	95% C.I.	Odds
	Copro	Sero	(P value)	Lower limit	Upper limit	ratio
Sex	30.09	31.49	20.5320	26.89	36.01	1.39
(male/female)	41.49	43.08	(<0.005)	35.98	45.29	
Age	40.98	41.69	25.8885	37.63	47.93	2.89
(young/adult)	38.13	40.02	(<0.005)	33.89	43.13	
Parasite species	31.86	33.15	78.6788	28.76	38.49	1.71
(*F. hepatica/ F. gigantica*)	6.92	7.48	(<0.005)	1.59	11.89	
Feeding sys	57.72	58.62	24.1896	53.24	63.44	2.89
(Grazing/Ground/Trough)	38.26	39.96	(<0.005)	32.79	42.89	
	4.02	5.92		0.69	9.93	
Watering sys (River water/ Pond	66.03	69.01	23.2230	61.83	71.96	3.97
water/ Tap water)	33.97	35.09	(<0.005)	28.69	38.22	
	5.34	6.02		0.68	10.89	
Tehsil (Bhalwal/ Sargodha/	40.88	41.03	13.0920	35.19	45.92	2.03
Silanwali Sahiwal / Shahpur /Kot	41.82	43.33	(0.018)	36.97	46.41	
momin)	45.83	48.23		40.16	50.79	
	42.86	46.73		37.35	47.59	
	34.09	37.19		29.93	39.49	
	33.73	35.06		28.18	38.84	

Goat screened for Fascioliasis (N=312)
Infested animals (n/N) = (121/312); Prevalence (%) = n/N×100 = 38.78%

& 49.36%, respectively) as compared to coprological examination (prevalence=36.46% & 38.78%). Both tests were not found equally sensitive and 100% sensitivity was recorded by commercial ELISA kit (Table 1).

DISCUSSION

Fascioliasis in small ruminants generally occurs as an acute, sub acute or chronic infection and causes considerable losses, particularly in endemic areas. Early diagnosis, i.e. during the migratory phase of the parasite, has therefore long been recommended. In the present study, higher prevalence of fascioliasis in small ruminant populations can directly be related to the geographical characteristics of the Sargodha district. This district consists of low lying and swampy areas that provide a favorable habitat for the snail population. Presence of low lying and swampy areas is the most important cause of higher prevalence rate in Sargodha district. Furthermore, unhygienic measures adopted by farmers are another reason for higher prevalence of fascioliasis in Sargodha (Khan *et al*., 2009).

A relatively lower prevalence (Asif *et al*., 2008; Gadahi *et al*., 2009; Ijaz *et al*., 2009; Khan *et al*., 2011; 2013) in Pakistan and other countries (Hussein and Khalifa, 2010; Hassan *et al*., 2011) has been observed. This low prevalence might be attributed to variation in agro-ecological conditions being less favorable for disease propagation.

In contrast to our study, higher abattoir based prevalence of small (40.51%) and large (43.63%) ruminant fascioliasis was reported by Ahmad (2012) and Rehman (2013) respectively in the same study area (district Sargodha). Higher prevalence was also observed by Mazid *et al*. (2006), Paz-Silva *et al*. (2007), Chanie and Begashaw (2012), Khan *et al*. (2013) and Anjum *et al.* (2014) in different parts of the world. This higher prevalence might be associated with non-adoption of control measures, development of resistance against flukicides (personal communication), indiscriminate animal trade, and availability of favorable agro-climatic conditions which help disease propagation (Jabbar *et al*., 2006). The possible reason for higher prevalence in rainy season might be the availability of most favorable environmental conditions for the growth, transmission and completion of parasitic life cycle stages including temperature and humidity which helps in maximal growth of parasite and snail (Khan *et al*., 2009; 2011; 2013; Anjum *et al*., 2014). The rainy season is considered as the most risky time of the year with regard to the prevalence of fascioliasis followed by spring, autumn and winter, which is similar to our study. The highest prevalence in rainy season is due to the availability of most favorable environmental conditions for the completion of parasitic life cycle including temperature humidity which helps in maximal growth of parasite and snail (intermediate host) (Khan *et al*., 2009, 2013).

Host species is an important determinant for disease occurrence. Some species are more prone to acquire infection due to their different grazing habits. Our results indicated higher prevalence in goats than sheep (Ahmad *et al*., 2005, 2008; Yadav *et al*., 2008; Ahmadi and Meshkehkar, 2010). Contrary to our findings, reports are available describing higher prevalence in sheep than goats (Asif *et al*., 2008; Ijaz *et al*., 2009, 2008; Garg *et al*., 2009; Gadahi *et al*., 2009; Dagnachew *et al*., 2011).

Prevalence in small ruminants ranges from 27-54% in six tehsil of Sargodha. The highest prevalence was determined in Silanwali tehsil and lowest in Shahpur tehsil in sheep while highest prevalence was determined in Silanwali tehsil and lowest in Kot momin tehsil in goat. Similar type of survey was performed by Ahmad (2012) reported highest prevalence in Shahpur tehsil (52.0%) and the lowest in Sahiwal tehsil (27.5%). Difference in prevalence rate of fascioliasis in tehsils of study area attributed to difference in study plan (our study is based on whole area survey as compare to Ahmad (2012) who collected slaughter house based prevalence of disease.

Association of different categories of age and sex variables was studied among risk factors which can influence the prevalence of small ruminant fascioliasis in district Sargodha. Both sex and age categories were found significantly associated with the risk of disease in small ruminants. Current study results are in line with Maqbool *et al*. (2000), Mazid *et al*. (2006), Ahmad *et al*. (2008), Talukde *et al*. (2010), and Khan *et al*. (2013) who reported higher prevalence in female than males. Some previous reports by Khan *et al*. (2009), and Chanie and Begashaw (2012) indicated sex as non significant determinant for the occurrence of disease. Higher prevalence in female was may be due to long rearing of female animal for milk production would change in physiological state thus poses stress on animals. Prevalence of fasciola was the highest in young age group than in adult in both sheep and goat. Few reports of higher prevalence in young animals was only limited to areas where fasciolosis was hyper-endemic and animals get infection shortly after birth. Dagnachew *et al*. (2011) reported higher prevalence in young animals. The results are different from the findings of Ahmad (2012), Hassan *et al*. (2011) and Mbaya *et al*. (2010). Higher prevalence in adult age group was due to long subclinical phase of disease in host which takes 4-6 months for the completion of complete life cycle and manifestation of clinical disease in animals. Another reason of higher prevalence in adults might be due to compromised immunity.

Current study revealed that grazing practice and provision of river water are strongly associated husbandry practices with the prevalence of ovine/caprine fascioliasis in the Sargodha district. Small ruminants often feed on grazing throughout the year. Some portions of year have reduced pasture availability that forces the animals to graze in low lying

swampy areas thus exposing them to heavily infested pasture with immature stages of Fasciola (Soulsby, 1987; Khan *et al*., 2009).

Diagnosis of fascioliasis has been relied for many years on the microscopic examination of Fasciola eggs in faeces. However, sensitivity of this method is very low because Fasciola eggs do not appear in faeces before the fluke has become adult and reached biliary system of liver. Fasciola takes 10-14 weeks after infection to mature and release eggs in faeces (Anderson *et al*., 1999). Up to that time most of hepatic damage has been occurred. Hence early detection of infection is necessary to avoid economic losses caused by small ruminant fascioliasis. To overcome deficiencies of faecal examination, serological detection of antibodies by ELISA was adopted as an alternative approach.

In present study, commercial ELISA reveals higher prevalence compared to coprological examination. It is clearly illustrated that predictive value for a positive egg count was higher (100%) than predictive value for a negative count (sheep, 70.04%; goat, 78.14%). This low value was due largely to the zero counts from animals with flukes in their liver.

An essential characteristic of ELISA based assays is higher sensitivity than microscopy. Commercial ELISA was found 100% sensitive in both species of small ruminant and 75.83%, 82.86% specific in sheep and goats, respectively. Martinez *et al.* (1996) recorded that 26% of animals shedding eggs of Fasciola were ELISA-negative and Hillyer *et al.* (1996) found 7 animals as ELISA negative which were positive in faecal examination. In all sheep and goat examined, none of the animals that yielded Fasciola eggs in coprological examination were found negative in ELISA. Also all animals positive in faecal examination yielded positive results by ELISA. With help of commercial ELISA, we were able to detect antifasciola antibodies in sera of 15 sheep and 12 goats, coprological examination of whose faeces could not identify Fasciola eggs. Infection in these animals may have been up to the level that the egg count was too low to be detected by the method employed or the disease may be subclinical as Fasciola starts on releasing egg after reaching maturity. A major disadvantage of the indirect ELISA test is that the presence of antibodies is not indicative of recent infection, as it has been stated that antibody titers will remain high in hosts even after treatment (Ibarra *et al.,* 1998). Three sheep were found false positive while only one goat serum was wrongly detected as positive. This may be due to low infection which have been missed during faecal examination or due to sharing of antigenic epitopes with other helminthes (Fagbemi and Obarisiagbon, 1991). Recent administration of fasciolicide may have eliminated fluke from bile ducts. The antibodies will persist in serum for 2-7 months (Zimmerman *et al*., 1983; Santiago and Hillyer, 1988). However, these may increase/ decrease if this information is used in conjunction with the test result.

***Conclusions*:** The above mentioned observations revealed that fascioliasis is infecting small ruminants of study area. Associated risk factors and unhygienic husbandry practices also influence the disease transmission, suggesting that more attention should be paid to these associated risk factors for the control of the infection in endemic areas like Sargodha. Grazing practice and provision of river water are strongly associated husbandry practices with the prevalence of ovine/caprine fascioliasis in the study area. Application of commercial ELISA provides cost effective tool for early diagnosis of fascioliasis in small ruminants. A multidimensional control and diagnostic methods may prove as an effective control strategy of fascioliasis in heavily infested areas of Pakistan. Continuous education and extension programs are need of the day for awareness regarding the transmission dynamics of fascioliasis. This kind of multi dimensional approach can provide better sustainable control.

***Acknowledgements*:** Authors acknowledge the financial assistance provided by Higher Education Commission Pakistan through support under Indigenous Ph.D. Fellowship Program Batch VII (117-6620-AV7-085).

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