Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Serological and Coprological Studies of Bovine Fasciolosis in the Pothwar Region, Pakistan

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ARTICLE HISTORY (14-353)

Received: July 09, 2014
Revised: December 29, 2014
Accepted: January 03, 2015

Key words:
Excretory secretory antigen
Fasciolosis
Indirect ELISA
Large ruminants
Pakistan

ABSTRACT

The present study was aimed to evaluate the diagnostic value of enzyme linked immunosorbent assay (ELISA) using excretory-secretory antigen (ESA) and Zinc sulphate sedimentation technique for coprodiagnosis. A comparison was made between both techniques for rapid and accurate diagnosis based on 200 serum and faecal samples from cattle (n=117) and buffaloes (n=83). In the first step, ELISA was standardized by using sera from *Fasciola* infected and non-infected animals. The level of sensitivity observed was 100%, while 95% specificity against *Gigantocotyle explanatum* and *Paramphistomum* spp. or a mixed infection with both parasites was noted. Fasciolosis rate was significantly ($P<0.05$) higher in buffaloes in which 60% prevalence was found for indirect ELISA and 53% in case of coprological analysis, while on other hand in cattle prevalence rate was 50 and 37%, respectively. The results showed a significant ($P<0.05$) breed difference in cattle. Furthermore, the significant difference was noted in buffaloes with reference to age and sex groups. After contrasting data from ELISA and faecal analysis, 11% of the sera analyzed had positive values of indirect-ELISA and negative by faecal analysis. The findings suggest that indirect ELISA is an efficient diagnostic technique and combination of both techniques could be useful for accurate diagnosis of fascioliasis in endemic areas. It is recommended that both cattle and buffaloes should be screened by applying both serological and coprological techniques in other agro-ecological zones of Pakistan.

To Cite This Article: Mufti S, K Afshan, IA Khan, S Irum, IZ Qureshi, SSR Rizvi, M Mukhtar, M Mushtaq, Z Iqbal and M Qayyum, 2015. Serological and coprological studies of bovine fasciolosis in the Pothwar region, Pakistan. Pak Vet J, 35(2): 178-182.

INTRODUCTION

Fasciolosis is an important food borne zoonotic disease caused by *Fasciola* trematode parasites. There are two types of *Fasciola* spp. namely *F. hepatica* and *F. gigantica*, widely distributed across the globe, affecting both human and animal hosts (Ali *et al.,* 2012; Charlier *et al.,* 2012). The economic losses attributed to fasciolosis in livestock animals occurred in the form of mortalities, reduced fertility, abortions, slow growth and reduction of milk and meat production, infected livers and withered carcasses (Rokni *et al.,* 2010; Hossain *et al.,* 2011). Fasciolosis in general, causes huge economic losses of 200 million US$ annually to the agriculture budget and affects the productivity of large ruminants (Ramajo *et al.,* 2001). In Pakistan, different prevalence records of fascioliasis in livestock were reported in various parts of the country (Khan *et al.,* 2009; Kakar *et al.,* 2011; Afshan *et al.,* 2013a). The accurate economic losses due to fascioliasis in livestock are not yet available. However, fasciolosis has been found a disease of prime concern on account of prevailing agro-climatic conditions that may facilitates occurrence of the infection.

Previous diagnostic studies on bovine fasciolosis were based on coprological examinations however; it has limitations in early diagnosis (Rokni *et al.,* 2003).

Therefore, rapid and accurate diagnosis of the infection is of prime importance for early chemotherapy. The control of fasciolosis was limited in the past due to lack of appropriate early diagnostic test. Recent studies have confirmed the presence of *F. hepatica*/*F. gigantica* intermediate forms in Punjab through adult morphological and molecular characterizations (Mufti *et al.,* 2011; Afshan *et al.,* 2013b; Mufti *et al.,* 2014; Zaman *et al.,* 2014). This has necessitated the development of rapid diagnostic test by using local fasciolid strains as compared to commercially available kits which may lead to misdiagnosis because of antigenic variations. In present study an indirect ELISA was developed for the serodiagnosis of *Fasciola* infection by using excretory secretory (ES) antigen derived from adult fasciolids of Pothwar region.

MATERIALS AND METHODS

Experimental site and Animal data: The area under study lies in the northern part of the Punjab province of Pakistan and comprises of the Pothwar region, lying between 32º-30" N to 34º latitude and from 71º- 45" E to 73º-45" E longitudes (Fig. 1). The large ruminants grazing in Pothwar region follow extensive and semi-extensive farming production systems. The study population consisted of 200 animals, buffaloes belonging to Nili Ravi breed (n=83) and cattle (n=117) belonging to Red Sindhi (n=34), Achai (n=15), calves (Red Sindhi) (n=10), Cross (Jersey x Sahiwal) (n=17), Jersey (n=12) and Desi breeds (n=29). A stratified random sampling method was used to select animals according to their breeds, sex and age groups.

Blood and Adult worm collection: Blood samples were taken in non-EDTA coated vacutainers, centrifuged at 3000 rpm for 15 min, sera was separated and kept at -20 °C until used. Adult worms were collected from infested bovine livers and washed 3-5 times at room temperature with 0.01 M phosphate buffered saline (PBS, pH=7.4) (one worm per five ml) at 37°C for 1 hr. To remove particulate materials, the prepared antigens centrifuged at 12,000 rpm at 4°C for 1 hr. The antigen was aliquoted and stored at –70°C until used. The protein concentration of the ES antigen was measured by the Bradford assay (Bradford, 1976).

Enzyme linked immunosorbent assay: The ELISA was performed on 96 well micro-titration plates, filled with 200ml of 4 g/ml antigen with coating buffer and incubated overnight at 4°C. The excess antigens were washed out 3 times, with 0.05% of PBS-Tween; blocked with BSA (200µl/well) for 1 h; reacted with (100µl/well) of animal sera for 1 h; incubated with 1:2,000 rabbit anti-bovine horseradish peroxidase-conjugate IgG (Sigma Immunochemicals) for 1 hr at 37°C. The plate was washed 3 times with PBS-Tween between every change of solution. Finally, 100µl/well of substrate solution containing ortho-phenylenediamine (OPD) (Sigma Immunochemicals) was added. The reaction was allowed to occur in the dark at room temperature for 5 min. The reaction was stopped by adding l00µl/well of 1 M H2SO4. Optical density (OD) at 490 nm was measured with an ELISA reader. The limit for discriminating negative from positive results was determined by mean value of the negative controls plus 2 standard deviations. The sensitivity and specificity of antigen was calculated as follows (Mandal *et al.,* 1998):

Sensitivity % = (True positive − false negative) / true positive × 100

Specificity % = (True negative − false positive) / true negative × 100

In order to determine possible cross-reactivity of *Fasciola* antibodies, monospecific sera each of *Gigantocotyle explanatum* and *Paramphistomum* spp. and mixed infection of both parasites were tested.

Parasitological Techniques: Three grams of faeces were taken from the rectum and examined by using a modified Zinc sulphate sedimentation technique to determine the presence of fluke eggs along with McMaster technique for faecal egg count (EPG). Trematode eggs were identified on the basis of morphology (Soulsby, 1982; MAFF, 1986).

Statistical Analysis: The association between prevalence and examined animals' data (age, sex and breed) were compared using Pearson's chi-square (χ^2) test. Statistical comparisons were carried out using SPSS 17.0 statistical software. A significant level of $P<0.05$ with 95% confidence level was adopted.

RESULTS

Serology (ELISA): *Fasciola* antibodies level was above the cut-off value in all infected cattle and buffalo (OD≥1) (Fig. 2). The overall seroprevalence of *Fasciola* infections was 60 and 50% in buffalo and cattle, respectively and difference was non-significant ($P>0.05$) (Table 1). A significant ($P<0.05$) difference was found among cattle breeds ($P=0.0042$, $\chi^2=17.143$), indicating high susceptibility to fasciolid infections. Achai breed and Red Sindhi calves were found most susceptible to infestation (Table 3). The seroprevalence of fasciolid infection among sex and age groups of buffalo and cattle indicated statistically significant difference (Table 2 and 3). The results also indicated that susceptibility was the highest in young and 1-5 year old bovines. Seroprevalence was found greater in males both in buffaloes and cattle. The sensitivity of the antigen and test in detecting bovine fasciolosis was calculated as 100%. The specificity calculated against *G. explanatum, Paramphistomum* spp. and against mixed infection of both parasites was 95%.

Coprological study: The number of positive samples obtained from buffaloes was higher than that from cattle, representing of 53 and 37%, respectively. There was a significant ($\chi^2=5.223$, $P=0.022$) difference in FEC between bovine species (Table 1). In case of cattle breeds, the infection was breed dependent ($\chi^2=13.753$, $P=0.017$) and the highest infection was found in Achai and in young calves (Table 3). The minimum mean FEC in cattle was 50, while the highest was 1000, whereas in buffalo FEC ranged from 50-1600. Age-wise difference

Table 1: Prevalence (%) of *Fasciola* species infection with respect to buffaloes and cattle

Variable		Prevalence	χ^2	P Value
ELISA test	Buffaloes (n=83)	60	2.225	0.136
	Cattle (n=117)	50		
Faecal analysis	Buffaloes (n=83)	53	5.223	0.022*
	Cattle (n=117)	37		

Data analysis by Pearson's Chi-square (χ^2) test; NS=non-significant difference (P>0.05); *Significant difference (P<0.05).

was also significant in buffalo (Table 2) and cattle (Table 3). There was a significant difference in FEC with cattle age (χ^2=9.805, P=0.007). The age group below 5 years had the highest FEC as compared to other two age groups (Table 3). There was also significant difference between sex of animal and prevalence of fasciolosis in case of cattle (χ^2=6.686, P=0.010) as males were more prone to infection than females (Table 3), while in buffalo (χ^2=2.387, P=0.122) results were non-significant (Table 2).

Comparison of two diagnostic tests: A comparison of the prevalence of fasciolosis in buffalo and cattle by faecal egg count and of positive animals by indirect ELISA test has been presented in Table 4. The results showed that in buffaloes the prevalence of infection based on ELISA and FEC, respectively was 60 and 53%, while in cattle it was 50 and 37%. The prevalence detected by serological analysis was consistently higher than that detected by the coprological test. After contrasting data from ELISA and faecal analysis, it was noted that 45.5% cattle and buffaloes were free from *Fasciola* infection. It was also that 11% of the sera analyzed had positive values of indirect-ELISA and negative by faecal analysis (Table 5).

DISCUSSION

In the present study, both coprological examination and immunodiagnosis were applied for seroepidemiology of bovine fasciolosis in Pothwar region. The conventional method of diagnosis of liver fluke infection was traditionally based on presence of eggs in faeces (Boray, 1985). However, coprological examination has its own limitations with reference to the quantity of faecal sample used and the misidentification of other trematode eggs having similar morphology (Hillyer, 1997; Anderson *et al*., 1999). Conducting faecal screening in the early stages of infection is not beneficial as eggs are usually shed in faeces until about three months after infection and immature liver flukes are in the liver besides this infection due to *Fasciola* residing elsewhere, besides the liver which makes it difficult to detect (Soulsby, 1982). Early diagnosis of fasciolosis using antibody detection tests, especially during the prepatent period makes ELISA practical for curbing the negative impact of infection on productivity and hence to avert huge economic losses (Sanchez-Andrade *et al*., 2000). Therefore, ELISA has become a commonly used diagnostic test because it is easily automated with high precision. This study emphasized on the evaluation of an indirect ELISA for diagnosis of bovine fasciolosis. *Fasciola* ES antigens used in this assay were isolated from fresh flukes recovered from bovines grazing in Pothwar, Pakistan.

In this study, serology (ELISA) gave a higher prevalence than coprology. This was due to the fact that in seroprevalence antibodies against *Fasciola* antigen are formed within 2 weeks and specific serum antibody titre indicates the animal to be positive. In contrast faecal analysis by egg detection is not possible until after 8-12 weeks post infection, ELISA for early detection is preferred (Bossaert *et al*., 2000). However, the results are not in agreement with Aliyu *et al.* (2014). The reason for the differences in result is they detected *F. gigantica* infection with the antigen and monoclonal antibody of *F. hepatica*. So much lower infection level was recorded with ELISA, as the two species might have marked dissimilarities in their antigenic epitopes.

In current study higher infection rate was in buffaloes as compared to cattle as they reside in closer proximity to water bodies. Their habit of grazing in wet areas close to soil leading to maximum exposure to acquire infection from the snails serving as intermediate host. The results are in agreement to Preston and Allonby (1979) who indicated that the difference in infection level could be due to natural resistance, such that some breeds can mount a better resistance than others. The present study showed mixed results of infection among breeds of cattle and buffaloes studied. These results are not in agreement with the findings of Aliyu *et al*. (2014). The higher prevalence obtained for the endemic and foreign breeds could be because of the fact that these breed compels to graze in areas that could be heavily infested with the intermediate hosts of the liver fluke when in dry season there is shortage of forage.

The age is also an important factor among large ruminants. In the present study prevalence of infection was higher in younger bovines (below 5 years) than older ones (above 5 years), this is in agreement with the results of earlier investigations (Kuchai *et al.,* 2011; Aliyu *et al.*, 2014). The reason for this could be the development of acquired immunity in the older animals which results in resistance (Phiri *et al.,* 2005). To the contrary some studies have reported an increased prevalence of infection in buffalo and cattle with advancing age (Khan *et al.,* 2009).

Table 2: Prevalence (%) of *Fasciola* species infection by ELISA test and faecal analysis with respect to age and sex of the buffaloes

Variable	ELISA test			Faecal analysis		
	Prevalence (%)	χ^2	P Value	Prevalence	χ^2	P Value
Age						
<1-5 (n=14)	100	11.36	0.003*	93	12.609	0.002*
6-10 (n= 19)	47			32		
>10 (n=50)	54			50		
Sex						
Male (n=6)	100	4.27	0.039*	83	2.387	0.122NS
Female (n=77)	57			51		

Data analysis by Pearson's Chi-square (χ^2) test; NS=non-significant difference (P>0.05); *Significant difference (P<0.05).

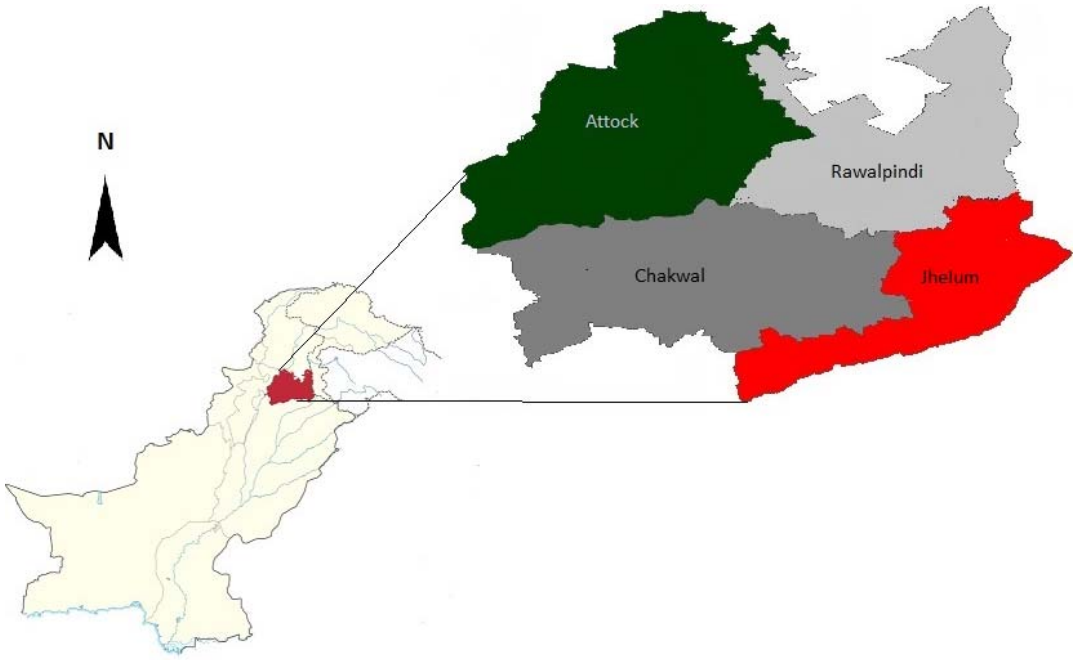

Fig. 1: Map of Pakistan showing Pothwar region.

Table 3: Prevalence (%) of *Fasciola* species infection by ELISA test and faecal analysis with respect to breed, age and sex of the cattle

Variable	ELISA test			Faecal analysis		
	Prevalence (%)	χ^2	P Value	Prevalence	χ^2	P Value
Breed						
Red Sindhi (n=34)	50	17.143	0.0042*	38	13.753	0.017*
Achai (n=15)	80			60		
Crossbreed (n=17)	53			35		
Jersey (n=12)	42			42		
Calves (n=10)	80			25		
Desi/Local (n=29)	24			17		
Age						
<1-5 (n=10)	80	12.17	0.002*	70	9.805	0.007*
6-10 (n= 78)	55			40		
>10 (n=29)	24			17		
Sex						
Male (n=17)	76	5.75	0.016*	65	6.686	0.010*
Female (n=100)	45			22		

Data analysis by Pearson's Chi-square (χ^2) test; NS=non-significant difference (P>0.05); *Significant difference (P<0.05).

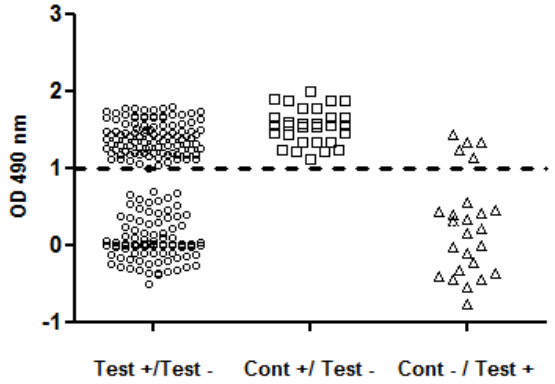

Fig. 2: Indirect ELISA by using Excretory Secretory antigen measuring the level of *Fasciola* antibodies in serum sample of bovines. The cut-off values represented with horizontal line, dotted lines indicate the mean OD values.

The present study shows a higher infection rate in males as compared to females, the results are not in agreement with findings obtained in Nigeria (Aliyu *et al.*, 2014). This could be due to very small sample of males as compared to large number of female samples, which are usually maintained for breeding and milk purpose (Phiri *et al.*, 2005). Whereas, Maqbool *et al.* (2002) reported that infection rate was almost equal in both sexes.

In Pakistan prevalence of fasciolosis in bovines by both *Fasciola* spp. was previously been reported to be 25.46 percent (Khan *et al*, 2009), 14.71 percent (Maqbool *et al.*, 2002), 10.48 percent (Sahar, 1996), 17.68 percent (Chaudhry and Niaz, 1984) and 23.97 percent (Masud and Majid, 1984). This study reported the highest incidence to date (55 percent). One possible reason for the high infection rate may be the inability of farmers to treat the disease due to lack of local available drugs (Jabbar *et al*., 2006). If a certain drug is frequently used for a longer time with wrong doses than resistance against fasciolicides is likely to develop (Fairweather and Boray, 1999).

Conclusion: Results indicated that indirect ELISA could be an efficient rapid diagnostic method as compare to coprology limits the diagnosis in latent period. Therefore

Table 4: Prevalence (%) of *Fasciola spp* infection in cattle and buffaloes with respect to ELISA test and faecal analysis

Variable	Prevalence	χ^2	P-value
Buffaloes (n=83)			
ELISA test	60	0.886	0.347^{NS}
Faecal analysis	53		
Cattle (n=117)			
ELISA test	50	3.919	0.048*
Faecal analysis	37		

Data analysis by Pearson's Chi-square (χ^2) test; NS=non-significant difference (P>0.05); *Significant difference (P<0.05).

Table 5: Diagnostic efficiency of ELISA test and faecal analysis against fasciolid infection in cattle and buffaloes

ELISA	Faecal analysis	Total number	Percentage (%)
-	-	91	(45.5)*
-	+	0	(0.00)
+	-	22	(11)
+	+	87	(43.5)
Total		200	(100)

*Parentheses represent percentages

the use of both methods together provides excellent information about the real situation of infection, and can be strongly recommended for further epidemiological surveys in other agro-ecological zones of Pakistan.

Author's contribution: MQ was involved in study design, sampling, ELISA and interpretation of the results. SM, KA, ZI and IAK performed ELISA and coprological tests. SSRR analyzed the data analysis. Mukhtar, Mushtaq and IZQ provided technical assistance in conducting the experiment and involved with *Fasciola* antigen preparation. All authors approved the submission.

Acknowledgement: The research work presented in this article is the part of studies carried out in research project (No. HEC 338 R & D 2nd Phase 05/280) funded by Higher Education Commission, Pakistan.

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