DIAGNOSTIC POTENTIAL OF 36-55 kDa SOMATIC ANTIGENS OF *Fasciola gigantica* FOR BOVINE FASCIOLOSIS

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ABSTRACT

Fascioliasis causes huge economic losses in livestock industry by reducing the growth, fertility rate, meat and milk yield. In the present study, somatic antigens isolated from *Fasciola gigantica* were assessed for the early detection of infection by developing antibody detection enzyme immunoassay. The somatic antigens extracted from adult *F. gigantica* worms was separated on SDS-PAGE and immunogenicity was determined by Western blot (WB). The results showed polypeptides bands between 15 to 95 kDa, and most prominent bands were 15, 28, 36, 38, 55, 72 and 95 kDa polypeptides. The WB revealed a single polypeptide of size between 36 to 55 kDa as antigenic. The sensitivity and specificity of ELISA test established for 36 to 55 kDa somatic antigens was 95.45% (95% CI: 77.16% to 99.88%) and 87.1% (95% CI: 70.17% to 96.37%), respectively. Kappa value revealed that the strength of agreement is considered to be very 'good'. In house established ELISA was implemented in the field and 12.1% (31/256) large ruminants were found positive, while 4.68% (12/256) with postmortem examination. The fasciolosis did not show significant (P>0.05) association with host type, breed and age groups, while significant (P<0.01) association with host sexes. The result confirmed that in-house established ELISA test had good value for serodiagnosis of fasciolosis in cattle and buffaloes for large scale epidemiological studies.

Keywords: *Bubalus bualis*, buffaloes, fascioliasis, ruminants, antigens, western blotting, ELISA, SDS-PAGE, Pakistan

INTRODUCTION

Fascioliasis is a cosmopolitan helminth infection, caused by digenetic trematodes species, *Fasciola hepatica* and *Fasciola gigantica*. The disease is one of the most significant and destructive liver damaging diseases of ruminants (Mas-Coma *et al*., 2009) caused by liver fluke species of the genus *Fasciola*, has always been well recognized because of its high veterinary impact but it has been among the most neglected diseases for decades with regard to human infection. However, the increasing importance of human fascioliasis worldwide has re-launched interest in fascioliasis. From the 1990s, many new concepts have been developed regarding human fascioliasis and these

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have furnished a new baseline for the human disease that is very different to a simple extrapolation from fascioliasis in livestock. Studies have shown that human fascioliasis presents marked heterogeneity, including different epidemiological situations and transmission patterns in different endemic areas. This heterogeneity, added to the present emergence/re-emergence of the disease both in humans and animals in many regions, confirms a worrying global scenario. The huge negative impact of fascioliasis on human communities demands rapid action. When analyzing how better to define control measures for endemic areas differing at such a level, it would be useful to have genetic markers that could distinguish each type of transmission pattern and epidemiological situation. Accordingly, this chapter covers aspects of aetiology, geographical distribution, epidemiology, transmission and control in order to obtain a solid baseline for the interpretation of future results. The origins and geographical spread of *F. hepatica* and *F. gigantica* in both the ruminant pre-domestication times and the livestock post-domestication period are analyzed. Paleontological, archaeological and historical records, as well as genetic data on recent dispersal of livestock species, are taken into account to establish an evolutionary framework for the two fasciolids across all continents. Emphasis is given to the distributional overlap of both species and the roles of transportation, transhumance and trade in the different overlap situations. Areas with only one *Fasciola* spp. are distinguished from local and zonal overlaps in areas where both fasciolids co-exist. Genetic techniques applied to liver flukes in recent years that are useful to elucidate the genetic characteristics of the two fasciolids are reviewed. The intra-specific and inter-specific variabilities of 'pure' *F. hepatica* and 'pure' *F. gigantica* were ascertained by means of complete

sequences of ribosomal deoxyribonucleic acid (rDNA). It is estimated that more than 700 million ruminants and above 180 million people are in danger of fascioliasis (Rehman *et al*., 2016). The economic losses caused by fascioliasis is estimated to be 3.2 billion USD dollars worldwide in term of reduction in the body weight, reduction in milk and the cost of treatment. The clinical symptoms range from the asymptomatic stage to severe episodes of bleeding within the abdomen and acute surgical abdomen (Torres *et al*., 2004). Damage of liver and other metabolic changes are due migration and hematophagic activities of *Fasciola* (Mas-Coma *et al*., 2014).

Conventional parasitological methods used to determine the nature and level of infection in animals required arduous laboratory extraction methods, culture and microscopic examination of ova and parasites from faeces (Anderson *et al*., 1999). The microscopic examination of parasite eggs is not effective until 10 to 12 weeks post-infection (PI). The use of more efficient and reliable methods is required by using advanced molecular technologies. The control of fasciolosis was limited due to lack of early diagnostic test. Several investigations were performed on development of early immunological tests against fasciolosis in bovine (Phiri *et al*., 2006). The antigenic fractions identified from *Fasciola* include whole metabolic or excretory secretory antigens (Salimi-Bejestani *et al*., 2005), somatic antigens, and purified recombinant cathepsins antigens (Sriveny *et al*., 2006). The cathepsins L are most frequently used antigen to detect fasciolosis antibodies (Muiño *et al*., 2011). Other recombinant antigens are also identified and successfully been applied for immuno detection (Arias *et al*., 2006). The limitation of these so far developed assays is their low sensitivity and specificity. Most of these assays

are not commercially available and not able to detect low intensity infections (Mezo *et al.*, 2007). However, the performance of such methods is still unsatisfactory and development of diagnostic test, using different subunit antigens of *Fasciola* is needed.

Studies conducted in Pakistan were based on microscopic and slaughterhouse-based screening of animal fascioliasis. Limited studies were conducted on serology of animals by using commercial kits which are very costly and have less sensitivity and specificity due to antigenic variations. Despite the enormous economic importance of fasciolosis, no attempt has been made in the country so far towards identification of protein markers of fasciolids which could be used in future for the development of rapid cost effective early diagnostic tools. Furthermore, identification, isolation and characterization of parasite antigens are required for vaccination, immunological diagnosis, analysis of immunopathology and for the quantitation of various immune responses of the host. Considering the aforementioned importance and need, the current study was aimed to characterize somatic antigens of *Fasciola gigantica* by SDS-PAGE analysis and assess the diagnostic potential of 36 to 55 kDa immunodominant antigens by using indirect ELISA test.

MATERIALS AND METHODS

Adult flukes collection and extraction of somatic antigen

The adult *Fasciola gigantica* flukes were collected from the bile ducts of the infected cattle and buffaloes, shortly after these animals were slaughtered. The Sihala slaughterhouse received animals from adjoining areas of Rawalpindi, while

in Peshawar these are brought from Charsada and Mardan (Figure 1). After removal from the bile duct, the flukes were washed many times with 0.01 M phosphate buffered saline (PBS) pH 7.2. For somatic extracts, worms were homogenized in chilled tissue lysis buffer, added according to the weight of tissue in a ratio of (1,000 µl buffer/100 mg of tissue). The tissue grinding was carried out on ice to avoid the denaturation. This homogenized mixture was centrifuged at 1,000 rpm for 10 minutes at 4°C. Protein concentration of somatic products was estimated through the process described by Bradford (1976).

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE was performed according to method described by Laemmli (1970). Briefly, the somatic antigens were pre heated with loading buffer for 5 to 10 minutes in water bath at 95°C and loaded in 12% and 10% separating gel. After running, the gel at 90 V for 2 h the gel was fixed for 1 h and then stained with 0.1% coomassie brilliant blue (Sigma). The 'Image analysis' software determined the molecular weight of each polypeptide.

Western blotting

The immunoblotting followed for the transfer was according to Hongbao and Kuan (2006). Briefly, after running the gel the stacking gel was separated, the nitrocellulose membrane pre-soaked in transfer buffer was added and at the top of this membrane the gel was placed. The pre-soaked 6 filter papers were added at the top and bottom and rolled to remove air bubbles. The blotting was carried out at 10 V constant voltages for 30 minutes in a semi-dry blotting apparatus (Bio-Rad). The membrane with transferred proteins was then

treated with blocking solution (5% skimmed milk) for 1 h, changing the solution at 15 minutes interval. After blocking, the membrane was incubated with primary antibody which is the sera obtained from infected animals (1:500) at 4°C overnight with shaking to allow maximum binding. The incubated membrane was washed with PBST 3 times, giving each wash 10 minutes. The membrane was treated with secondary antibody (Anti-goat antibody) for 2 h with shaking. After washing substrate (BCIP/NBT) was added and stored in dark and incubated for 15 minutes until the bands became visible.

Isolation of immunodominant antigen from the polyacrylamide gels

After running the SDS-PAGE gels, different protein bands obtained were eluted. Each piece was crushed in elution buffer and left overnight, centrifuged at 10,000 rpm for 10 minutes. The supernatant was separated and protein concentration in each sample was determined by Bradford (1976) and stored at -20°C.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA was performed according to method described by Guobadia and Fagbemi (1997); Ferre et al. (1997). Each eluted antigen was mixed with coating buffer in equal proportion (1:1) and 100 µl was added to each well of microtiter plate and incubated overnight at 4°C. After washing with 0.05% PBS-Tween 20 blocked with 0.05% BSA for 2 h at room temperature. Then washed three times and 100 µl of sera from infected cattle and buffaloes was added to each well and incubated for 2 h at 37°C. After 3 times washing, 100 µl of secondary antibody (horseradish peroxidase conjugated anti-bovine IgG antibodies) was added to each well and plate was incubated for 1 h at room temperature. The plates were washed and 100 µl of

substrate (TMB Tetramethylbenzidine) was added in each well and plate was incubated in the dark for 15 minutes at room temperature. The reaction was stopped by adding 100 µl of 1 M H_2SO_4. The OD values were determined by ELISA reader at 450 nm.

Evaluation of ELISA using naturally infected buffaloe sera

Developed ELISA was validated with 53 blood samples collected from positive and control buffaloes confirmed with postmortem examination. Blood samples were taken from buffaloes positive for *Fasciola gigantica* (n=22) and cross reactivity was tested with control sera collected from buffaloes positive for *Gigantocotyle explanatum* (n=10) and *Paramphistomum* spp. (n=10). Negative control sera (n=11) were obtained from 2 weeks old kids born to herd having history of stall feeding. Sera were separated and stored at -20°C. The sensitivity and specificity of indirect ELISA using 36 to 55 kDa somatic antigen of *F. gigantica* was computed in comparison to postmortem examination by following formulae:

$$\text{Sensitivity} = \text{true positive}/(\text{true positive} + \text{false negative}) \times 100$$

Where 'true positive' is animal number positive by ELISA and postmortem examination while 'false negative' is animals positive by postmortem examination but negative by ELISA.

$$\text{Specificity} = \text{true negative}/(\text{false positive} + \text{true negative}) \times 100$$

Where 'true negative is animals negative by ELISA and postmortem examination while 'false positive' is animals negative by postmortem

examination but positive by ELISA.

Field implementation of ELISA

After evaluation and standardization of ELISA test, postmortem examination of 256 randomly selected cattle and buffaloes were carried, and blood samples were collected to determine the prevalence of fasciolosis.

Statistical analysis

The prevalence percentages and their association with risk factors were determined by Chi-square analysis by using SPSS version 20. The online software 'QuickCalcs (https://www.graphpad.com/quickcalcs/) used to calculate the Kappa value of developed ELISA. The cut-off value was set at 0.6. The cut-off was calculated by the mean optical density (OD) of the negative reference serum, plus three times standard deviations (0.36+3*0.074=0.582).

RESULT AND DISCUSSION

SDS-PAGE and immunoblotting

The SDS-PAGE analysis, of somatic antigen of *F. gigantica* resolved 7 polypeptide bands of molecular weight ranged between 15 to 95 kDa. Other polypeptides separated in somatic extracts were 15, 28, 36, 38, 55, 72 and 95 kDa. The intensity of band ranged between 36 to 38 and 55 to 72 kDa polypeptides was highest among all other somatic extracts (Figure 2). Morales and Espino (2012) separated 40 polypeptides bands from the *F. hepatica* tegument antigens ranged 10 to 150 kDa. Allam *et al.* (2012) found polypeptide bands of 12 to 117 kDa. Meshgi *et al.* (2008) separated 11 polypeptide bands in somatic extracts of *F. gigantica* using SDS PAGE ranged 18 to 68 kDa. In the same study he resolved 8 different polypeptide bands of molecular weight ranged 18 to 62 kDa from the somatic extracts of *F. hepatica*. Kumar *et al.* (2008) showed different bands in range of molecular weights 14 and 76 kDa. Awad *et al.* (2009) fractionized 17 different polypeptides from the somatic extracts with molecular weights 13 and 262.3 kDa. Velusamy *et al.* (2004) separated different bands of somatic extracts of *F. gigantica* in range 8 and 54 kDa. These differences in molecular weight of polypeptides may be due to the isolation methods of somatic extracts. This may be explained with presence of different strains of flukes, collected from different host species or it may be because of geographic variations (Gupta *et al.*, 2003).

Immunogenic detection of *F. gigantica* somatic extract by western blotting method in current investigation showed a single polypeptide with molecular weight in range between 36 to 55 kDa or more close to 35 kDa (Figure 3). Anuracpreeda *et al.* (2013) identified a 37 kDa polypeptide by using western blotting method. Gonenc *et al.* (2004) observed antigenic polypeptides in somatic extracts of *F. hepatica* were 33 and 66 kDa. Morales and Espino (2012) recorded that 12 to 14, 24 to 26, 38 and 52 kDa polypeptides were immunogenic. De Almeida *et al.* (2007).

ELISA

The present results of indirect ELISA showed that highly immunogenic proteins are present in molecular weight that range between 36 to 55 kDa. Guobadia and Fagbemi (1997) detected immunogenic polypeptides of molecular weights of 17, 21, 53 and 57 kDa using indirect ELISA method. Velusamy *et al.* (2004) detected a 54 kDa immunogenic polypeptide from the somatic extracts of *F. gigantica.* Kumar *et al.* (2008) found

that 27 kDa polypeptide was 100% immunogenic to detect infection by *F. gigantica*. The sensitivity and specificity of currently developed diagnostic test for 36 to 55 kDa somatic antigens were 95.45% and 87.1% respectively on 53 serum samples from cattle and buffaloes. The current results of developed 36 to 55 kDa ELISA showed high sensitivity values this may be due to two dominant bands (molecular weight 36 and 55 kDa). The cattle and buffaloes postmortem examination showed positivity with other trematodes along with fasciolid infections and cross reactivity of 10 to 20% was observed for developed ELISA (Table 1). This may also explain with some common antigenic epitopes that are capable of detecting antigens from other trematodes (Arora *et al*., 2010).

The postmortem examination and 36 to 55 kDa somatic antigen indirect ELISA (Table 2) showed an increased level of seroprevalence rate in cattle and buffaloes (31/256=12.1%), indicative of *F. gigantica* either in the stage of active or passive infection. However, the prevalence of *F. gigantica* infection based on postmortem examination was 4.68% in cattle and buffaloes brought from different districts of Pakistan. The fascioliasis did not show significant (P>0.05) association with host type, breed and age of animals examined by postmortem and ELISA test (Table 2). The estimated prevalence record of fasciolosis in country appeared from less than 1% in northern hilly areas (Gadahi *et al*., 2009) to 70.6% in Punjab plain where irrigation system promotes stagnant water channels (Iqbal *et al*., 2007). Howerver, lower prevalence rates (2 to 9%) were recorded in the southern part of the province (Raza *et al*., 2007). Apart from Punjab, fascioliasis has also been reported in other provinces with prevalence of 11.5 to 16.2% in Quetta (Kakar *et al*., 2011), 5.9% in Dir district (Azam *et al*., 2002) and 42.06% in Sindh (Bhutto *et al*., 2012).

The overall seroprevalence of *F. gigantica* infection in cattle and buffaloes respectively, was 8.97% and 13.48%, similarly prevalence in postmortem examined buffaloes (5.62%) were more than cattle (2.56%). Breed wise seroprevalence of fasciolosis in *Azi kheli* (25%) was greater than *Nili Ravi* (15.71%) and Kundhi (5.5%), while in cattle Dhani (12.5%) and Sahiwal (11.11%) breed showed greater seroprevalence. The postmortem examined Azi khaeli breed showed incidence of infection 11.1% followed by *Nili Ravi* 5.6% and 2.8% Kundhi, while in cattle highest prevalence was in Sahiwal (5.6%) breed. The higher rate of infection in buffaloes may be attributed to their habit to reside in swampy areas, where availability of intermediate host and metacercarial stages of *F. gigantica* are abundant. However, in cattle low infection may be due to their grazing habit and genetically resistant breed.

Significantly (P<0.01) higher seroprevalence and post examination of fasciolosis in female animals were observed, which can be explained by assuming hormonal influence and other physiological aspects such as milk production, and stress during pregnancy and parturition (Spithill, 1999). The age of animals did not show significant (P>0.05) association with fasciolosis, while young animals of 2 to 10 years showed maximum infection, however the antibody titer was higher in case of age group >11 years of buffaloes. The possible reason may be that mature animals develop resistance and strong immunity towards re-infections due to maximum exposure to parasitic stages (Khedri *et al*., 2015).

CONCLUSION

In conclusion, western blot result showed

Table 1. Kappa value prediction of ELISA established for 36 to 55 kDa antigen with animals postmortem examination.

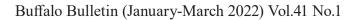| Test	ELISA test			Sensitivity	Specificity	
Postmortem examination	Positive	Negative	Total	95% CI	95% CI	KAPPA
Positive	21	1	22	95.45 (77.16-99.88)	87.1 (70.17-96.37)	Kappa = 0.809
Negative	4	27	31			SE of kappa = 0.081

- 95% confidence interval: From 0.652 to 0.967.

- The strength of agreement is considered to be 'very good'.

Table 2. Prevalence (%) of fascioliasis based on postmortem examination and ELISA test with respect host type, breed, gender, and age groups.

Variable	Postmortem examination			P-value	ELISA test		
	Sample size	Infected n (%)	χ^2		Infected n (%)	χ^2	P-value
Host type			1.13	0.287[NS]		1.036	0.309[NS]
Buffalo	178	10 (5.62)			24 (13.48)		
Cattle	78	2 (2.56)			7 (8.97)		
Buffaloe breed			6.204	0.516[NS]		11.395	0.122[NS]
Azi kheli	36	4 (11.1)			9 (25)		
Nili Ravi	70	4 (5.71)			11 (15.71)		
Kundhi	72	2 (2.78)			4 (5.5)		
Cattle breed							
Bhagnari	8	0			0		
Dhani	16	0			2 (12.5)		
Lohani	4	0			0		
Mix breed	14	0			1 (7.14)		
Sahiwal	36	2 (5.56)			4 (11.11)		
Gender			3.95	0.047*		11.519	0.001*
Female	142	10 (7.04)			26 (18.3)		
Male	114	2 (1.75)			5 (4.38)		
Age (years)			0.258	0.612[NS]		0.818	0.366[NS]
2 to 10	198	10 (5.05)			22 (11.11)		
≥ 11	58	2 (3.44)			9 (15.51)		

χ^2Pearson's chi-square test; [NS] non-significant difference (P>0.05); *Significant difference (P<0.05).
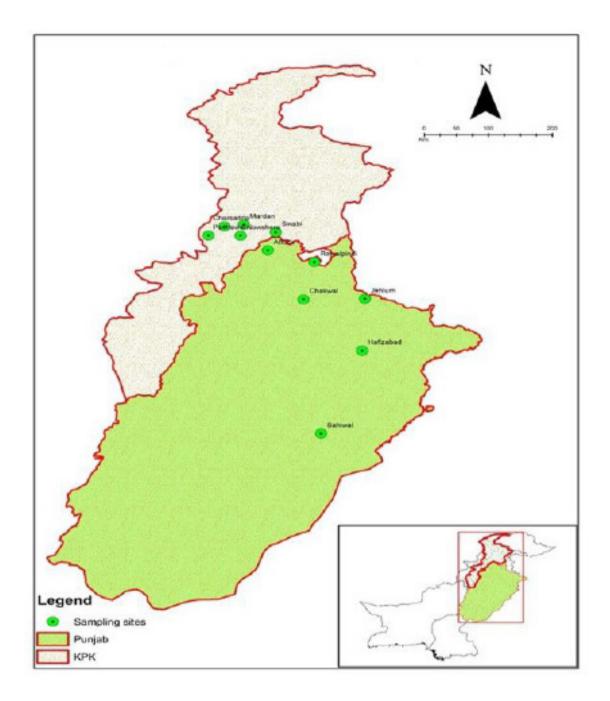
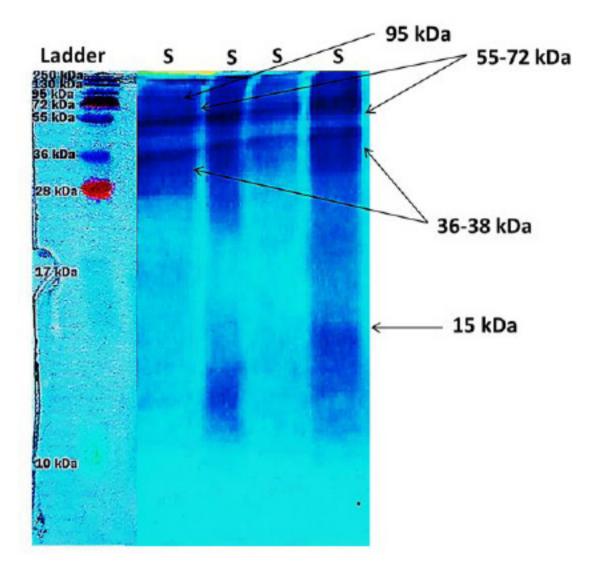Figure 1. Map of Pakistan indicating study areas in Khyber Pakhtunkhwa and Punjab province.

Figure 2. Protein bands separated by SDS-PAGE in somatic (S) extracts of adult worms of *Fasciola gigantica* collected from cattle and buffaloes.
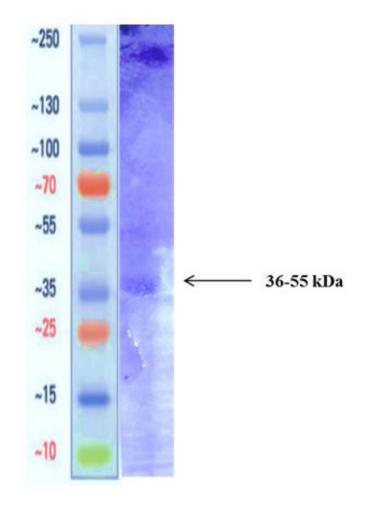
Figure 3. Immunoblotting of adult *Fasciola gigantica* antigen against naturally infected bovine sera.

that 36 to 55 kDa somatic antigens are highly immunogenic and the in house established ELISA was found highly sensitive and specific. Further studies on the identification of more antigenic proteins having the diagnostic potential are required for epidemiological surveys.

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