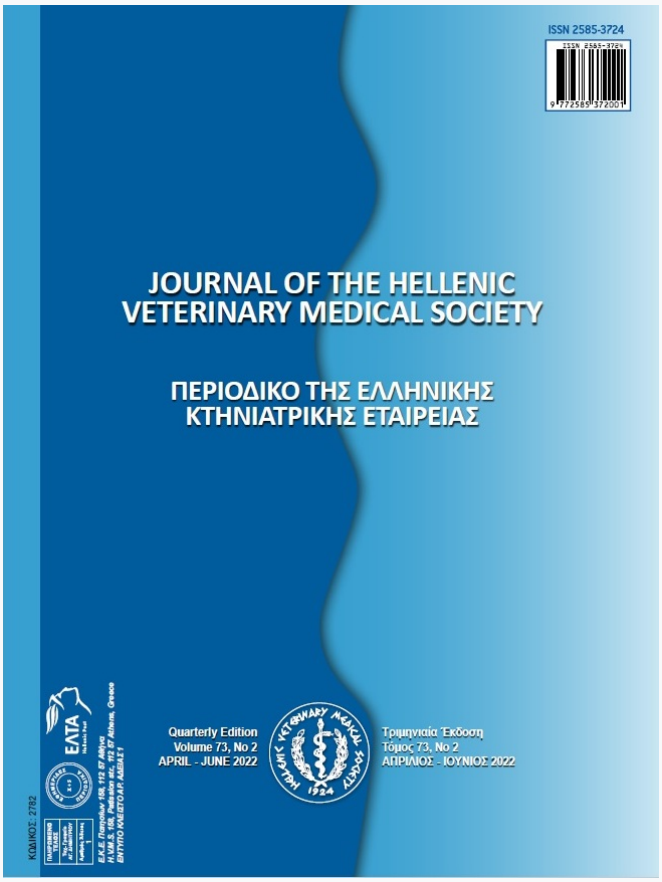
Journal of the Hellenic Veterinary Medical Society

Vol 73, No 2 (2022)



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doi: 10.12681/jhvms.26565

To cite this article:

Ali, R., Afshan, K., Fuentes, M. V., & Firasat, S. (2022). Serological diagnostic potential of the 38-72 kDa somatic antigen of Gastrothylax crumenifer in buffaloes using the Indirect Enzyme-Linked Immunosorbent Assay. *Journal of the Hellenic Veterinary Medical Society*, *73*(2), 4105–4112. https://doi.org/10.12681/jhvms.26565

J HELLENIC VET MED SOC 2022, 73 (2): 4105-4112
ΠΕΚΕ 2022, 73 (2): 4105-4112

Research article
Ερευνητικό άρθρο

Serological diagnostic potential of the 38-72 kDa somatic antigen of *Gastrothylax crumenifer* in buffalos using the Indirect Enzyme-Linked Immunosorbent Assay

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ABSTRACT: Paramphistomosis, caused by digenetic trematodes of the superfamily Paramphistomoidea, causes heavy economic losses in terms of reduced fertility, milk, and meat production in the livestock industry. In the present study, somatic and excretory-secretory antigens isolated from 500 live *Gastrothylax crumenifer* were assessed for their diagnostic potential by using an antibody detection enzyme immunoassay. Before the enzyme immunoassay, the somatic and excretory/secretory (ES) antigens of *G. crumenifer* were subjected to SDS-PAGE and Western blot (WB) for the detection of immunogenic proteins. Indirect ELISA analysis was performed on sera from buffalos naturally infected with *G. crumenifer*, with control sera of buffalos infected with *Gigantocotyle explanatum, Fasciola* spp., *Cotylophoron* /*Paramphistomum* spp. The SDS-PAGE results of the somatic products of *G. crumenifer* identified proteins were between 10-123 kDa, showing a maximum abundance of 10, 15, 25-28, 36, 38-72, 95-123 kDa proteins. The most abundant proteins recorded in ES products were ≥95, 72, 55, and 40 kDa. The antigenic analysis of somatic proteins using WB revealed reactive polypeptides of a size between 55-70 kDa, while metabolic extracts did not show reactivity with naturally infected buffalo sera. The sensitivity and specificity of the ELISA test for 38-72 kDa somatic antigens were 85.71% and 89.74%, respectively. The cross-reactivity with other trematode sera was 16-20%. Antibodies were tested against the 38-72 kDa somatic antigens, and 19.69% (39/198) of buffalos were found positive, while 12.1% (24/198) presented infection in the faecal/postmortem examination. The study confirmed that ELISA established for the 38-72 kDa somatic antigens of *G. crumenifer* had diagnostic potential against paramphistome infections.

Keywords: Paramphistomes, buffalos**,** Antigens, Western blotting, ELISA, SDS-PAGE

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Date of initial submission: 25-03-2021
Date of acceptance: 17-07-2021

INTRODUCTION

Paramphistomes are conically shaped digenetic trematode parasites belonging to the superfamily Paramphistomidae (Hafeez, 2013), having a wide geographical range including tropical and sub-tropical countries. The economic loss caused by paramphistomes infections has not been estimated, and the cost is in terms of reduced feed conversion ratio, milk, and meat production in the livestock industry (Horak, 1967; Kilani *et al.,* 2003). In some areas of India, the Republic of South Africa, and Australia, the mortality is 80-90% in domesticated ruminants (Boray, 1959, Soulsby, 1987). In Pakistan, the four most commonly occurring genera infecting ruminants are *Paramphistomum, Gastrothylax, Cotylophoran*, and *Gigantocotyle* (Yusuf and Chaudhry, 1970). The adults of the first three genera occur in the rumen and reticulum, while those of *Gigantocotyle* are found in the bile ducts.

A coprological examination is considered the gold standard for the diagnosis of paramphistome infections but may often result in the misdiagnosis of the disease and a lack of sensitivity, especially in light infections (Prasad and Singh, 2014). Most paramphistomes, including *Gastrothylax crumenifer*, present seasonal reproduction with the highest egg output taking place in the monsoon and post-monsoon period, whereas gonadal regression leads to ceased egg production in the winter season (Hanna *et al.,* 1988). The coprological examination in the non-reproductive phase of paramphistomes is not a useful diagnostic test as eggs appear 13-17 weeks post-infection (Anuracpreeda, 2016). Moreover, paramphistomes eggs resemble those of other trematode parasites and differentiation is not possible with the coprological examination (Kumar, 1998). The adult flukes residing in the rumen release the eggs in faeces, but do not cause severe harm to their hosts. Immature paramphistomes cause pathogenicity in the intestine but are incapable of laying eggs. Therefore, early diagnosis is required for timely treatment before irreversible damage to the intestine occurs (Wang *et al.,* 2006). Immunological diagnosis would be considered an effective tool for early detection of infections during the prolonged prepatent period. Antibody detection occurs with accuracy and precision as early as 2 weeks after infection (Santiago & Hillyer, 1988). Thus, numerous attempts have been made in recent years to determine the potential antigens of different trematodes, including paramphistomes, throughout the world (Gomez-Morales *et al.,* 2013; Anuracpreeda *et al.,* 2013; Arunkumar *et al.,* 2014; Salib *et al*., 2015; Dar *et al.,* 2017; Jadav *et al.,* 2018).

In Pakistan, studies conducted on the prevalence and the distributions of paramphistomes in ruminants are based on coprological examination. However, immunodiagnostic studies on these infections have never been conducted. The present study aims at characterizing the somatic and excretory/secretory (ES) antigens of *G. crumenifer* by SDS-PAGE analysis and to assess their somatic antigen diagnostic potential using the antibody detection enzyme immunoassay, particularly during the prepatent period.

MATERIALS AND METHODS

Research Ethics Committee

The study was conducted by following the ethical guideline approved by the Ethical Committee of Quaid-i-Azam University Islamabad, Pakistan. All animals used in the study were slaughtered for other purposes to fulfill the protein demand of local population.

Sample collection

Adult paramphistomes (n= 281) were removed from buffalos (n= 21) taken to be slaughtered at Sihala abattoir, Rawalpindi (Punjab), and local abattoirs in Peshawar (Khyber Pakhtunkhwa), Pakistan (Fig. 1). The rumens were examined, and parasites were removed with the help of forceps, carefully following all precautions to avoid any damage to the parasites. Helminths were meticulously washed with 0.01M phosphate-buffered saline (PBS) pH 7.2.

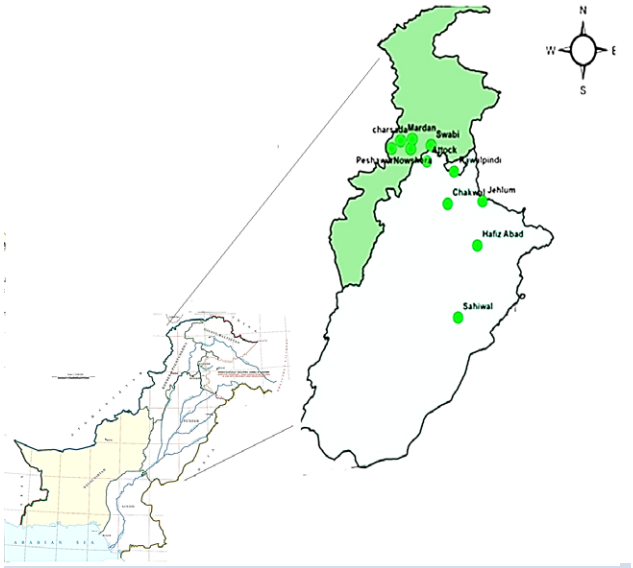

Figure 1: Map of Pakistan indicating study areas in Khyber Pakhtunkhwa and Punjab Province

Extraction of excretory/secretory and somatic products

To obtain excretory/secretory products, live helminths were kept in 0.01M PBS (1 helminth / 5 ml) for 24 hours. Flukes were removed and the extract was centrifuged at 10,000 rpm for 10 minutes and the supernatant was separated. To obtain somatic (whole helminth) extract, the fluke was homogenized in chilled tissue lysis buffer, added according to the weight of tissue in a ratio of (1000 µl buffer/100 mg of tissue). The cold condition was maintained by keeping the tissue grinder in an icebox throughout lysis. The homogenate was then transferred to pre-chilled Eppendorf tubes and centrifuged at 10,000 rpm at 4°C for 10 minutes. The Bradford assay (Bradford 1967) was used for protein quantification. The supernatant was transferred to pre-chilled Eppendorf tubes and stored in aliquots at -20°C.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE was performed according to the method described by Laemmli (1970). Briefly, the somatic and ES antigens were preheated with loading buffer for 5-10 minutes in a water bath at 95°C and loaded in 12 % and 10% separating gels, respectively. After running the gel at 90V for 2 hours, the gel was fixed for 1 hour and then stained with 0.1% Coomassie brilliant blue (Sigma). The molecular weight of each polypeptide was calculated using the protein ladder (Thermo Scientific™).

Immunoblotting

Immunoblotting was carried out according to Hongbao & Kuan (2006). Briefly, after running the gel, the stacking gel was separated, the nitrocellulose membrane presoaked in transfer buffer was added and the gel was placed at the top of this membrane. The presoaked 6 filter papers were added at the top and bottom and rolled to remove air bubbles. The blotting was carried out at 10V 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 hour, changing the solution at 15-minute intervals. The primary antibody (1:500) from naturally infected sera was incubated at 4°C overnight. After incubation, the membrane was washed with PBST 3 times, each wash for 10 minutes. The membrane was incubated with a secondary antibody (anti-goat antibody) for 2 hours on rotation. After washing, the substrate (BCIP/NBT) was added and stored in the dark, and incubated for 15 minutes until the bands became visible.

Enzyme-Linked Immunosorbent Assay (ELISA)

After running the SDS-PAGE gel, different protein bands of somatic antigen 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 the protein concentration in each sample was determined with Bradford assay (1967) and stored at -20°C. The checkerboard method was used to determine the working dilutions of antigens and test sera. Different concentrations of antigens (20 µg, 15 µg, 10 µg, 5 µg, and 1 µg, per 100 µl per well) and positive and negative pooled sera (1:10, 1:20, 1:50, 1:100, 1:200, 1:400) were tested. The optimum concentrations of antigen and natural sera dilution were found to be 10 µg/100 µl and 1:10, respectively. ELISA was performed according to the method described by Fagbemi *et al.* (1997) and Ferre *et al.* (1997). Each eluted antigen was mixed with coating buffer in equal proportion (1:1) and 100 µl were added to each well of the microtiter plate and incubated overnight at 4°C, then washed with 0.05% PBS-Tween 20 and blocked with 0.05% BSA for 2 hours at room temperature. Next, 100 µl of sera from infected and control animals were added to each well and incubated for 2 hours at 37°C. After 3 washes, 100 µl of secondary antibody (horseradish peroxidase-conjugated anti-bovine IgG antibodies) were added to each well and the plate was incubated for 1 hour at room temperature. The plates were washed and 100 µl of the substrate (TMB Tetramethylbenzidine) was added to each well and the plate was incubated in the dark for 15 minutes at room temperature. The reaction was stopped by adding 100 µl of 1M H_2SO_4. Optical density (OD) values were determined by an ELISA reader at 450 nm. The cut-off value was determined with the mean absorbance value of negative serum added by three standard deviations. Sera with OD values higher than the cut-off value were considered positive.

Evaluation of ELISA using naturally infected buffaloe sera

The ELISA developed was validated with 60 blood and faecal samples collected from buffalos. Blood and faecal samples were taken from buffalos positive for *G. crumenifer* (n=21), and cross-reactivity was tested with control sera collected from buffalos positive for *Gigantocotyle explanatum* (n=10)*, Fasciola* spp. (n=7), *Cotylophoron* spp./*Paramphistomum* spp.

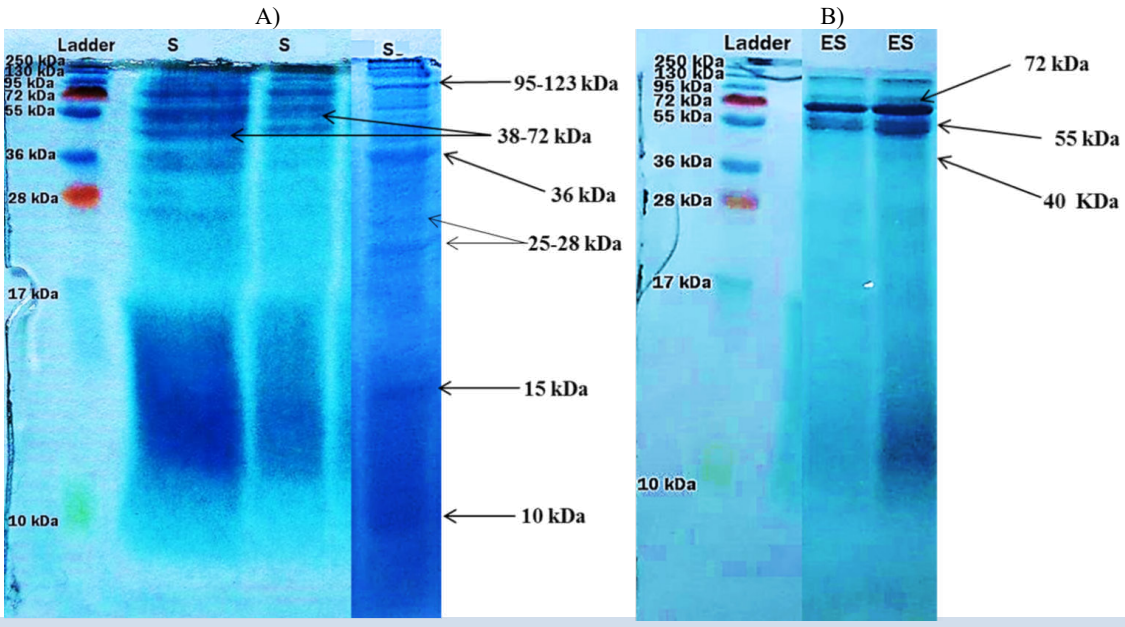

Figure 2: Protein bands separated by SDS-PAGE in A) somatic (S) and B) excretory secretory (ES) extracts of adult worms of Gastrothylax crumenifer collected from buffalos

(n=12). Negative control sera (n=10) were obtained from 2-week-old kids born to a herd having a history of stall feeding only. Sera were separated and stored at -20°C, and faecal samples were collected per rectum into well-labeled sterile polythene bags, transported in ice packs to the laboratory, then stored at 4°C and analyzed within two days. The sensitivity and specificity of the ELISA using somatic antigen of *G. crumenifer* were calculated in comparison to faecal/postmortem examination by the following formulae:

Sensitivity = [a/ (a+c)]×100

where 'a' is the number of animals positive by ELISA and faecal/postmortem examination (true positive), while 'c' is the number of animals positive by faecal/postmortem examination but negative by ELISA (false negative);

Specificity = [d/ (b+d)] ×100

where 'd' is the number of animals negative by ELISA and faecal/postmortem examination (true negative), while 'b' is the number of animals negative by faecal/postmortem examination but positive by ELISA (false positive).

Field implementation of ELISA

After the evaluation and standardization of the ELISA test, a total of 198 randomly collected blood and faecal/postmortem samples were examined.

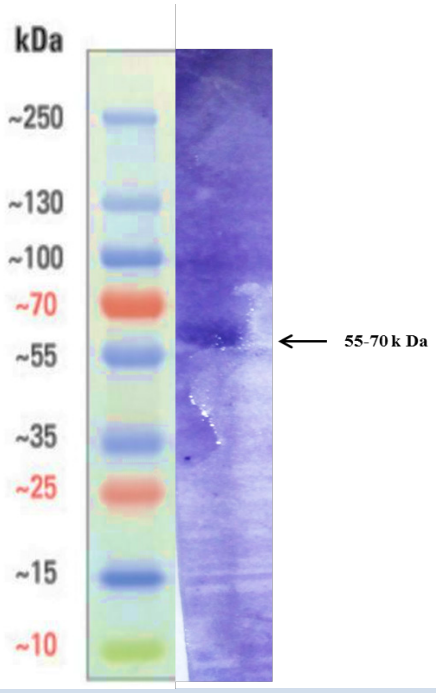

Figure 3: Immunoblotting of Gastrothylax crumenifer somatic antigens in naturally infected buffalos

Statistical Analysis

Statistical analysis was performed using SPSS version 20. Kappa value of ELISA was calculated by using online software 'QuickCalcs (https://www.graphpad.com/quickcalcs/).

RESULTS

SDS-PAGE and Western blot analysis

The SDS-PAGE profile of the separated somatic polypeptides of adult *G. crumenifer* flukes revealed a total of 10 polypeptides in the molecular weight range of 10-123 kDa. The molecular weights of the most visible bands in the somatic extracts were 10-20, 21-37, 38-72, 95-123 kDa polypeptides (Fig.2A). The excretory/secretory extracts of *G. crumenifer* adults showed polypeptides of molecular weights ≥95, 72, 55, and 40 kDa (Fig. 2B).

The Western blot analysis revealed that somatic extract polypeptides of a size between 55-70 kDa were antigenic against sera obtained from buffalos infected with *G. crumenifer* (Fig. 3). However, in the case of excretory/secretory extract, no distinct band was observed.

ELISA

The cut-off was set by the mean optical density (OD) of the negative reference serum, plus three times standard deviations (0.24+3*0.07=0.45). Eluted antigens of somatic extract of *G. crumenifer* coated on microtiter plates were tested against sera from buffalos to check the antigenicity of the proteins. The sensitivity and specificity of the diagnostic test for 38-72 kDa somatic antigens were 85.71% and 89.74%, respectively. The Kappa value calculated for the test was 0.746 (Table 1), which revealed that the strength of agreement was 'good'.

The cross-reactivity with *G. explanatum* positive sera was 20% and 16.6% was recorded for *Cotylophoron* /*Paramphistomum* infected sera. Sera from buffalo suffering from fasciolosis and normal control sera did not show reactivity with any of the antigens tested (Table. 2).

Prevalence of *Gastrothylax crumenifer* among buffalos

A total of 198 serum samples were tested for anti-IgG against 38-72 kDa somatic antigen of *G. crumenifer* in buffalos, and 19.69% (39/198) were found positive. The faecal/postmortem examination showed a 12.1% (24/198) infection rate. Paramphistomosis did not show a significant association with the breed, sex nor age of the animals examined by faecal/postmortem and ELISA test.

DISCUSSION

In the present study, somatic and excretory/secretory antigens of *G. crumenifer* were separated and subjected to a protein profiling study. The somatic antigens were used in antibody detection ELISA. The SDS-PAGE analysis of somatic antigens of *G. crumenifer* revealed ten polypeptide bands of molecular weight in the range of 10-123 kDa. Among the prominent bands, the intensity was stronger for high molecular weight bands (38-72 and 95-123 kDa) and weaker for low molecular weight bands, indicating that separation was not very discrete, maybe due to the presence of a non-proteinaceous complex nature

Table 1: Diagnostic efficacy of ELISA established for 38-72 kDa somatic antigen with faecal/postmortem examination

Test	ELISA Test			Sensitivity	Specificity	KAPPA
Faecal/ Postmortem Examination	**Positive**	**Negative**	**Total**	**95% CI**	**95% CI**	
Positive	18	3	21	85.71% (63.66%-96.95%)	89.74% (75.78%-97.13%)	Kappa= 0.746
Negative	4	35	39			SE of kappa = 0.090
			60			95% confidence interval: 0.570 to 0.922

Table 2: Evaluation of cross-reactivity of ELISA test established for 38-72kDa somatic proteins of G. crumenifer against animal sera infected with other trematodes along with positive and negative control sera

Sera Positive for Trematodes	Total Sera tested n=60	Test Positive n (%)	Test Negative n (%)
Gastrothylax crumenifer	21	18 (85.7)	3 (14.3)
Fasciola spp.	7	0 (0)	7 (100)
Gigantocotyle spp.	10	2 (20)	8 (80)
Cotylophoron/ Paramphistomum spp.	12	2 (16.6)	10 (83.3)
Negative control	10	0 (0)	10 (100)

of these proteins (Arunkumar *et al.,* 2014). The results are in agreement with the study by Saifullah *et al.* (2000) who recorded eight major fractions ranging from 14 to 205 kDa in *G. crumenifer*.

Similarly, studies on *P. epiclitum* recorded molecular weight bands in the ranges of 4-100 kDa (Jadav *et al.,* 2018) and 14.9-95.5 kDa (Arora *et al.*, 2010); in *P. cervi* the molecular weight band ranged from 12 to 100 kDa (Dar *et al.,* 2016), and in *Paramphistomum* spp. the molecular weight band was in the 11.5-174 kDa range (Salib *et al.*, 2015). However, the results were comparable with mixed paramphistomes bands ranging from 25 to 120 kDa (Meshgi *et al.*, 2009) and *F. hepatica* and *F. gigantica* protein bands in the ranges of 18-62kDa and 18-68 kDa, respectively (Meshgi *et al.,* 2008). The variations in the relative molecular weight of the polypeptides in the current research and other reported studies may be due to the influence of the geographical location of the parasite and antigen isolation methods (Jadav *et al.,* 2018). The ES antigens found in the present study were polypeptides with major bands of 55 kDa and 72 kDa and diffused bands of <10-15 kDa. Similar studies conducted in *G. crumenifer* recorded eight polypeptides in a molecular weight band of 240-30 kDa (Arunkumar *et al.*, 2014) and seven polypeptides in the range of <14 to 165 kDa, respectively (Saifullah *et al.,* 2011). Other studies on the separation of ES antigens of paramphistomes recorded polypeptide bands of different molecular weights (Irving and Howell, 1982; Yadav and Gupta, 1995; Saifullah *et al.,* 2000; Ridi *et al.,* 2007; Saifullah *et al.,* 2011; Anuracpreeda *et al.,* 2013; Jadav *et al.,* 2018). This difference in the number and molecular weights of polypeptides found may be explained by the method of isolation of ES antigens and the species of paramphistomes and other trematodes selected.

Western blot analysis of somatic extracts of *G. crumenifer*, using pooled positive sera samples of naturally infected buffalos, revealed immunoreactive molecular weight bands of 55-70 kDa. No polypeptide band showed immunoreactivity with the verified pooled negative serum Previous studies on Western blot analysis of somatic antigens of *G. crumenifer* (Saifullah *et al.,* 2000; Saifullah *et al.,* 2011), recorded low molecular weight polypeptides, ranging from <14 to 50 kDa. The results of studies on other trematodes are comparable with the current research, having identified somatic antigenic polypeptides of: *F. hepatica* in the range of 33 and 66 kDa (Gonenc *et al*., 2004); *Paramphistomum cervi* 52 kDa (Anuracpreeda *et al.,* 2008); mixed infection with paramphistomes 90 kDa (Meshgi *et al.,* 2009); and *P. epiclitum* with two lower molecular weight protein bands of 37.6 and 39.8 kDa (Arora *et al*., 2010). The studies on *Paramphistomum spp*. revealed immune-dominant polypeptides of molecular weights of 27, 39, 58, 63, 71, and 87 kDa, with a maximum reactivity of 63kDa (Salib *et al*., 2015), and *P. cervi* revealed six major positive antigenic bands, of which the 90 kDa polypeptide showed the maximum antigenic reactivity (Dar *et al.,* 2017). The results of the current study may be explained by geographical and ecological variation and the host type as causal factors of differences between protein bands. However, the differences between our results and the aforementioned studies may be attributed to single and mixed paramphistome infections (Dar *et al.,* 2017). ES antigens used in serodiagnosis of trematode infections have been well documented (Choi *et al.,* 2003; Narain *et al.,* 2005; Awad *et al.,* 2009; Gomez-Morales *et al.,* 2013; Anuracpreeda *et al.,* 2013). However, in the present study, no ES immunoreactive antigen band was detected by Western blot analysis using naturally infected positive buffalo sera infected with *G. crumenifer*. The results may be explained by the different isolation methods of ES antigens used, as well as the low molecular weight of the precipitated proteins by the higher concentration of alcohol (Jaiswal *et al.,* 2018).

Indirect ELISA is considered a reliable tool for early diagnosis of paramphistomosis in different animal species (Anuracpreeda *et al.,* 2013). Herein, using the indirect ELISA, the somatic antigen in the 38-72 kDa range was found to display sensitivity and specificity against *G. crumenifer* infections of 85.71% and 89.74%, respectively, using 60 serum samples from buffalos. This high sensitivity might be due to four immunoreactive bands (molecular weights of 36, 38, 55, and 72 kDa). However, four samples were found to be false positive (ELISA positive and faecal/post-mortem examination negative), indicating that disease was likely to be in the early stage (immature paramphistomosis) or that animals had been treated for paramphistomosis. The likelihood of cross-reactivity with other trematode infections was found in about 10-20% of the cases when other trematode flukes/eggs were found in postpartum examinations/faeces of the buffalos under study. This may also be explained with some common antigenic epitopes that develop immune responses in their hosts, generating antibodies that can detect antigens from other in-

fections (Fagbemi & Obarisiagbon, 1991; Ghosh *et al.,* 2005; Arora *et al.,* 2010 Kanjuga *et al.,* 2015). The phosphoryl-choline epitope is the main element of cross-reaction, an important conserved epitope of trematodes (Sloan *et al.,* 1991).

Similar results to our study were reported by Jadav *et al.* (2018) for somatic antigens of *P. epiclitum* in postpartum negative groups for indirect ELISA with a specificity of 89.83% in goats and 89.23% in sheep, respectively. Salib *et al.* (2015) evaluated indirect ELISA against somatic antigens of paramphistomes and revealed that the sensitivity, specificity, and accuracy were 74%, 82.4%, and 79.76%, respectively. Kumar *et al.* (2008a) developed ELISA using immunoaffinity column chromatography purified antigen from the somatic antigen of *F. gigantica* in buffalos and found a specificity of 97, 95, and 98% (in indirect) / 92.3, 94.7, and 90% (in dot) against *G. crumenifer*, *G. explanatum* and mixed infection of both parasites. In another study, Kumar *et al.* (2008b) observed 97-98% specificity of the indirect ELISA test against purified 27 kDa glycoprotein from the somatic antigen of *F. gigantica* against *G. crumenifer, G. explanatum*, or a mixed infection with both parasites in buffalos. Jadav *et al.* (2018) recorded a high level of specificity against PeSAg in a faecal negative animal group with indirect ELISA (86.11% for somatic antigens in goats and 66.67% in sheep) and dot-ELISA (83.33% for somatic antigens in goats, 83.33% in sheep). A comparatively high level of specificity was also observed by Kumar *et al.* (2008 a, b) in negative animal groups.

In the current study, after combining the faecal/postmortem examination and 38-72 kDa somatic antigen indirect ELISA results, an increased prevalence was found in buffalos (19.69%), indicative of *G. crumenifer* either in the stage of active (prepatency and patency) or passive infection. The higher positivity prevalence observed in the current study using indirect ELISA may be due to early infections or to cross-reactivity with other closely related helminth parasites (Maji *et al.,* 1999; Yadav *et al.,* 2003; Salib *et al.,* 2015). These findings are in agreement with other studies on paramphistome infections (Kumar *et al.*, 2008 a, b; Kaur *et al.,* 2008; Jadav *et al.,* 2018). However, the prevalence of *G. crumenifer* infection based on faecal/postmortem examination in buffalos from some districts of Punjab and Khyber Pakhtunkhwa in the present study was 12.1%, which is in the range of the prevalence reported from other studies carried out in Pakistan (Zaman *et al.,* 2014; Iqbal *et al.,* 2014).

CONCLUSIONS

The results of the current study confirm that polypeptides of 38-72kDa in the somatic extract of *G. crumenifer* can be used as a specific diagnostic antigen for paramphistomiasis in buffalos of Pakistan. However, the limitation of these assays is that present and past infection cannot be differentiated as the positive titers can also be observed even after elimination of infection. Additional studies on IgM and IgG antibody titers are required to differentiate between current and past infections.

COMPETING INTERESTS

None.

ACKNOWLEDGEMENTS

The research work presented in this article is under internal research funds of Quaid-i-Azam University, Islamabad.

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