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SHORT COMMUNICATION

Diagnostic Efficacy of Copro-ELISA for Detection of Fasciolosis in Cattle and Buffaloes in Punjab Province, Pakistan

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Abstract

Fasciolosis is a food borne zoonotic trematode disease that causes liver damage in ruminants and humans. The information recorded on the prepatent diagnostic methods is limited with low sensitivity. The study aimed to investigate the diagnostic efficacy of coproantigens by using in house developed indirect enzyme linked immunosorbent assay (ELISA). Adult helminths were collected from the bile duct of buffaloes for the extraction of excretory secretory (ES) and somatic (SA) antigens. The polyclonal antibodies were produced by immunization of rabbits with SA and ES antigens of Fasciola. Kappa value of developed ELISA was calculated to check the diagnostic performance of the test. The mean absorbance values at different concentrations of coproantigens were significantly different (P≤0.001) from controls. However, the difference was not significant among the concentrations of coproantigen. A positive linear relationship was observed to the concentration of antigens used in fecal supernatant and the absorbance values. The sensitivity and specificity of diagnostic test with ES-polyclonal antibodies were 100% (95% CI: 89.42%-100.00%) and 76.19% (95% CI: 52.83.30%-91.78%), respectively. Kappa value revealed that the strength of agreement is almost substantial. The SA-polyclonal antibodies showed the specificity and sensitivity of diagnostic test were 100% (95% CI: 89.42%-100.00%) and 90.00% (95% CI: 68.30%-98.77%), respectively. However, Kappa value of the test revealed that the strength of agreement is perfect. The result provides information that will add sensitive diagnostic methods for the detection of fasciolosis.

Keywords: *Copro-ELISA, Fascioliasis, Ruminants, SA antigens, ES antigens*

Pakistan'ın Pencap Eyaletinde Sığır ve Mandalarda Fasciolozis'in Tespitinde Copro-ELISA'nın Tanısal Etkinliği

Öz

Fasciolozis, geviş getiren hayvanlarda ve insanlarda karaciğer hasarına neden olan gıda kaynaklı zoonotik bir trematod hastalığıdır. Prepatent tanı yöntemleri ile elde edilen diagnostik bilgiler düşük hassasiyetli ve sınırlıdır. Bu çalışma, in house geliştirilen indirekt Enzyme Linked Immunosorbent Assay (ELISA) kullanılarak koproantijenlerin tanısal etkinliğinin araştırılmasını amaçlamıştır. Ekskretuar sekretuar (ES) ve somatik (SA) antijenlerin ekstraksiyonu için kullanılacak erişkin helmintler bufaloların safra kanallarından toplandı. Fasciola'nın SA ve ES antijenleri ile tavşanların immunizasyonunu takiben poliklonal antikorlar üretildi. Geliştirilen ELISA testinin tanısal performansını kontrol etmek için Kappa değeri hesaplandı. Koproantijenlerin farklı konsantrasyonlardaki ortalama absorbans değerleri, kontrollerden önemli ölçüde farklı saptandı (P≤0.001). Fakat, koproantijen konsantrasyonları arasındaki fark önemli değildi. Fekal süpernatanda kullanılan antijenlerin konsantrasyonu ile absorbans değerleri arasında pozitif linear bir ilişki gözlemlendi. ES-poliklonal antikorlarla yapılan diagnostik testin duyarlılığı %100 (%95 CI: %89.42-100.00) ve özgüllüğü %76.19 (%95 CI: %52.83-91.78) saptandı. Kappa değeri, uyum gücünün neredeyse önemli derecede olduğunu ortaya koydu. SA-poliklonal antikorlarlarla yapılan diagnostik testin özgüllüğü %100 (%95 CI: %89.42-100.00) ve duyarlılığı %90.00 (%95 CU: %68.30-98.77) saptandı. Ancak, testin Kappa değeri, uyum gücünün mükemmel olduğunu ortaya koydu. Bulgular, fasciolozisin tespiti için hassas tanı yöntemlerine ek bilgi sağlayacak niteliktedir.

Anahtar sözcükler: *Copro-ELISA, Fasioliazis, Ruminant, SA antijenleri, ES antijenleri*

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Introduction

Fascioliasis is a food-born zoonotic infection having etiological agent *Fasciola* species, commonly acquired by eating metacercaria encysted on vegetation [1]. Fasciolosis is a critical problem to the livestock population, having high morbidity, considerable mortality, and tremendous economic losses to the livestock sector [2,3].

The fecal egg counting (FEC) techniques with zinc sulfate floatation and sedimentation methods are most frequently used for the diagnosis of chronic fasciolosis [4]. The limitations of FEC reduction tests (FECRT) includes the long pre-patent period, low worm burden, and irregular egg shedding of adult *Fasciola spp.* [5]. In live animals, for the detection of early-stage infection and precautionary control, preventive measures appropriate diagnostic methods are very pivotal. Various molecular, biochemical, and metabolic processes are necessary for these diagnostic tools [6]. The diagnostic tools like immunological and coprological methods not only detect the circulating antibodies and antigens but also help in quantifying the coproantigens. A highly sensitive antibody specific test is critical to help reduce the losses caused by fasciolosis in the livestock sector in different regions of the globe [7]. ELISA and immunoblots based on antibody detection are the preferred techniques for the diagnosis of fasciolosis on account of their relative simplicity and detection of seroconversion in primary infection. Serological and coprological detection of fasciolosis in live animals has an advantage over other techniques due to its low cost and high specificity [8]. Nevertheless, collecting sera is a tough routine with a large population of animals, and methods like these are of less diagnostic value in areas where the disease is highly prevalent because antibody concentration remained at high even after animals have been treated [9]. On the other hand, active fasciolosis can be diagnosed by the detection of eggs shed into feces. It is conclusive that the collection of feces at the herd level is a comparatively easier process than the collection of blood samples. Studies had centralized the idea of the establishment of ELISA tests for the diagnosis of trematode antigens in feces, i.e., coproantigens [10]. The present study aimed to evaluate the suitability of coproantigen ELISA established for the detection of fasciolosis.

Material and Methods

Adult Helminths and Feces Collection

Adult helminths were collected from the bile duct of cattle and buffaloes which were brought to the local abattoirs to slaughter. The study was conducted by following the ethical guideline approved by the Ethical Committee of Quaid-i-Azam University Islamabad, Pakistan. Slaughtered animals (n=252) were examined for confirmation of the *Fasciola* helminths among which thirty-three were found positive. Collected helminths were subjected to 0.01M Phosphate buffer saline (pH-7.2) washed several times to clear any contaminated material. Fecal samples from animals positive (n=33) and negative i.e. no history of prior infection or infected with other helminths (n=21) were collected.

Extraction of Excretory Secretory and Somatic Antigens

Fasciola excretory secretory (ES) antigen was prepared according to the method described by Mezo et al.[11]. Briefly, mature live helminths were kept in 0.01M PBS (1 helminth/5 mL) for 24 h. The medium containing the flukes was removed and centrifuged for 20 min at 10000 g and 4°C by adding protease inhibitors. To obtain somatic (SA) 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 homogenate was centrifuged at 10.000g and the resultant pellet was removed, and the supernatant refrigerated at -70°C. The protein concentrations of the ES and SA products were calculated by the method described by Bradford [12].

Generation of Polyclonal Antibodies in Rabbits

Four New Zealand white rabbits were injected by *Fasciola* SA and ES extracts to produce polyclonal IgG antibodies. The rabbits were given four doses of 200 mg of *Fasciola* ES and SA antigens at a 3-week interval by subcutaneous, parenteral route. Freund's complete adjuvant was used as an immune stimulator and sera were collected from all four rabbits a week after the last booster dose and stored at -20°C for further analysis.

Extracting Antigens from Fecal Samples

To detect the *Fasciola* coproantigens, the collected fecal samples were mixed individually with distilled water at 1:1 (3 mL water containing 3 g of faces) and subjected to centrifugation for 15 min at 1000 g. Then supernatants were collected from each sample and maintained at -20°C until further investigation by ELISA.

Coproantigen ELISA

The assay was performed according to the method described by Ahmad and Nizami [13]. Briefly, 50 µL/well of fecal supernatant in coating buffer were added in microtiter plates and left overnight at 4°C. The plates were washed with PBS 0.1% Tween and blocked with bovine serum albumin (100 µL/well) for 2 h at room temperature. In the next step, plates were washed again and serially diluted 100 µL/well rabbit anti-*Fasciola* sera generated against ES and SA antigens (1: 2000) were incubated for 1 h at room temperature. The plates were washed three times and 100 µL/well goat anti-bovine IgG secondary antibodies (1:10.000), conjugated with alkaline phosphatase (Invitrogen™ Cat. nos. WP20006, WP20007) was incubated for 1 h at room temperature. After washing the plates 100 µL of the substrate

para-Nitrophenyle Phosphate (PNPP) (Thermo Scientific™ Cat. No. 37621) was incubated at room temperature for 20 min. Finally, the reaction was stopped by the addition of 50 µL of 3N NaOH solution, and OD was recorded at 405 nm on an ELISA reader.

Statistical Analysis

The online software 'QuickCalcs (https://www.graphpad.com/quickcalcs/) was used to calculate the Kappa value of developed ELISA. The sensitivity and specificity of indirect ELISA was computed by using Online Statistical Software MedCalc *(https://www.medcalc.org/calc/diagnostic_test.php)*. Univariate analysis was performed between concentration and absorbance values and the level of significance was set at P=0.001. The relationship between coproantigen concentration and absorbance values was calculated with a regression equation.

Results

The detectable level of coproantigens (OD value 0.2) by using indirect ELISA was observed in a total of 33 samples out of 252. The cut-off point ≥0.22 was considered positive for *Fasciola* coproantigens. The ELISA O.D. value for the buffalo population tested for the coproantigens of *Fasciola* was high with increasing the antigen concentrations, indicating a high level of coproantigens. From the 50 µL of fecal supernatant, the mean absorbance values were significantly different (P<0.001) from controls *(Table 1)*. However, the difference was not significant among the animals tested positive along with different concentrations of coproantigen *(Fig. 1-a,b)*. A positive linear relationship was observed to the concentration of antigens used in fecal supernatant and the absorbance values *(Fig. 2-a,b)*.

Coproantigen ELISA with ES Polyclonal Antibodies

The cut-off point was calculated by the average optical density (OD) of the negative reference feces, plus three times standard deviations (0.045+3*0.0168=0.095). The specificity of the assay was determined by using *Fasciola* negative feces from 21 buffaloes, although some of the buffaloes (7/20) harbored other gastrointestinal parasites. The cross-reactivity was observed with helminths parasitizing these domesticated animals.

Table 1. *OD values obtained with the Copro- ELISA testing fecal samples from animals infected with Fasciola species and controls. The absorbance values are presented a Mean± SD of different concentrations of coproantigens*

Concentration of Coproantigens	ES Polyclonal Antibodies		SA Polyclonal Antibodies	
	Mean± SD	95% CL	Mean±SD	95% CL
50 µL	0.42±0.08[a]	0.40±0.44	0.46±0.07[a]	0.44±0.48
80 µL	0.45±0.07[ab]	0.43±0.48	0.46±0.06[a]	0.44±0.48
100 µL	0.44±0.08[ab]	0.42±0.47	nc*	
120 µL	0.46±0.06[ab]	0.43±0.48	nc	
140 µL	0.48±0.05[b]	0.46±0.51	nc	
Cross reactive	0.11±0.04[c]	0.05±0.18	0.11±0.04[b]	0.05±0.17
Negative Controls	0.6±0.04[c]	0.03±0.09	0.08±0.04[b]	0.06±0.11
P-value	>0.001		>0.001	
F calculated	107.500		217.800	

***nc:** not calculated*

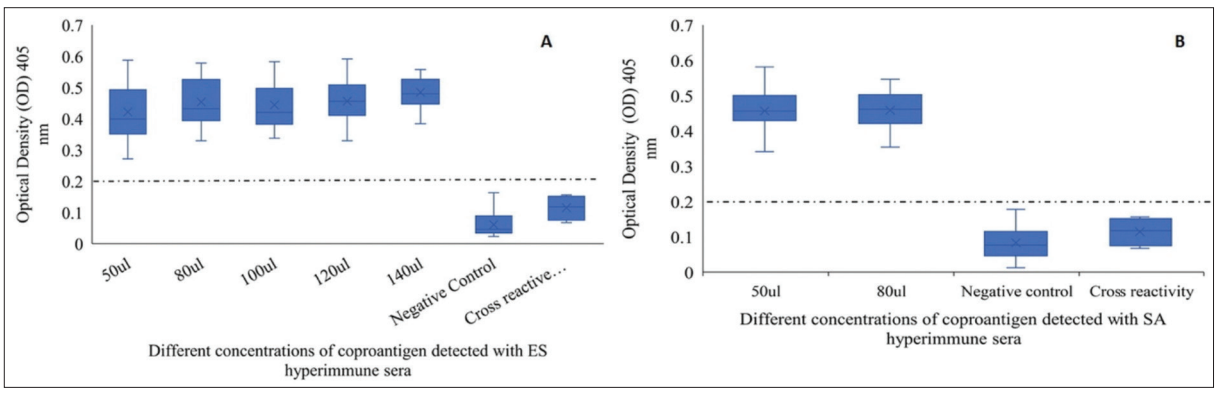

Fig 1. Detectability of Copro ELISA performed with **A)** ES polyclonal antibodies **B)** and SA polyclonal antibodies raised in rabbits from animals infected with *Fasciola* species. Cut-off point was set at 0.2

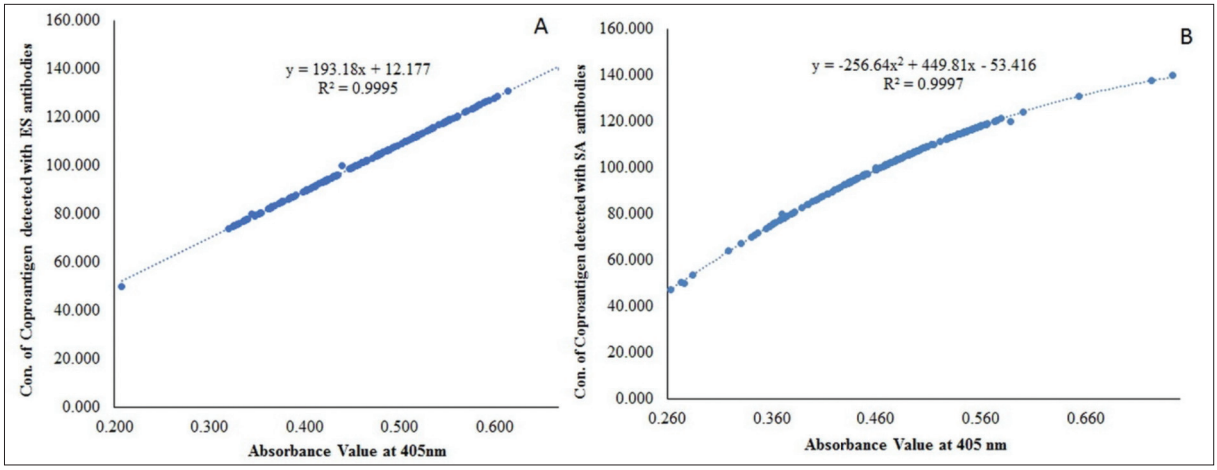

Fig 2. Correlation between absorbance values and different concentrations of coproantigens with Copro ELISA testing fecal samples **A)** with ES **B)** and SA polyclonal antibodies raised in rabbits from animals infected with *Fasciola* species

Table 2. *Diagnostic efficacy of copro-ELISA using hyperimmune sera of Fasciola ES antigens*

Diagnostic Methods	**ELISA Test**			**Sensitivity**	**Specificity**	**KAPPA Value**
Fecal/Postmortem Examination	**Positive**	**Negative**	**Total**	**95% CI**	**95% CI**	
Positive	33	0	33	100.00 (89.42-100.00)	76.19 (52.83-91.78)	Kappa = 0.796 SE of Kappa = 0.085 95% Confidence interval: 0630 to 0.963
Negative	5	16	21			
Total	38	16	54			

Table 3. *Diagnostic efficacy of copro-ELISA using hyperimmune sera of Fasciola somatic antigens*

Diagnostic Methods	**ELISA Test**			**Sensitivity**	**Specificity**	**KAPPA Value**
Fecal/Postmortem Examination	**Positive**	**Negative**	**Total**	**95% CI**	**95% CI**	
Positive	33	0	33	100 (89.42-100.00)	90 (68.30-98.77)	Kappa = 0.918 SE of kappa = 0.057 95% confidence interval: 0.807 to 1.00.
Negative	2	18	20			
Total	35	18	53			

The sensitivity and specificity of diagnostic test were 100% (95% CI: 89.42%-100.00%) and 76.19% (95% CI: 52.83.30%-91.78%), respectively *(Table 2)*. Kappa value of the test calculated that the strength of agreement is almost substantial.

Coproantigen ELISA with SA Polyclonal Antibodies

The cut-off point was calculated by the mean optical density (OD) of the negative reference feces, plus three times standard deviations (0.083+3*0.044=0.22).

The specificity and sensitivity of diagnostic test were 100% (95% CI: 89.42%-100.00%) and 90.00% (95% CI: 68.30%-98.77%), respectively *(Table 3)*. The results of Kappa value showed that the strength of agreement is approaching to perfect. A total of 20 negative control sera were used and two of the fecal samples showed cross-reactivity with *Fasciola* SA antigen polyclonal antibodies.

Discussion

In the present study, the efficacy of coproantigens ELISA based on polyclonal antibodies against SA and ES antigens was tested for the diagnosis of fasciolosis. The *Fasciola* coproantigens ELISA was found highly sensitive, which may reflect the stability of the antigens being excreted out along the feces. Several studies have been conducted on the detection of coproantigen in feces for a

number of helminths [14-17]. Monoclonal antibodies based coproantigen ELISA method was reported several times for detection of fasciolosis, which may compromise sensitivity due to high variability in the concentration of cathepsins in feces of infected animals [18].

In the present study 50 µL of fecal supernatant was used for detection of fasciolosis, the values containing 10 ng/mL of coproantigen in feces. The detection values in feces were found lower than reported previously [19,20]. These differences may be due to the capture antibody used since in our work a polyclonal serum was used whereas in the two other studies two different monoclonal antibodies were used.

The detection limits were different in the current study for the somatic and ES polyclonal antibodies based coproantigen ELISA, which may attribute to components present in the feces which resulted in an elevation of the non-specific limit of the assay [21]. The difference in detection limit as compared to previous studies may be because of diluting fecal samples in PBS containing BSA [17].

The cross-reaction occurred with other helminths when detected in the assay. However, the possibility of false positives would be very low, thus making the test highly specific. Similarly, 100% specificity was recorded by Espino et al.[22], however, Deplazes et al.[23] observed that a polyclonal antibody against ES antigen of tapeworms can detect different parasites of the same genus. The limitation of the current assay was, did not use helminth species i.e. *Schistosoma* and *Dicrocoelium* to check the cross-reactivity of the assay. Previous work had reported that *Fasciola* spp. shares antigens with these trematode parasites i.e. *Schistosoma* and *Dicrocoelium* [11,13,24]. Therefore, possible cross-reactions with antigens of these trematodes probably are not a factor that would affect the use of this test to diagnose fasciolosis in Pakistan.

In conclusion, the developed coproantigen detection methods are a good alternative to the conventional fecal examination microscopic techniques for fasciolosis detection in ruminants. However, further studies are required for field implementation of this indigenous coproantigen ELISA to detect fasciolosis and the cross-reactivity with other helminths species which may shares antigens with *Fasciola*.

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Conflict of Interest

Authors state no conflict of interest.

Author's Contribution

KA designed the study. KA, IA, MK performed the experiment. SF, IAK and MQ advised on methods, experimentation, and interpretation of findings. KA and MQ conducted literature search, data analysis and manuscript preparation. KA and MQ reviewed the manuscript. All authors participated in the study and concurred with the submission and subsequent revisions submitted by the corresponding author.

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