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Isolation and Characterization of Immunodiagnostic Antigen from *Strongylus vulgaris* Infecting Horses

Soad E. Hassan, A.A. Ghazy and Eman H. Abdel-Rahman

Parasitology and Animal Diseases Department, National Research Center, Dokki, Giza, Egypt

Abstract: Isolation of immunodiagnostic fraction from *Strongylus vulgaris* adult worms was described by CNBr-Sepharose 4B affinity column chromatography. The isolation process resulted in a fraction with 2161.5 fold increase in binding activity compared to its crude extract. Characterization of the isolated fraction by SDS polyacrylamide gel electrophoresis and isoelectric focusing showed that the fraction consists of only two bands of 39 and 31 KDa with isoelectric points of 6.8 and 6.7. Comparative evaluation of the immunogenic binding activities of the crude extract, unbound and bound fractions by ELISA proved the potency of the bound fraction over the other two antigens. Diagnosis of *S. vulgaris* infection in horses by ELISA in which the bound fraction was utilized, recorded high infection percentage (73.7) as compared with the parasitological examination (27.6). The isolated fraction in the current study could be utilized successfully in the diagnosis of *S. vulgaris* infection using ELISA, particularly in the prepatent period.

Key words: *Strongylus vulgaris* · Horses · Affinity purification · ELISA

INTRODUCTION

Nematode parasites of the horse include more than 50 species of Strongylidae from the subfamilies Strongylinae (large strongyles) and Cyathostominae (small Strongyles). *Strongylus vulgaris* is the most pathogenic member of the large strongyles, the migrating larval stages cause verminous arteries, thrombosis, weight loss, diarrhea, colic, peripheral edema, while the adult stages cause anemia, poor performance and/or hypoalbuminaemia [1]. The use of modern anthelmintics has decreased the prevalence of *S. vulgaris*, but has increased anthelmintics resistance and prevalence of cyathostomes [2,3]. Adult worms can be detected by fecal egg count analysis. However, horses with high mucosal and arterial larval burdens commonly have low or negative fecal egg counts [4], with no larvae in their feces. Clinical haematological parameters such as albumin: globulin ratios have been used in attempts to estimate the level of mucosal and arterial larval infection [5]. An immunodiagnostic test, capable of detecting the infection even at larval stage, would have a substantial impact on equine welfare by allowing therapy to be based on the definitive diagnosis of heavily parasitized horses. Furthermore, the availability of such an assay would promote the development of dynamic epidemiological field studies and less invasive anthelmintic trials.

Previously, Derbala and Ghazy [6] recorded infection percentage with *S. vulgaris* of 97.3% among horses using somatic antigen of adult worms in ELISA. Moreover, two antigens complexes (25 and 20KDa) with immunodiagnostic potential were identified in somatic antigen preparations of larval strongyles [7]. IgG(T) antibody subclass responses to these antigen complexes significantly increased within 5 weeks of an experimental infection [8]. Additionally, specific IgG(T) responses to gel - purified extracts of both antigen complexes in sera from naturally and experimentally infected horses were studied [9]. Recently, interests were focused on *S. vulgaris* proteases, especially cathepsins as appropriate targets for chemotherapy and vaccines [10] which in turn are successful diagnostic candidates.

In participation of successful diagnosis of *S. vulgaris* infection in horses, the current study aimed to utilize the affinity isolated and characterized fraction of *S. vulgaris* mature worms in the diagnosis of infection using ELISA.

MATERIALS AND METHODS

Animals and Samples: A total number of 76 farm horses (Arabian and Thoroughbred), up to three years age, from some private and governmental

Corresponding Author: H. Eman Abdel-Rahman, Parasitology and Animal Diseases Department, National Research Center, Dokki, Giza, Egypt

horse farms was investigated in this study. These horses showed different clinical signs in the form of diarrhea, emaciation, fever, recurrent colic and lameness.

Fecal and serum samples were collected from each animal for parasitological and serological studies.

S. vulgaris horse infected sera were collected from slaughtered horses at Giza-Zoo abattoir from which *S. vulgaris* adult and larvae were detected.

Parasitological Examination: Faecal samples were obtained from horses and examined using concentration floatation method [11]. Eggs of *S. vulgaris* and other large and small strongyles can not be differentiated. Therefore, it was necessary to make faecal culture [12] for obtaining and differentiation of the infective third stage larvae (L_3). The third larval stage of *S. vulgaris* is characterized by presence of 28-32 intestinal cells with well defined long column shape. Identification of the collected larvae and adult worms was performed according to Lichtenfels [13].

Preparation of Somatic Adult Worm Antigen: Adult worms of *S. vulgaris* were obtained from caecum and colon of infected animals during post mortem examination. The somatic antigen was prepared as the modified method of Klei *et al*. [14]. Adult worms were washed several times in saline, chopped and homogenized in PBS pH 7.2. Particulate materials were removed by centrifugation at 10000 g for 30 min., the supernatant was decanted and used as somatic adult antigen. Protein concentration was determined as the method of Lowry *et al*. [15].

Antibody - Sepharose 4b Affinity Column Chromatography: *S. vulgaris* horse infected sera (collected from slaughtered horses at Giza-Zoo abattoir from which *S. vulgaris* adult and larvae were detected) were dialyzed against 100mM $NaHco_3$ buffer pH 8.3 containing 500mM Nacl and 0.02% NaN_3 and coupled to Cyanogen bromide - activated Sepharose - 4B (CNBr-Sepharose 4b) at the ratio of 2mg/ml swollen beads by strictly following the manufacturer instructions. *S.vulgaris* mature worm extract was applied to the column. The column was washed with 0.015M PBS containing 0.02% NaN_3 pH 8 and bound materials were eluted with 50mM glycine - 500 mM Nacl - 0.02% w/v NaN_3 pH 2.3 [16]. Protein content of isolated antigen was measured according to the method described by Lowry *et al*. [15].

SDS- Polyacrylamide Gel Electrophoresis (SDS-PAGE): SDS-PAGE was performed in polyacrylamide gels according to Laemmli [17]. Both crude antigen and bound fraction were separately mixed with sample buffer containing 2-mercaptoethanol before loading to the gel. After separation, slab gel was stained with commassie brilliant blue dye. Relative molecular weights of bands were calculated using marker supplied by Fermentas International INC. Canada.

Isoelectric Focusing (IEF): IEF was performed as described by O´Farrell [18] in a slab urea gel supplemented with ampholine pH 3.5-10. Gel was stained with Commassie blue and photographed wet. Isoelectric point (PI) of a particular band can be determined by running mixture of proteins of known isoelectric points on the same gel (IEF- USB, USA; 3.6 -9.3).

Enzyme Linked Immunosorbent Assay (ELISA): The immunogenic binding activities of the crude, unbound and bound fractions were compared by ELISA against infected sera collected from slaughtered horses from which *S. vulgaris* adult and larval stages (4^{th} and 5^{th} larval stages) were collected. The assay was also utilized to assess the diagnostic potentials of the isolated fraction against 76 serum samples collected from *S. vulgaris* infected and non-infected farm horses. The optimum antigen concentration, serum and horse radish peroxidase conjugate dilutions were determined by checkerboard titration. The test procedures were carried out according to Dumenigo *et al*. [19]. The cut off values were determined according to Murrell *et al*. [20].

RESULTS

Isolation of Immunogenic Fraction from *S. vulgaris* Adult Worm Extract: A quantitative summary of the protein content and yields of activities are presented in Table 1. 79.4% of the initial immunogenic activities in the crude extract were recorded in the bound and eluted fraction which represents only 6% of the total protein applied to the column giving 2161.5 fold increases in the immunogenic binding activities as compared to the crude extract.

Characterization of the Isolated Fraction:

SDS-PAGE: The electrophoretic profile of *S. vulgaris* crude extract is shown in Figure 1, whereas it was resolved into 12 bands of molecular weight ranged

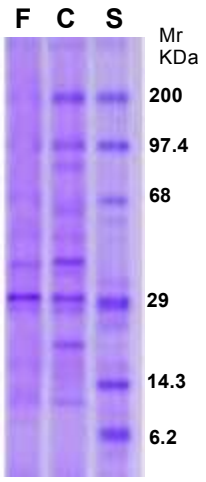

Fig. 1: Electrophoretic profile of *S.vulgaris*. Lane S; molecular weight standards. Lane C; crude adult extract. Lane F; bound fraction

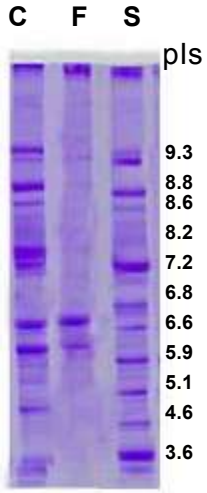

Fig. 2: Isoelectric focusing of *S.vulgaris*. Lane S; standards. Lane C; crude adult extract. Lane F; bound fraction

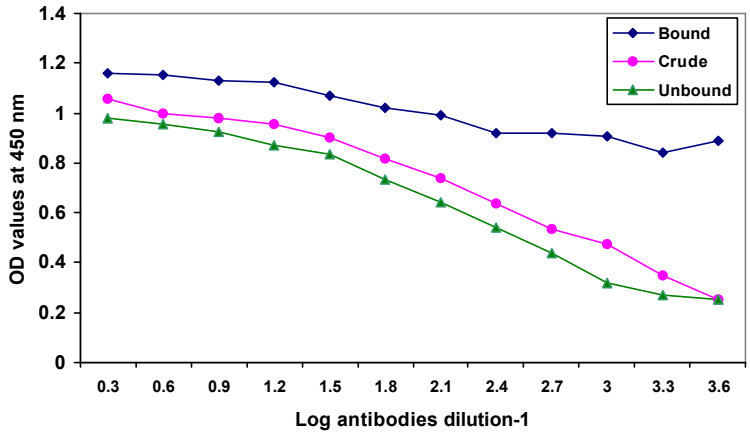

Fig. 3: The binding activities of *S. vulgaris* crude, bound and unbound antigens towards infected horse sera

Table 1: Isolation of *S. vulgaris* immunogenic fraction

Antigen	Total protein (µgx10^3)	Activity unit[a] (Aux10^6)	Specific activity [b] (Au/µg)	Purification fold	Yield (%)
Crude extract	330	33	100	1	100
Isolated fraction	20	26.2	13100	2161.5	79.4

a.A unit of activity is defined as the amount of protein required to give one well of agglutination

b. Specific activity is the number of activity per µg of protein and is related to the starting crude extract

Table 2: Comparative diagnosis of *S. vulgaris* infection in farm horses using parasitological examination and ELISA

Parasitological examination	Positive	Negative	Total
Positive	21	35	56
ELISA	(27.6%)	(46.1%)	(73.7%)
Negative	0.0	20	20
	(0.0%)	(26.3%)	(26.3%)
Total	21	55	76
	(27.6%)	(72.4%)	

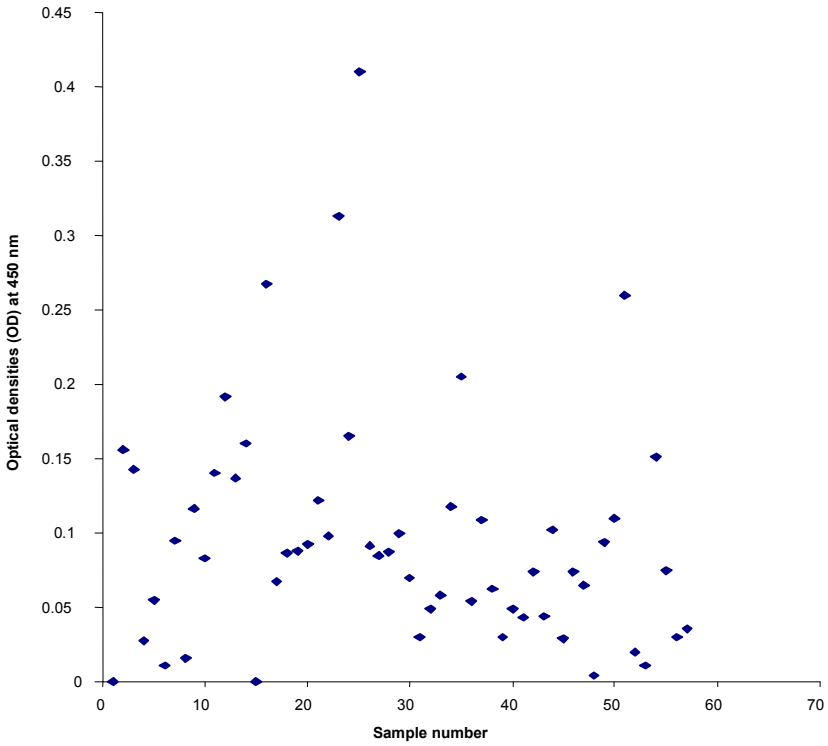

Fig. 4: Evaluation of the immunodiagnostic potentials of *S. vulgaris* isolated fraction against horse sera

between 200 and 11KDa. Of these bands, only two of 39 and 31KDa were bound and eluted from the column (Figure1).

IEF: For further characterization of the isolated fraction, isoelectric point of the two bands was identified by isoelectric focusing technique. As shown in Figure 2, the two bands are acidic in nature and have pIs of 6.8 and 6.7. Figure 2 also shows the pIs of crude extract bands, whereas it varies from acidic to basic ranges (3.6 to 9.5).

Diagnostic Potentials

Parasitological Examination: Parasitological examination revealed that 21 faecal samples from 76 (27.6%) were positive for *S. vulgaris* infection (Table 2).

ELISA: Comparative evaluation of the diagnostic potentials of the crude extract, unbound and bound fractions by ELISA proved the potency of the bound fraction over the other two antigens (Figure 3).

The isolated fraction showed high diagnostic potency (73.7%) when tested against 76 horse sera. Out of 56 positives by ELISA, 35 (46.1%) were negative by parasitological examination (Table 2).

DISCUSSION

Accurate diagnosis of *S. vulgaris* infection is a basic step in the determination of its epidemiology and design the successful therapeutic strategy. While, fecal egg counts estimate adult luminal burdens [21], they provide no information on *S. vulgaris* larval infection. Hence, there is a need for a diagnostic antigen that will allow clinicians and researchers to establish the prepatent mucosal and arterial larval burden in a relatively non invasive manner. Whereas, the prepatent period of *S.vulgaris* extends to about 6 months during which larval migration and destruction occurs in the arterial system, especially the cranial mesenteric arteries.

In the current study, a fraction was isolated from *S. vulgaris* worm extract to accurately diagnose the infection in both patent and prepatent periods. ELISA using the isolated fraction proved higher potency in diagnosis infection with *S.vulgaris* larval stages as compared with parasitological examination. ELISA succeeded to detect infection (positive cases) in 35 (46.1%) of the negative cases examined by parasitological techniques. ELISA as a sensitive diagnostic technique was repeatedly adopted [6, 7,9, 22]. But, the advantage in the current research , is the fraction which was isolated

from crude extract by antibodies of infected sera using affinity column chromatography. This fraction has 2161.5 fold increases in the immunodiagnostic potentials than crude extract.

The fraction was composed of two bands of molecular weight 39 and 31 KDa as obtained from SDS-PAGE. Previous characterization of *S. vulgaris* antigen was performed by Nichol and Masterson [23] who used specific rabbit anti-*S. vulgaris* sera to identify *S. vulgaris* worm antigen by Western blotting. Their study revealed bands of molecular weights 100, 52 and 36KDa. The identified bands in the current study were recognized by horse infected sera coupled with CNBr-Sepharose-4B which supports its immunodiagnostic potentials rather than rabbit hyperimmune sera raised against the parasite. Using immunoblot in which rabbit hyperimmune sera against excretory-secretory products (ESP) of adult worms were utilized, [23] identified most ESP subunits (80, 60, 54, 42, 35, 30, 20 and 15 KDa). Although, the antigen was the worm products not somatic worm antigen as that investigated in the current study, low molecular weight bands were identified in both antigens. Despite of the studies concerned with larval antigens of *S. vulgaris* and its immunodiagnostic antigens [9], the existence of common antigens between adult worms and larvae was previously documented [14].

In conclusion, the current study introduced an antigen isolated from *S. vulgaris* worm by CNBr- activated Sepharose-4B affinity column chromatography with immunodiagnostic potentials in both patent and prepatent periods of infection with *S. vulgaris* in horses.

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