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Value of *Haemonchus longistipes* Purified Antigens in Diagnosis of Gastro-intestinal Nematodes Infection in Camels

[1]M.M. El-Bahy, [2]N.M. El-Bahy and [3]H.A. Shalaby
[1]Department of Veterinary Medicines, Faculty of Agriculture and Veterinary,
Medicine El-Qassium University, KSA
[2]Department of Parasitology, Faculty of Veterinary Medicine, Monfiya University, Egypt
[3]Department of Parasitology and Animal Diseases, National Research Center, Egypt
P.O. Box 12622, El-Tahrir Street, Dokki, Giza, Egypt

Abstract: Fractionated *Haemonchus longistipes* crude antigen revealed 7 protein fractions at molecular weights of 93, 74, 67, 56, 32, 26 and 16 kDa. The bands at 56, 32 and 26 kDa reacted specifically with *H. longistipes* hyper-immune sera prepared in rabbit (1:100 dilution) using EITB technique. The eluted concentrated fractions as well as crude worm antigens were used in diagnosis of natural infection of Gastro-Intestinal Nematodes (GIN) only or associated with other parasites in camels using ELISA technique. *H. longistipes* crude antigen and fraction of 26 kDa induced high sensitivity in diagnosis of infection in animals harboring GIN only (sensitivity was 95-100%), while it was 85.0-93.3% with 32 kDa fraction and 55.5-73.3% with that of 56 kDa using ELISA technique. The two *H. longistipes* eluted protein fractions of 26 and 32 kDa showed higher sensitivity than their crude antigens in diagnosis of GIN infection mixed with other parasites. While, Protein bands of 26 kDa appeared more sensitive than the other protein bands in detection of anti- *H. longistipes* antibodies at higher serum dilution. With special superiority for fraction of 26 kDa, in comparison with the other antigens, sharp specificity or sensitivity in diagnosis, could not be achieved in this study especially in case of mixed infection with other parasites, where these animals might be immune-compromised.

Key words: *Haemonchus longistipes*, EITB, ELISA, electro elution

INTRODUCTION

Gastrointestinal nematodes (GIN) are one of the most important widely spread parasites of camels and other animals. They cause significant economic losses worldwide due to its blood-feeding behavior (*Haemonchus* suck 0.05 mL blood/worm/day, Soulsby, 1986).

H. longistipes is the most common abomasal nematodes of camels, its prevalence rate reached to 94% (in Ethiopia).The infection rate was higher for older animals (13-22 years old) compared to younger ones (3-7 years), for females compared to males and for rainy compared to dry season (Bekele, 2002).

Diagnosis of infection is usually carried out by means of coproscopy, fecal culture and identification of infective larvae. Besides the restriction to patent infections, this method is time consuming. Detection of genetic material in parasites' eggs by PCR (Christensen *et al*., 1994) could solve some of the problems but are also restricted to patent periods of the infection. The detection of parasite antigens would be an ideal method. This approach however was unsuccessful when determining circulating *H. contortus* antigens in infected sheep (Petit *et al*., 1981). The detection of antibodies with total extracts of the parasite using serology with different immunological methods did not yield consistent results (Adams and Beh, 1981). Since, the lack of sensitivity of the methods assayed was probably more related to complex nature of the antigens used than to the inability of the animal to recognize the parasite (Charly-Poulain *et al*., 1984). Therefore, purified and specific immunodiagnostic antigens are needed. Indeed, Gomez-Munoz *et al*. (1996) described the identification and partial isolation of 26 kDa antigen of adult *H. contortus* by means of gel filtration and anion exchange chromatography and evaluated the diagnostic value of this antigen in sheep. This antigen was apparently specific for the diagnosis of *H. contortus* infections in sheep. Besides, a 24 kDa component of

Corresponding Author: Dr. H.A. Shalaby, Department of Parasitology and Animal Diseases, National Research Center, P.O. Box 12622, El-Tahrir Street, Dokki, Giza, Egypt
Tel: 002-0103554567 Fax: 00202-3371362

secretory/excretory products of adult parasites useful for diagnosis of haemonchosis had been reported (Schallig *et al*., 1994). Also, a 66 kDa *H. contortus* excretory secretory antigen was identified in Western blot by reaction with sera from infected goats. The absence of this protein in the free living L3 larvae suggested that the expression of this protein coincided with the adaptation to the parasitic life (Rathore *et al*., 2006).

The present study aimed to identify the specific *H. longistipis* (the common GIN of camels) protein fractions using SDS-PAGE and enzyme linked immunotransfer blot (EITB) as well as ELISA techniques in diagnosis of GIN infections in field collected camels' sera.

MATERIALS AND METHODS

The selected animals and control samples: A total of 30 and 36 natural infected rectal fecal and jugular blood samples were selected from natural infected camels examined in Al-Qassium area, KSA and Cairo abattoir, Egypt, where this study was conducted, respectively. The samples were selected according to types of parasitic infections in feces and its culture. Five samples from young camels proved to be free from nematodes infection were selected for the trial.

According to the fecal history of these samples, their serum samples were arranged in 7 groups (Table 1), as group (I), (G-I) was 15 animals shedding 100-500 eggs per gram of feces (epg) with high percentage of *Haemonchus* larvae recorded after fecal cultivation. G-II was 20 animals shedding over 500-1000 epg and large variety of larvae in fecal culture. G-III was 15 animals shedding over 1000 epg as a mixture of parasites included different levels of GIN and *Moniezia* spp. G-IV was 10 animals harboring over than 3000 epg and contained a mixture of GIN, *Fasciola*, *Moniezia*, *paramphistomum* species eggs. G-V was 3 animals infected with *Paramphistomum* spp. only (2, 3 and 5 epg) and G-VI was another 3 animals harboring *Moniezia* sp. only (3, 5 and 5 epg). The last group was G-VII contained 5 young animals with feces free from parasitic infection. Also, three hyper immune rabbit sera were prepared and used as reference control positive sera.

Examination of the samples: Using the Mc-Master technique, according to Soulsby (1986) a total number of different gastro-intestinal nematodes epg were calculated for each sample. Cultivation of the collected fecal samples was done using the modified Baermann technique and the detected larvae were identified according to Burgur and Stoye (1968). The larvae were counted relatively for each individual animal where the mean number per animal in each group was calculated.

Fasciola and *Paramphistomum* species infection was diagnosed using fluke finder technique according to Welch *et al*. (1987). The animals were considered positive if one *Fasciola* egg per gram of feces was recorded.

Antigen preparation: According to Smith and Smith (1996), *H. longistipes* worms were extracted from the abomasum of naturally infected slaughtered camels. They were identified according to Yamaguti (1959) with the aide of their characteristic long spicules. The anterior and posterior parts of the male were cut out, homogenized, sonicated and exposed to high speed centrifugation (20,000 rpm) for one hour at 4°C. The supernatant was separated as crude soluble antigen. Its protein content was determined before its storage in small vials at -70°C using method of Lowry *et al*. (1951).

Crud antigens of *Trichostrongylus* sp., *Moniezia* sp. scolexes and *Paramphistomum* sp. suckers were prepared as before, from fresh living worms collected from animals slaughtered in Cairo abattoir. *Fasciola* excretory-

Table 1: Level of infection by different parasites and type of GIN larvae detected in feces of selected camels

		Mean % of the different larvae in 100 counted larvae									
		Total GIN eggs Detected (epg)		*Haemonchus* sp.	*Trichostrongylus* sp.	*Nematodirus* sp.	*Cooperia* sp.	*Ostertagia* sp.	Other parasites		
Group No.	Animal No.	Low-high	Mean No.	Mean (%)	Mean (%)	Mean (%)	Mean (%)	Mean (%)	*Paramphistomum* sp.	*Monezia* sp.	*Fasciola* sp.
Group I	15	100-500	350	85	10	0.0	0.0	5.0	-	-	-
Group II	20	>500-1000	700	57	21	12	0.0	10	-	-	-
Group III	15	Over 1000	1650	36	26	14	8.0	16	-	++	-
Group IV	10	Over 3000	3400	38	30	5.0	11	16	++	++	++
Control Group V	3	*Paramphistomum* 2, 3 and 5 epg		0.0	0.0	0.0	0.0	0.0	++	-	-
Group VI	3	*Monezia* infection by 3, 5 and 5 epg		0.0	0.0	0.0	0.0	0.0	-	++	-
Group VII	5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	-	-

e.p.g. = eggs per gram of feces

secrotory (ES) antigen was prepared according to River Marrero *et al*. (1988).

Preparation of reference hyper-immune sera: Three hyper immune sera raised against *H. longistipes*, *F. gigantica* ES and *Moniezia* crude antigens were prepared in rabbits (2 animals per antigen), according to Langley and Hillyer (1989).

Fractionation of *H. longistipes* crude antigen using SDS-PAGE: SDS- PAGE was performed according to Laemmli (1970) in 12% polyacrylamide gel slabs in Tris-glycine buffer, pH 8.3. The stacking gel consisted of 5% acrylamide prepared in 12.5 mM Tris-HCL buffer (pH 6.7) (Sigma chemical Co.). Low molecular weight (MW) standard was employed (Sigma SDS-100B).

Electrophoretic transfer of protein fractions onto nitrocellulose sheet: Electrophoretic transfer of fractionated proteins onto nitrocellulose sheet (NC) for EITB technique was performed according to Towbin *et al.* (1979) using transfer buffer (25 mM tris-base, 192 mM glycine, 20% (v/v) methanol at pH 8.3). Transferring was carried out at 10V, 100 mA overnight at 4°C. Ponceau S (Sigma) stain was used for primary identification of different fractions with the aid of MW standard using its standard curve.

Determination of *H. longistipes* specific protein fractions using EITB technique: According to Towbin *et al.* (1979), *H. longistipes* specific protein fractions were that reacted with its homologous rabbit HIS, at the meantime, no reaction appeared with the control negative sera.

Isolation of selected protein fraction by electro elution: Once the SDS-PAGE finished, strips with the MW standard were cut out fixed and stained with Coomassie Blue stain according to Tsai and Frasch (1982) in order to determine the region where the antigens of interest would be. Regions in the gel containing protein fractions of 56, 32 and 26 kDa were cut out horizontally across the whole gel. Then, isolation of selected protein fraction was done by electro elution according to Katrak *et al.* (1992). The eluted concentrated materials were aliquoted in 1 mL vials; their protein contents were measured, and kept at -20°C until used in coating of ELISA plate as purified *H. longistipes* protein fractions.

Indirect ELISA technique: ELISA test was done as described by Espino *et al.* (1987). Condition of the test and values of control serum were adjusted after checkerboard titration. The test was applied to determine the diagnostic value of different antigens versus the tested sera at 1:100 serum dilution. Sensitivity of the ELISA with tested *H. longistipes* antigen fractions was evaluated via serial dilutions of the tested sera from 1:250 to 1:1000, according to Abdel-Rahman *et al*. (1998), where specificity is percentage of positive samples among the total no. of tested samples at standard serum dilution and sensitivity is percentage of the positive sera at high dilution among the total number of the original positive samples.

RESULTS

Identification of *H. longistipes* specific diagnostic protein fractions using EITB technique: Ponceau-s stained longitudinal strips, blotted with fractionated *H. longistipes* crude antigen revealed 7 protein fractions that were identified using the MW standard curve as 93, 74, 67, 56, 32, 26 and 16 kDa. From these protein fractions, only three at MW of 56, 32 and 26 kDa were reacted specifically with homologous HIS (1:100 dilution) (Fig. 1, lane 2). Additionally, nonspecific protein fraction was recognized, in the previous strip and also in that reacted with negative rabbit sera, at MW of 74 kDa (Lane 3).

Diagnosis of GIN infection in camels using eluted protein fractions by ELISA technique: The three specific, previously recorded, protein bands (56, 32 and 26 kDa) as well as *H. longistipes* crud antigens were evaluated in diagnosis of current infection with different levels of GIN and other parasites in camels' sera after elution and concentration of these fractions using ELISA technique, with the aid of reference sera and antigens of *Moniezia*, *Paramphistomum* and *Fasciola* as control. The data in Table 2 revealed that, *H. longistipes* crude antigen induced higher sensitivity (100%) than that of each individual fraction in diagnosis of infection in animals harboring GIN only (G-I and G-II). This level of sensitivity was 100 and 95% for 26 kDa, while it was 93.3 and 85% for 32 kDa and 73.3 and 55% for 56 kDa in animals of G-I and G-II, respectively. In the meantime, Crude antigen of *Trichostrongylus* detected 66.6 and 60.0% of infection in these two groups, respectively. No cross reaction could be recorded in these serum samples versus *Moniezia*, *Paramphistomum* and *Fasciola* antigens in these two groups (Table 2). The fractionated *H. longistipes* crude antigen induced higher sensitivity in diagnosis of GIN infection in animals harboring other types of parasitic infections (G-III and G-IV) in comparison with its crude antigen. The diagnostic value of 26 kDa fraction was (86.6 and 80.0%), followed by that of 32 kDa (80.0 and 70%). The crude antigen of *H. longistipes* induced rate of

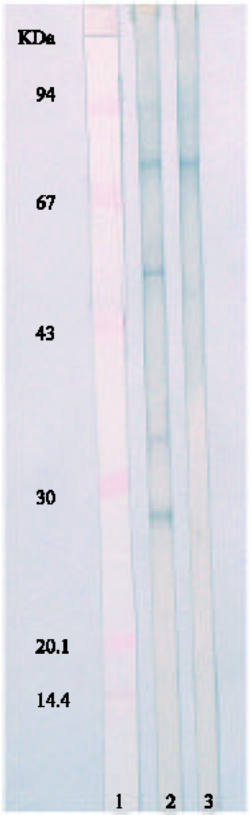

Fig. 1: Recognition of specific fractions of *H. longistipes* antigen on N.C. strips using EITB technique, Lane 1. Low molecular weight marker stained with Ponceau s solution, Lane 2. NC strip reacted with *H. longistipes* HIS, Lane 3. NC strip reacted with negative rabbit sera

sensitivity reached to 73.3 and 70.0% in animals of both groups, respectively. The lowest value in diagnosing mixed infection was recorded for the fraction of 56 kDa (46.6 and 40%) in the above two groups, respectively.

In the meantime, antigen fraction of 26 kDa appeared as the most specific one, where it did not react non-specifically with any of the other antibodies in sera of animals harboring other parasitic infection (*Paramphistomum* and *Moniezia*). While, *H. longistipes* crude antigen, 32 and 56 kDa antigens cross reacted by 33.3% with anti-*Paramphistomum* antibodies. *H. longistipes* crude antigen and that of 56 kDa fraction cross reacted also by (33.3%) with anti-*Moniezia* antibodies in control positive animals (Table 2). On the other hand, those antigens showed absolute specificity in detection of homologous antibodies with the exception of 56 kDa. Where, the later displayed reactivity with anti-*Fasciola* antibodies. None of all tested antigens showed positive reaction when testing versus the control negative camels' sera.

In conclusion, the two *H. longistipes* eluted protein fractions of 26 and 32 kDa showed marked potency than their crude antigens in diagnosis of GIN infection mixed with other parasites.

Sensitivity of those three *H. longistipes* antigens (26 kDa, 32 kDa and crude one) In detection of low level of anti-GIN antibodies through serial sera dilutions of animals infected with GIN only (G-I), GIN with different types of parasites (G-IV) as well as *H. longistipes* HIS was illustrated in Table 3. The data revealed that protein fraction of 26 kDa appeared more sensitive in detecting of anti-nematodes anti-bodies in sera of camels infected with

Table 2: Value of *H. longistipes* crude and purified antigens in diagnosis of infection using ELISA technique at (1:100) serum dilution

Group No.	Animal No.	Total GIN eggs Detected (epg) Low-high	Mean No.	No. and % of positive samples using crud antigens *H. longistips*	*Trichostrongylus*	*Moniezia*	*Paramphis-tomum*	*Fasciola*	No. and % of positive samples using *H. longistip* spurified protein fraction 56 kDa	32 kDa	26 kDa
Group I	15	100-500	350	15/ 100%	10/ 66.6%	0.0	0.0	0.0	11/ 73.3%	14/ 93.3%	15/ 100%
Group II	20	>500-1000	700	20/ 100%	12/ 60.0%	0.0	0.0	0.0	11/ 55.0%	17/ 85.0%	19/ 95.0%
Group III	15	Over 1000	1650	11/ 73.3%	10/ 66.6%	4/ 26.6%	0.0	2/ 13.3%	7/ 46.6%	12/ 80.0%	13/ 86.6%
Group IV	10	Over 3000	3400	7/ 70.0%	5/ 50.0%	4/ 40.0%	4/ 40.0%	6/ 60.0%	4/ 40.0%	7/ 70.0%	8/ 80.0%
Control groups											
Group V	3	*Paramphistomum* 2, 3 and 5 epg		1/ 33.3%	0.0	0.0	3/ 100%	1/ 33.3%	1/ 33.3%	1/ 33.3%	0.0
Group VI	3	*Monezia* infection by 3,5 and 5 epg		1/ 33.3%	0.0	3/ 100%	0.0	0.0	1/ 33.3%	0.0	0.0
Group VII	5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Rabbit HIS*		*H. longistipes* crude antigen		100%	50.0%	0.0	0.0	0.0	100%	100%	100%
versus		*F. gigantica* ES antigen		0.0	0.0	0.0	50.0%	100%	50.0%	0.0	0.0
		Monezia Crude antigen		0.0	0.0	100%	0.0	0.0	0.0	0.0	0.0

epg = eggs per gram feces Rabbit HIS*, Laboratory prepared rabbit hyper-immune serum

Table 3: Sensitivity of *H. longistipes* purified protein fractions in detection of anti-*Haemonchus* antibodies in known serum samples

			at 1:250 serum dilution						at 1:500 serum dilution						at 1: 1000 serum dilution			
Tested serum samples			32 kDa		26 kDa		Crude Ag.		32 kDa		26 kDa		Crude Ag.		32 kDa		26 kDa	
Animal groups	Animal infected by	No.	No. of +ve	Sensitivity %	No. of +ve	Sensitivity %	No. of +ve	Sensitivity %	No. of +ve	Sensitivity %	No. of +ve	Sensitivity %	No of +ve	Sensitivity %	No. of +ve	Sensitivity %	No. of +ve	Sensitivity %
Group (1)	GIN only	15	11	73.3	15	100	9	60.0%	8	53.3%	14	93.3%	3	20.0%	3	20.0%	10	66.6%
Group (1v)	GIN and other parasites	10	5	50.0%	8	80.0%	5	50.0%	3	30.0%	7	70.0%	2	20.0%	0	0.0	5	50.0%
Rabbit anti *H. longistips*		2	2	100%	2	100%	2	100%	2	100%	2	100%	2	100%	1	50.0%	2	100%
Control non-infected animals		5	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0

nematodes only, in decreasing manner associated with increasing serum dilution in G-I as 100, 93.3 and 66.6% corresponding to sera dilutions of 1:250, 1:500 and 1:1000, respectively. Sensitivity of the same fraction decreased to 80.0, 70.0 and 50.0% in animals infected with GIN and other parasites (G-IV) with the previous sera dilutions, respectively. The above figures became lower for the other tested protein fraction (32 kDa) as 73.3, 53.3 and 20% for animals in G-I and 50.0, 30 and 0.0% for animals in G-IV as in Table 3. Besides, antigen of 26 kDa fraction reacted specifically (100%) with anti-*H. longistipes* antibodies in hyper immune sera till 1:000 serum dilution, but only till 1:500 serum dilution, on reaction with 32 kDa fraction.

On the other hand, *H. longistipes* crud antigen induced low sensitivity for detection of GIN antibodies in tested sera with increasing sera dilutions as it was only 60.0 and 20.0% in G-I, decreased more to 50.0 and 20% in animals infected with different parasites (G-IV).

DISCUSSION

Diagnosis of GIN in animals by detection of eggs in feces is an easily subject, but presence of arrested larvae in the tissue of the intestine act as a source of new infection even with application of a restricted control measures around the animals (Jacquiet *et al*., 1996). In this respect, sero-diagnosis is the other way for assessment presence of infection by arrested larvae with absence of eggs in feces. Identification of specific protein fraction, able to induce an accurate specific diagnosis of the disease under filed level, is the required goal for designing a control plane used in eradication of special parasite. More accurate results could be obtained using EITB, but this technique was non-practical for current field application in comparison with ELISA technique (Ibarra *et al*., 1998). Moreover, De Morilla *et al.* (1989) mentioned that ELISA technique was a sensitive serological test, able to analyze many samples simultaneously.

The present study aimed to identify the specific *H. longistipes* (the common GIN of camels) protein fractions using SDS-PAGE and EITB technique versus rabbit hyper immune sera. Then estimate the diagnostic value of each specific fraction, after elution from the gel, in diagnosis of GIN infections in field collected camels' sera using ELISA technique.

Fractionation of *H. longistipes* crude antigen using SDS-PAGE revealed 7 protein fractions at the molecular weight rang of 93, 74, 67, 56, 32, 26 and 16 kDa. These bands were in the range previously described by Fetterer (1989), Smith and Smith (1996) and Haig *et al.* (1989). Reaction of separate NC stripes blotted with the above mentioned protein fractions (EITB) demonstrated that fractions of 56, 32 and 26 kDa were more specific in catching of anti *H. longistipes* anti-bodies in laboratory prepared rabbit hyper-immune sera with high specificity towered that of 26 kDa fraction. This was constant with Gomez-Munoz *et al.* (1996) on *H. contortus* in sheep and Haig *et al.* (1989) who mentioned that the most common fractions diagnosed in *H. contortus* 3rd larval antigens were 15-18, 25-29, 70-80 and that greater than 100 kDa after fractionation by SDS-PAGE. They added that not all of them reacted specifically with sera of *H. contortus* infected sheep.

Clarification the diagnostic values of the above 3 fractions (56, 32 and 26 kDa) in comparison with *H. longistipes* crud antigens in detection of different GIN antibodies in camels' sera of known fecal history using ELISA technique revealed that *H. longistipes* crude worm antigen appeared suitable for accurate diagnosis of the disease in animals had GIN infection only, followed by the fraction of 26 kDa. This concurred with Gomez-Munoz *et al.* (2000) in closely related work on

H. contortus in sheep. They revealed that *H. contortus* 26 kDa protein fraction succeeded to diagnose infection in animals at prepatency as well as at patency, but failed to diagnose infection in animals with the lowest worm burdens or during early patency (day 28 post-infection). High affinity of the crude antigen in diagnosis of GIN infection (100%) in comparison with (95-100%) for fraction of 26 kDa might be related to presence of non-specific epitopes in the crud one, could catch antibodies of other GIN recorded in sera of those animal groups.

In animals of mixed parasitic infection (G III and G IV), the SDS eluted fraction of 26 and 32 kDa appeared more potent in diagnosis of GIN infection (80-86.6%) and (70-80%) in comparison with crud one (70-73.3%), respectively. Fraction of 26 kDa induced higher sensitivty in diagnosis of GIN infection than that of 32 kDa. This came in agreement with Fetterer (1998).

In the others' opinion and in agreement with Ford *et al.* (1987) and Khalil *et al.* (1990), none of all tested antigens could induce absolute specificity or sensitivity toward diagnosis of GIN infections especially in animals infected with other parasites. This might be due to those animals were immunocompromised and had exhausted immune system, exposed to large varieties of different epitopes; none of them was able to produce high level of specific antibodies.

In conclusion, *H. longistipes* crude antigen and the 26 kDa fraction induced high sensitivity for diagnosis of infection in animals suffering mainly from GIN. While, the 26 and 32 kDa fractions were more potent for diagnosis of mixed infection with GIN and other parasites as *Fasciola*, *Paramphistomum* and *Moniezia* sp.

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