

Antigenic and Immunogenic Components of *Haemonchus longistipes* Identified by Western Immunoblotting

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ABSTRACT

The present study aimed to examine the ways that the *Haemonchus longistipes* components interact with the host by investigating the components of the parasite which acted as antigens during infection and those who are capable of inducing immune response when administered as immunogens. Adult *H. longistipes* worms (300 females and 200 males) were obtained from the abomasums of naturally infected slaughtered camels. *H. longistipes* soluble extract was prepared and stored at -20°C until the time of analysis. Hyperimmune serum of *H. longistipes* crude soluble extract was raised in rabbits. *H. longistipes* soluble extract, were electrophoresed on 7-20% gradient polyacrylamide gels. Protein banding pattern on the gels was visualized by Coomassie Blue stain. After electrophoresis, Western blots were prepared by electro-blotting separated antigens in unstained gels onto nitrocellulose paper. Antigens were detected by western immunoblotting against infection serum and antisera raised against soluble extract. The antigenic components of the parasite were identified. Twenty three protein bands ranging from 126 to 18-kDa were detected in the parasite crude soluble extract using SDS-PAGE. Sixteen of these components were identified as immunogens using Western immunoblotting against sera from rabbits immunized with a range of soluble parasite materials. Three of these immunogens, 126, 76 and 18-kDa were also found to act as antigens during infection as revealed by immunoblotting against serum from infected camels. The 76-kDa and 18-kDa showed potent reaction with both types of antisera and necessitate further investigation in their role as diagnostic materials and as target antigens for production of vaccines. The present study suggests that the peptide bands 76 and 18-kDa represent useful candidates for serodignosis of cameline haemonchosis. The role of these peptides in serodiagnosis of cameline haemonchosis will be investigated by purifying these peptides and raising monospecific antisera against them which will be used to develop an antigen capture ELISA for detection of circulating *H. longistipes* antigens in camel serum. The ability of the developed assay to differentiate between infections with other helminthes parasites will also be investigated.

Keywords: Electrophoresis, Western Blotting, Anti-Camel Sera, Characterization

1. INTRODUCTION

The camel is the principal domestic animal in Saudi Arabia and its meat and milk constitute a vital source of

animal proteins to nomads and city dwellers. Cameline haemonchosis caused by *Haemonchus Longistipes* has an extremely wide geographical range particularly in tropical and subtropical areas including Saudi Arabia.

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The prevalence of this parasite in Saudi Arabia varies from 46.1% in Central region (Magzoub *et al.*, 2000) to 60% in Eastern region (Banaja and Ghandour, 1994).

The worm occurs in the abomasum of the infected camels sucking blood from the mucosal vessels leading to hemorrhagic anemia, a characteristic feature of the disease. The larvae develop the piercing lancet just before the final moult enabling them to obtain blood from the blood vessels (Armour *et al.*, 1996; Soulsby, 1986). Diagnosis is usually made by means of coproscopy, fecal culture and identification of infective larvae. This approach is, however, restricted to patent infection and is time consuming. Detection of genetic material in parasites, egg using PCR (Christensen *et al.*, 1994), although it offers many advantages over other tests in term of speed and sensitivity it also restricted to patent infection. No approach has been made to diagnose infection during prepatent period although the worm starts to suck blood just before the final moult. The detection of circulating parasite antigens in hosts, blood during prepatent period would be an ideal approach. Although El-Bahy *et al.* (2007) tested the immunodiagnostic ability of purified *H. longistipes* antigens in detection of anti-parasite antibodies in naturally infected camels, his approach does not discriminate between current and previous infection. Up to date no approach is made to detect circulating *H. longistipes* antigens in camel serum. The aim of this study was to determine the antigenic components of *H. longistipes* using western blotting as a preliminary step for selecting target protein candidates for use as diagnostic materials.

2. MATERIALS AND METHODS

2.1. Parasites

Adult *H. longistipes* worms (mainly females) were extracted according to Smith and Smith (1996) from the abomasums of naturally infected slaughtered camels at Al-Ahsa Central Abattoir, KSA. The examined abomasums contained only *H. longistipes* parasites. Five hundreds worms were collected and include 300 females and 200 males. The worms were identified according to Soulsby (1986) by barber pole appearance of the females and the long spicules of males.

2.2. Experimental Animals

Rabbits were used for the production of polyclonal antibodies against *H. longistipes* materials and production of anti-camel sera. They were female adult

New Zealand white rabbits weighing between 2-2.5 Kg. They were maintained as performed by national guidelines and protocols, approved by the University Scientific Research Ethics Committee. They were housed in clean and disinfected cages. Commercial basal diet and water were provided ad libitum. Rabbits were subjected to natural photoperiod of 12hr light:dark cycle throughout the experiment.

2.3. Preparation of Parasite Crude Soluble Extract

For preparation of *H. longistipes* soluble extract, all 500 males and females worms were homogenized in 5ml PBS, treated with a mixture of protease inhibitors and centrifuged at 13000 rpm for one hour at 4°C and the supernatant was collected as adult crude soluble extract. Its protein concentration was determined using QuantiPro™ BCA Assay kit (Sigma Chemical Company, UK) and stored at -20°C. Part of this extract was diluted with an equal volume of Bio-Rad Laemmli sample buffer, Cat. No. 161-0737 [62.5mM Tris-HCl (pH 6.8), 2% SDS, 0.01% Bromophenol blue, 25% glycerol, 5% β-mercaptoethanol] and heated to 100°C for 5 min. After cooling to room temperature and centrifugation at 10000 rpm for 5 min the extract was aliquoted into 200 μL volumes and stored at -20°C until the time of analysis.

2.4. Production of *H. Longistipes* Hyperimmune Serum

Polyclonal antibodies to *H. longistipes* crude soluble extract were raised in rabbits. The immunization regime for production of polyclonal antibodies was adapted from Harlow and Lane (1988). Two rabbits were first injected subcutaneously each with a total of 200 μg *H. longistipes* crude soluble extract emulsified in Freund's complete adjuvant. The rabbits were then boosted by s/c injection with a further 100 μg of the same materials in incomplete Freund's adjuvant at 28 and 56 days post immunization. A final boost of 100 μg of soluble extract in PBS was administered intravenously to each rabbit 68 days after the first immunization. The rabbits were bled for serum to monitor antibody production on days -1, 7, 10, 21, 34, 49, 61 and 67. The final bled was conducted on day 75 post first immunization.

2.5. Production of Anti-Camel Serum

Two rabbits were also immunized with camel serum in order to obtain anti-camel sera. The immunization protocol and serum collection was carried out as

described above. The antiserum was used as a secondary antibody in the immunoblotting test before the addition of the available anti-rabbit conjugate.

2.6. Infection Serum

This was obtained from a naturally infected camel harboring the parasite in its abomasums in Al-Ahsa Central Abattoir, KSA.

2.7. Electrophoresis and Western Blotting

H. longistipes soluble extract, stored frozen in SDS-sample buffer, was heated, immediately before use, to 100°C for 3 min to re-dissolve any crystallized SDS. Cooled samples were then electrophoresed on 7-20% gradient polyacrylamide gels as described by Laemmli (1970). Protein banding pattern on the gels was visualized by Coomassie Blue stain and their molecular weight were determined using a Technicurve software for evaluation of unknown. After electrophoresis, Western blots were prepared by electro-blotting separated antigens in unstained gels onto nitrocellulose paper according to the method described by Towbin *et al.* (1979) and Burnette (1981) using a Trans-Blot SD semi-dry transfer cell (Bio-Rad, USA). Antigens were detected by western immunoblotting against infection serum and antisera raised against soluble extract. The unbound sites on the nitrocellulose membranes were blocked overnight with 5% dried milk in blocking buffer (50 mM Tris, 150 mM sodium chloride, 1 mM EDTA, 0.05% NP40, 0.25% gelatin, 0.02% thimerosal, pH = 7.4) prior to each incubation. The blocked membrane was rinsed with PBS, cut into strips and incubated overnight with the above anti-*H. longistipes* antibodies diluted 1/50 in blocking buffer. Normal rabbit and camel sera diluted 1/50 in blocking buffer were also included in the run as negative controls. The incubation was carried out at room temperature. The unbound antibodies were then removed by washing the membranes with 7 changes of PBS over 2 h, before a peroxidase-labelled, goat anti-rabbit IgG (Bio-Rad Laboratories, 2000 Alfred Nobel Dr., Hercules, CA 94547) diluted to 1/500 in blocking buffer was added to strips containing antisera to soluble extract and incubated for 2 h. Strips containing infection serum and normal camel serum were incubated overnight with rabbit anti-camel antibodies diluted 1/50 in blocking buffer, washed as above and then incubated with the peroxidase-labelled, goat anti-rabbit conjugate as above. After washing the membranes in PBS as above to remove excess conjugates, the labeled antigen/antibody complexes were visualized by incubation in substrate solution (4-chloro-1- α -naphthol

0.5 mg mL⁻¹ in 20 mM Tris, 500 mM sodium chloride, pH 7.5, Sigma Ltd., UK) with hydrogen peroxide (0.06%) as the substrate. Color development was allowed to occur for 30 min and the reaction was stopped by washing in distilled water and permanent records made by photography.

3. RESULTS

3.1. SDS-PAGE Separation of *H. longistipes* Materials

Twenty three individual protein bands with molecular weight ranging from 126-kDa to about 18-KDa (Fig. 1, lanes 2-5 and Table 1) were resolved by Coomassie blue stain of the soluble extract of the parasite.

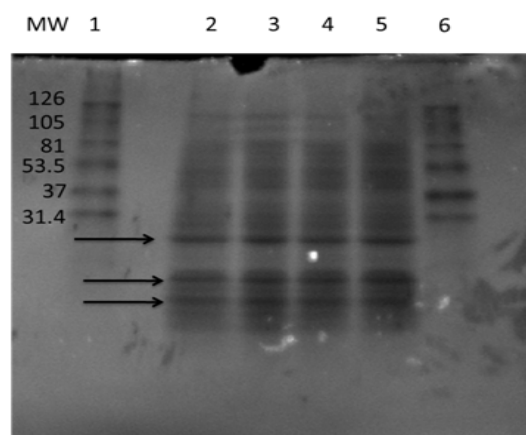


Fig. 1. Coomassie Brilliant Blue stained SDS-PAGE 7-20% gradient gel analysis of *H. longistipes* crude soluble extract (Documented using Gel DocTMXR System, Bio-Rad). (Lanes 1 and 6: Low molecular weight marker. Lanes 2-5: a repeated *H. longistipes* crude soluble extract. The arrows from up to down indicate prominent bands 29, 25 and 22-KD, respectively)

Table 1. SDS-PAGE fractionation of *H. longistipes* soluble extract visualized by Coomassie Brilliant Blue stain

No.	M.W. KD	No.	M.W. KD
1	126.0	13	32.0
2	119.0	14	31.0
3	105.0	15	29.0
4	99.0	16	27.0
5	87.0	17	26.0
6	81.0	18	25.0
7	76.0	19	24.0
8	65.0	20	22.0
9	53.3	21	21.5
10	48.0	22	20.0
11	42.0	23	18.0
12	37.0		

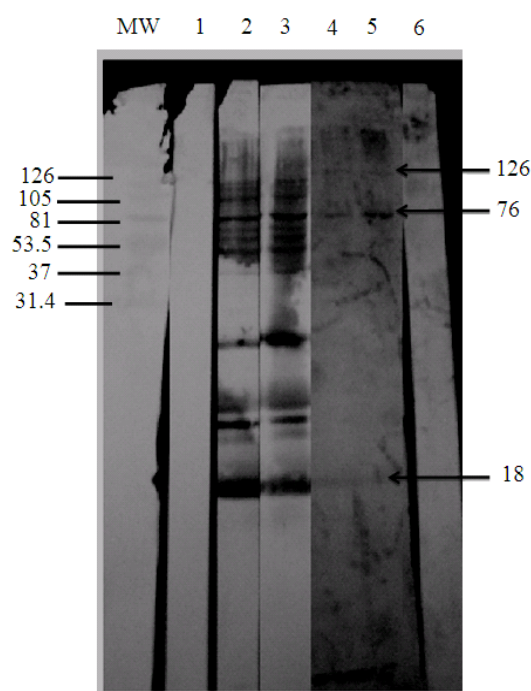


Fig. 2. *H. longistipes* proteins identified by infection serum and hyperimmune serum to the parasite soluble extract. MW: low molecular weight marker in kDa. Lane 1: normal rabbit serum. Lane 2: hyperimmune serum collected 67 days post first immunization. Lane 3: hyperimmune serum collected 75 days post immunization. Lane 4 and 5: repeated samples of infection serum. Lane 6: normal camel serum.

Table 2. Immunogenic and antigenic *H. longistipes* proteins (kDa) recognized by infection serum and hyperimmune serum to the parasite soluble extract

No.	Immunogenic as soluble extract	Antigenic during infection
1	126.0	126
2	119.0	—
3	105.0	—
4	99.0	—
5	87.0	—
6	81.0	—
7	76.0	76
8	65.0	—
9	53.5	—
10	48.0	—
11	37.0	—
12	29.0	—
13	24.0	—
14	22.0	—
15	21.5	—
16	18.0	18

The high molecular weight components in the range 126-kDa to 99-kDa showed the lowest staining intensities, while the low molecular weight components 29, 25 and 22-kDa showed the higher staining intensities (Fig. 1, lanes 2-5).

3.2. Immunological Characterization of *H. longistipes* Proteins

3.2.1. Reactivity of Infection Serum

Serum collected from the naturally infected camel recognized a total of three antigenic components with molecular weight of 126-kDa, 76-kDa and 18-kDa (Fig. 2, lane 4 and 5). The three components were also recognized by the hyperimmune serum and the 76-kDa and 18-kDa reacted strongly with both types of serum (Fig. 2, lanes 2-5 and Table 2).

3.3. Reactivity of Hyperimmune Serum

Serum raised in rabbits to *H. longistipes* soluble extract recognized a total of sixteen components out of the 23 protein components present in the parasite crude soluble extract, with a molecular weight ranging from 126-kDa to 18-kDa (Fig. 2, lane 2 and 3 and Table 2). The three antigens recognized during infection were also recognized by the hyperimmune serum and components 76-kDa, 29-kDa, 22-kDa and 18-kDa showed powerful reaction with this serum (Fig. 2, lane 2 and 3).

4. DISCUSSION

Detection of *H. longistipes* infection during the prepatent period is important for effective reduction of pasture infestation with these nematode eggs and consequently reduction of infection rate in camels following successful treatment. The parasite materials will be circulating in the host's blood during this period since 4th stage larvae and immature worms have the capability of sucking blood (Armour *et al.*, 1996; Soulsby, 1986). Helminthes antigens circulating in the blood of infected animals have been detected (Robertson *et al.*, 1988; Prasad *et al.*, 2008; Ishiyama *et al.*, 2009; Ibrahim *et al.*, 2010; Zibaei *et al.*, 2010; Schnyder *et al.*, 2011; Shane *et al.*, 2011). In order to select a diagnostic reagent from parasitic materials it is necessary to break the whole organism down into individual components. Therefore, the present paper represent an approach for selecting diagnostic reagents that can be used for designing a diagnostic test for detection of circulating *H. longistipes* antigen in infected animals. In the present study, a total of 23 proteins were revealed by Coomassie staining of crude soluble extract of *H. longistipes* electrophoresed on 7-20% gradient

polyacrylamide gels. The molecular weight of these proteins ranges from 126-kDa to about 18-kDa. The number of proteins detected in this study was much higher than that reported for *H. longistipes* by EL-Bahy *et al.* (2007) who reported only 7 protein fractions of molecular weight ranging from 93-16kDa. This variation in the number of fractions could be attributed to the type of gel and stain used in each study. El-Bahy *et al.* (2007) used a homogenous 12% polyacrylamide gel and transferred the fractions to nitrocellulose sheet and stained them with Ponceau S stain. Indeed the resolving power of a homogenous gel is less than that of a gradient gel since different parts of the gradient gel have different porosities (Walker, 2002). In addition, some of the proteins might not be transferred to the nitrocellulose sheet during electrophoretic transfer. On the other hand, however, high number of protein fractions was reported for *H. contortus*. Shoiab and Irshadullah (2009) reported 35 proteins in this parasite using SDS-PAGE and Coomassie blue stain. This increase in the number of fractions could be due to parasite species variation.

Three components of *H. longistipes* with molecular weights 126-kDa, 76-kDa and 18-kDa were recognized as antigens during infection as revealed by their reaction with the infection serum collected from the naturally infected camel in this study. These antigens also reacted with the hyperimmune serum raised in rabbits to the parasite soluble extract. Sixteen components of the parasite acted as immunogens when presented to the rabbits as soluble extract as revealed by their reaction to the hyperimmune serum. This difference in antigenicity of the parasite components released during infection and by physical disruption could be accounted for by difference in the way they were released from the parasite (Barriga, 1981) and the way in which they were presented to the host e.g., immunogenicity of those incorporated with adjuvant will be increased due to their presentation in an aggregated form (Goding, 1996). In addition the three antigens released during infection may represent excretory-secretory antigens and are expected to be less than those released by physical disruption since the latter contains both somatic and excretory-secretory components. Excretory-secretory antigens of similar molecular weights were reported in *H. contortus* (Joshi and Singh, 1999; Bakker *et al.*, 2004; Prasad *et al.*, 2008) and in other helminth parasites (Savigny, 1975; Ishiyama *et al.*, 2009; Schnyder *et al.*, 2011). Moreover, the use of rabbit anti-camel serum as a secondary antibody in the immunoblotting test, due to the lack of anti-camel conjugate, before the addition of anti-rabbit conjugate may also contribute to the low number of

antigenic components through increasing the possibility of dissociation of antibody bonds during washing cycles.

The 76-kDa, 29-kDa, 22-kDa and 18-kDa peptide bands appeared as immunodominant antigens in the present study. Similarly immunodominant antigens of 35, 40, 45 and 80-kDa were identified in *H. contortus* (Meshgi and Hosseini, 2007) in addition to 91.2-kDa antigen (Kaur *et al.*, 2002). Low molecular weight proteins were found useful in the protection and diagnosis of *H. contortus* (Prasad *et al.*, 2008; Garcia-Cloradas *et al.*, 2009). Similarly low molecular weight proteins of 32 and 26kDa were found to be specific to *H. longistipes* and useful in the diagnosis of infection (El-Bahy *et al.*, 2007). Although both the 32 and 26 KDa components were detected in coomassie blue stained extract and were reported by El-Bahy *et al.* (2007) as immunogens, they were not recognized by the hyperimmune sera raised in rabbits in the present study. This is possibly due to difference in parasite strains. These two components appeared as weak bands in the stained gel and possibly in a low concentration in the parasite crude extract to trigger an immune response. Although the 126-kDa component reported in the present study did not appear as an immunodominant antigen, nevertheless it reacted with both infection and hyperimmune sera. A 122-kDa polypeptide was reported in *H. contortus* as a surface antigen in the feces of infected sheep (Ellis *et al.*, 1993). The infected as well as control sera were collected from single animal because the goal of the study was to search for a possible candidate to be used as a diagnostic reagent and therefore any predominant antigen that reacted to both infection and immune serum will fulfill this goal. In addition, the camel serum used in this experiment was obtained from the same animal from which the parasite was collected for the preparation of the soluble extract. Regarding the control, the negative control serum used in the experiment was collected from a newly born camel and this serum was also used to produce rabbit anti-camel serum.

5. CONCLUSION

The present study suggests that the peptide bands 76 and 18-kDa represent useful candidates for serodiagnosis of cameline haemonchosis. The role of these peptides in serodiagnosis of cameline haemonchosis will be investigated by purifying these peptides and raising monospecific antisera against them which will be used to develop an antigen capture ELISA for detection of circulating *H. longistipes* antigens in

camel serum. The ability of the developed assay to differentiate between infections with other helminthes parasites will also be investigated.

6. ACKNOWLEDGEMENT

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6.1. Competing Interest

The researchers declare that they have no competing interests.

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