

Evaluation of different *Haemonchus contortus* antigens for diagnosis of sheep haemonchosis by ELISA and their cross reactivity with other helminthes

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Abstract *Haemonchus contortus* (*H. contortus*) remains important nematode that infecting sheep all over the world. Truthful diagnosis of haemonchosis needs reliable Enzyme linked immune sorbent assay test as well as the immunoreactive protein profile of different prepared *H. contortus* antigens; larval (L), excretory secretory product (ESP) and adult somatic *H. contortus* (AS). The current study fulfilled that L antigen is the talented antigen for such serological diagnosis. Immunodominant band at molecular weight 57 kDa were answerable for highest specificity and accuracy of positive predictive value of this antigen. Moreover, the highest apparent prevalence value was 92 and 75% obtained by L and ESP antigens, respectively. The results of cross reactivity among AS, *Monezia expansa* (*M. expansa*) and *Fasciola* spp. revealed that AS antigen appeared major cross reactivity with other cestode and

trematode. Best dilution of serum was (1:800) to rise above this phenomenon.

Keywords *Haemonchus contortus* · *Monezia expansa* · *Fasciola* spp · ELISA · Cross reactivity

Introduction

Haemonchus contortus is one of the most pathogenic gastrointestinal nematodes that affecting sheep. All larval, immature and mature worm stages are blood sucking thus, this abomasal nematode causing severe anemia which may be fatal particularly to young animals. So, haemonchosis is considered as life threatening disease disturbing sheep population (Kandil et al. 2015; Besier et al. 2016). Economically, *H. contortus* and/or other gastrointestinal nematodes cost the livestock sheep industry. The diagnosis of haemonchosis is usually based on clinical signs observation and microscopic examination of fecal samples (Meshgi and Hosseini 2007). The low sensitivity of fecal examination and unapparent clinical signs leads to limitation of accurate diagnosis of the disease. Therefore, reliable serological diagnosis assay is needed for more accurate diagnosis. ELISA enables detection of infection as well as sero-epidemiological studies in massive intensive breeding of sheep livestock (Li et al. 2007; Prasad et al. 2008; Demeler et al. 2012). The detection of anti-*H. contortus* antibodies in sheep sera using a dependable serological assay as ELISA circumvent the limitation of traditional methods depending on the use of specifically immunogenic antigens (Lone et al. 2012; Kandil et al. 2015). Many different antigens prepared from *H. contortus* nematode are used in serological diagnosis of haemonchosis with different impact on their sensitivity and specificity (Kandil

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et al. 2015). Therefore, our study aimed to characterize and evaluate the different prepared *H. contortus* antigens; L, ESP and AS antigens in serodiagnosis of haemonchosis in sheep.

Materials and methods

Collection of worms and serum samples

Adult worms of *H. contortus*, *M. expansa* and *Fasciola* spp. were collected from abomasa, intestine and liver of slaughtered sheep, respectively at El-Bassatin, El-warrak and El-Monieb abattoirs in Egypt. Worm recovery was carried out according to standard procedures described by MAFF (1986). Three hundred and twenty serum samples were collected by centrifugation (2000 rpm/15 min) blood of slaughtered sheep. All samples were divided and labeled as random, infected and non-infected sheep according to their post-mortem inspection.

Preparation of antigens and hyper immune sera

Adult somatic antigens of *H. contortus*, *M. expansa* and *Fasciola* spp. were prepared from collected adult worms according to Johnson et al. (2004). In addition, ESP and L antigens of *H. contortus* were prepared according to Prasad et al. (2008) and Tariq et al. (2008), respectively. Total protein content was estimated for all antigens according to Lowry et al. (1951).

Eighteen healthy white New Zealand males' rabbits weighing 1.5–2 kg. B. WT. each, were grouped and immunized (3 rabbits for each antigen) with all different prepared antigens (L, ESP, AS, *M. expansa* and *Fasciola* spp.) and keep one group as control. Hyper immune sera were prepared according to Fagbemi et al. (1995) in order to obtain their homologous mono-specific hyper immune sera.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting technique

Fifty µg of the three prepared *H. contortus* antigens; L, ESP, and AS were electrophoresed using 10% gel under reducing conditions according to Laemmli (1970). After that, the protein bands were transferred from SDS-PAGE gel to a nitrocellulose sheet according to Towbin et al. (1979).

Enzyme linked immuno sorbant assay (ELISA)

ELISA was applied in order to evaluate the diagnostic value of different prepared *H. contortus* antigens in

detection of anti-*H. contortus* IgG in sera of naturally infected and non infected sheep. As well as, sensitivity, specificity, negative and positive predictive values and apparent prevalence were calculated for each antigen according to Schallig et al. (1995). In addition, the cross reactivity with other cestode which is *M. expansa* and nematode spp represented by *Fasciola* spp. was estimated in comparison to AS antigen. Briefly, the optimal concentration of antigens and dilutions of tested sera and conjugate were estimated by checker-board titrations. The well were coated with 100 µl of each diluted antigen at the concentration of 2 µg/well in carbonate-bicarbonate buffer, pH 9.6 and incubated overnight at 4 °C. After blocking with 200 µl/well of 2% dry skimmed milk in PBS, pH 7.2. A 100 µl diluted sera of infected and non infected samples (1:200) in PBS-T and double fold serial dilution hyper immune sera raised against AS, *M. expansa* and *Fasciola* spp. (1:50–1:3200) was incubated at 37 °C/2 h. One hundred µl/well of appropriate conjugate diluted at 1:1000 were added and incubated for 1 h at 37 °C. After washing, 100 µl/well of substrate solution (O, phenylenediamine, one tablet (20 mg/ml) dissolved in 50 ml substrate buffer, pH 5 and 25 µl 30% H₂O₂) were added to all wells and the plates were incubated at 37 °C for 10–20 min. Finally, the optical densities (OD) were read at wave length of 450 nm with an ELISA reader (BIO-TEK, INC., ELx, 800UV). Positive values were assigned as those values with absorbance reading greater than the cut-off value which was calculated from mean OD value of the negative control sera plus three fold standard deviations. Data analysis parameters were calculated for each antigen as following according to Tabouret et al. (2001).

Statistical analysis

OD data were expressed as arithmetic mean with standard deviation. The apparent prevalence parameter was analyzed using Chi square test by statistical computer package for social science (SPSS) version 15.

Results

Protein profile of different *H. contortus* antigens using SDS-PAGE and western blotting technique

Electrophoretic protein profile of different *H. contortus* prepared antigens (L, ESP and AS) demonstrated variable protein bands with different molecular weights (Fig. 1). The L antigen showed 23 bands which detected at molecular weights 204, 173, 122, 115, 101, 92, 81, 73, 59, 54, 50, 47, 44, 43, 40, 38, 36, 33, 31, 28, 24, 22, and 18 kDa.

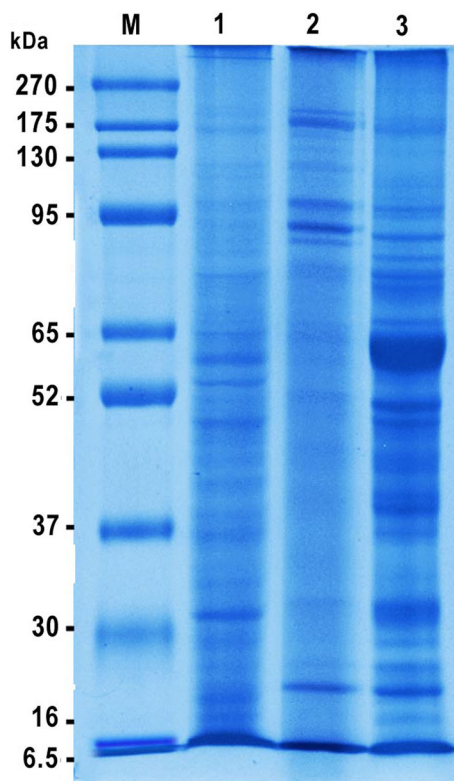


Fig. 1 Electrophoretic profile of different prepared *Haemonchus contortus* antigens (L, ESP and AS). *M* BLUUltra Prestained protein ladder, genedirex, *Lane 1* larval of *H. contortus* antigen, *Lane 2* excretory secretory product of *H. contortus* antigen, *Lane 3* adult somatic of *H. contortus* antigen

While, the ESP antigen demonstrated 15 protein bands at molecular weights 212, 188, 162, 122, 102, 91, 85, 79, 75, 58, 49, 44, 38, 31, and 22 kDa. Moreover, the protein bands of AS antigen were 22 protein fractions at molecular weights 267, 195, 178, 135, 112, 100, 87, 80, 73, 70, 61, 56, 49, 47, 43, 38, 37, 31, 28, 24, 22, and 19 kDa. Common protein bands at molecular weights 22 and 38 kDa were detected among the three *H. contortus* prepared antigens. Also, the bands at molecular weights 22, 24, 28, 31, 43 and 47 kDa appeared in both L and AS antigens. In addition to the band at 49 kDa was dominant between AS and ESP antigens. Also, the band at 44 kDa was dominant between L and ESP antigens (Fig. 1).

Immunogenic reactive bands profile of different *H. contortus* prepared antigens appeared when reacted with their homologous hyper immune sera (Fig. 2). The L antigen detected 13 reactive bands at molecular weights 170, 130, 124, 98, 88, 82, 70, 57, 49, 44, 39, 23, and 22 kDa. However, the ESP antigen gave only 4 bands at molecular weights 171, 61, 59, 57 kDa. The AS antigen showed 21 reactive bands at molecular weights 252, 222, 174, 138, 105, 98, 82, 69, 59, 51, 47, 46, 45, 38, 34, 32, 29, 28, 25, 22, and 17 kDa. The bands at molecular weights 98,

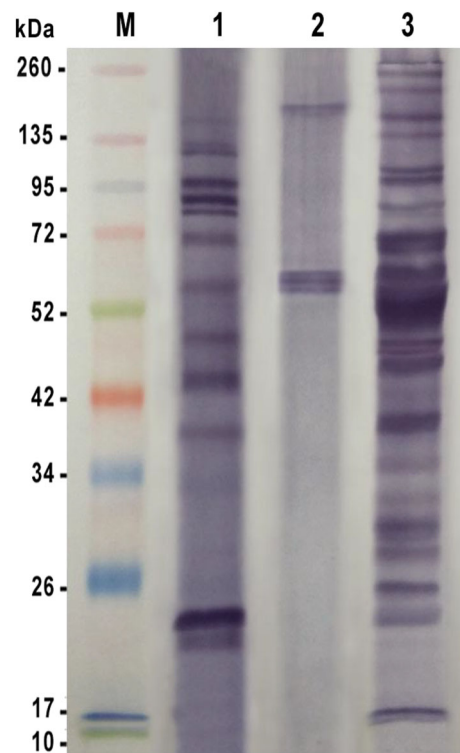


Fig. 2 Reactivity of different prepared *Haemonchus contortus* antigens against their homologous hyper immune sera (L, ESP and AS). *M* Prestained molecular weight protein ladder, Fermentas, *Lane 1* larval of *H. contortus* antigen, *Lane 2* excretory secretory product of *H. contortus* antigen, *Lane 3* adult somatic of *H. contortus* antigen

82 and 22 kDa were common between L and AS antigens. Also, the band at molecular weight 57 and 59 kDa were immune-dominant in ESP, L and ESP, AS antigens, respectively.

Immunogenic reactivity of different *H. contortus* antigens using indirect ELISA

Immunogenic reactivity of three prepared antigens (L, ESP and AS) were evaluated by ELISA using the collected sheep's sera. ELISA results indicated that OD values of detecting *H. contortus* antibodies were highly varied among different serum samples in their reactivity with different *H. contortus* prepared antigens. The apparent prevalence of *H. contortus* infection among sheep's sera differed according to the used antigen. The highest value was 92 and 75% obtained by L and ESP antigens, respectively and statistical analysis clarified that the higher percentage of L antigen was insignificantly versus ESP antigen ($\chi^2 = 1.731$, $p < 0.188$). However, AS antigen achieved 35.6% and the statistical analysis elucidated that lower percentage of AS antigen was significantly versus ESP antigen ($\chi^2 = 13.703$, $p < 0.001$) and also versus L antigen ($\chi^2 = 24.500$, $p < 0.001$). The maximum values of

Table 1 Data analysis parameter of different *Haemonchus contortus* antigens using indirect ELISA

Parameter	Types of antigens					
	L		ESP		AS	
	+ve	–ve	+ve	–ve	+ve	–ve
NIS sera	10/10	0/10	6/10	4/10	5/10	5/10
NNIS Sera	8/10	2/10	7/10	3/10	2/10	8/10
Random Sera	276/300	24/300	225/300	75/300	107/300	193/300
Total	294/320	26/320	238/320	82/320	114/320	206/320
Sensitivity (%)	55.55		46.15		71.4	
Specificity (%)	100		42.85		61.5	
Negative predictive value (%)	20		30		80	
Positive predictive value (%)	100		60		50	
Apparent prevalence (%)	92		75		35.6	

NIS Naturally infected sera, NNIS Naturally non infected sera, L larval of *H. contortus* antigen, ESP excretory secretory product of *H. contortus* antigen, AS adult somatic of *H. contortus* antigen

specificity and positive predictive value 100% were recorded by L antigen (Table 1). The antigens of AS and ESP were gained 61.5, 50 and 42.85%, 60% for specificity and positive predictive value, respectively. Otherwise, the lowest negative predictive value 20% obtained by L antigen followed with ESP antigen which achieved 30%. Nevertheless, the sensitivity of ELISA was 71.4, 46.15 and 55.55% when AS, ESP and L antigens were used, respectively (Table 1).

Cross reactivity of different adult somatic *H. contortus*, *M. expansa* and *Fasciola spp.* antigens using indirect ELISA

The obtained results in Figs. 3, 4 and 5 demonstrating the talent of the three antigens (*H. contortus* (AS), *M. expansa* and *Fasciola spp.*) in binding with their mono-specific antibodies of hyper immune sera raised against them as well as cross binding with each other revealed that there was cross-immune reactivity among the antigens and their sera still appeared even with rising serum dilutions. Moreover, these data showed comparable strong binding patterns with serially diluted sera with a common trait of high reactivity binding of antigens. The result showed that the highly diluted sera (1:3200) still specifically reacted with their respective antigens otherwise, at (1:800) serum dilution, the immunogenic potency of each antigen proved weakly with other heterologous sera.

Discussion

Haemonchosis is considered as life threatening disease disturbing sheep population (Kandil et al. 2015). For serological diagnosis of *H. contortus* infection, various ELISA based methods have been described in order to

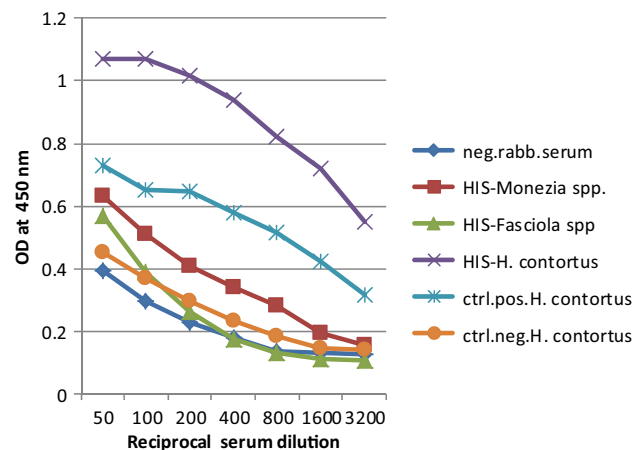


Fig. 3 Cross-reactivity of adult *H. contortus* (AS) antigen against different prepared hyper immune sera. *Neg. rabb. serum* negative rabbit serum, *HIS-Monezia spp.* Hyper immune serum raised against *M. expansa*, *HIS-Fasciola spp.* Hyper immune serum raised against *Fasciola spp.*, *HIS-H. contortus*: Hyper immune serum raised against *H. contortus*, *ctrl.pos. H. contortus*: control positive serum of adult *H. contortus*, *ctrl.neg. H. contortus*: control negative serum of adult *H. contortus*

detect the antibodies formed against *H. contortus* in sera of naturally and/or experimentally infected sheep (Li et al., 2007; Prasad et al. 2008; Lone et al. 2012; Arab et al. 2013). In this study, different prepared *H. contortus* antigens; L, ESP and AS were characterized and compared to precise the most reliable antigen that can be used in sero-diagnosis of haemonchosis in sheep.

Depending on the indirect ELISA, as well as the immuno-reactive protein profile, the present data appeared that the L antigen yielded good results for detection of *H. contortus* infection in naturally infected and non-infected sheep sera. It achieved the highest specificity followed by AS antigens and this result go along with the presence of

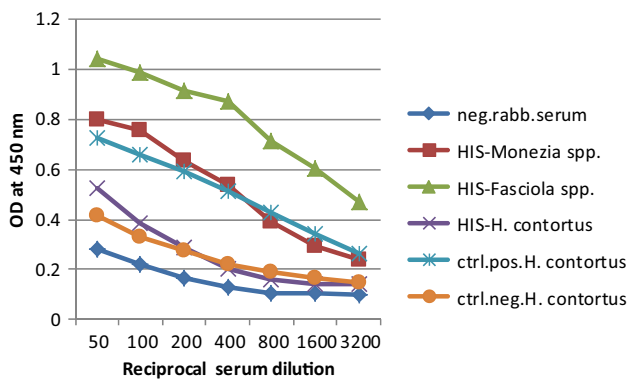


Fig. 4 Cross-reactivity of *Fasciola* spp. antigen against different prepared hyper immune sera. *Neg. rabb. serum* negative rabbit serum, *HIS-Monezia* spp: Hyper immune serum raised against *M. expansa*, *HIS-Fasciola* spp: Hyper immune serum raised against *Fasciola* spp, *HIS-H. contortus*: Hyper immune serum raised against *H. contortus*, *ctrl.pos. H. contortus*: control positive serum of adult *H. contortus*, *ctrl.neg. H. contortus*: control negative serum of adult *H. contortus*

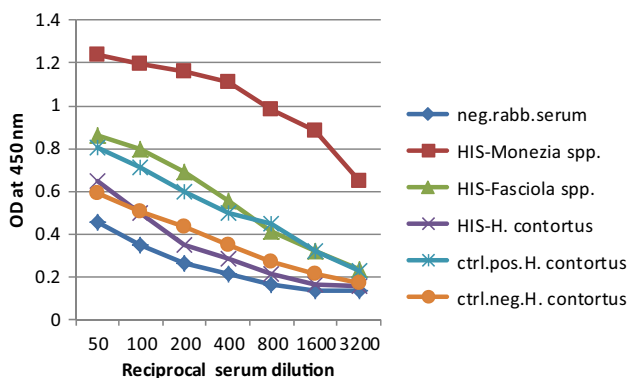


Fig. 5 Cross-reactivity of *M. expansa* antigen against different prepared hyper immune sera. *Neg. rabb. serum* negative rabbit serum, *HIS-Monezia* spp: Hyper immune serum raised against *M. expansa*, *HIS-Fasciola* spp: Hyper immune serum raised against *Fasciola* spp, *HIS-H. contortus*: Hyper immune serum raised against *H. contortus*, *ctrl.pos. H. contortus*: control positive serum of adult *H. contortus*, *ctrl.neg. H. contortus*: control negative serum of adult *H. contortus*

protein bands at the range 22–31 kDa which were reactive at performing immunogenic profile via western blotting technique. These results agree with the previous literature which stated that a 26 kDa antigen of adult worm is specific for diagnosis of *H. contortus* infection (Gomez et al. 1996; Molina et al. 1999; Arab et al. 2013; Kandil et al. 2015) and deviate with the result of (Meshgi and Hosseini 2007). Although, L and AS antigens showed good specificity but the L antigen achieved the best results in detecting the anti-*H. contortus* antibodies in all collected sheep's sera and recorded the highest apparent prevalence 92% followed by the ESP antigen which recorded 75%. These result may be attributed to that the AS antigen which prepared from the adult worm has the ability to escape host immunity by switching gene expression between variants

stored within each genome during the total time of infection. As host immunity builds against a common variant, one or more newly expressed variants can rise. The host must then build another specific immune response against the new variants so this switching may help to avoid the immunological memory of a previously infected host (Frank 2002; Gasser et al. 2016). As well as, the immune evasion mechanisms, formation of circulating immune complexes and differences in status of host immune response may be responsible for variation in antibody level (Lone et al. 2012). So, the present study studied cross reactivity among adult *H. contortus*, *M. expansa* and *Fasciola* spp. and revealed that AS antigen showed prominent cross reactivity with other cestode and trematode represented by *M. expansa* and *Fasciola* spp. antigen and to overcome this phenomenon best dilution of serum was (1:800) and This result agrees with the recorded investigation of Molina et al. (1999) and Kandil et al. (2015).

The highest positive predictive value and apparent prevalence percentages achieved by L and ESP antigens harmonize with the emergence of an immunodominant band at 57 kDa which may be responsible for accurate diagnosis. But this result was in difference with the result of Meshgi and Hosseini (2007) who stated that two major peptide bands belong to intestine and uterus of adult *H. contortus* with 35 and 40 kDa, respectively were specific for such diagnosis. Our result may be discussed due to the persistence of the host immune response of latent infections which may be treated by anti-helminthic drugs so there is no worm detected at post-mortem inspection or may be probably due to cross reactivity of *H. contortus* with other trematode or cestode helminthes (Lone et al. 2012). So, the present study concluded that L antigen is the promising antigen for serological diagnosis of *H. contortus* infection in sheep as the immunodominant band at molecular weight 57 kDa responsible for specificity and accuracy of positive predictive value of this antigen.

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