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Immuno-biochemical characterization of excretory secretory proteins of *Haemonchus contortus* isolated from goats

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Abstract
Haemonchus contortus releases certain excretory-secretory (E/S) proteins which are essential for their survival inside the host and can perform functions like tissue penetration and host protein degradation. The present study was carried out with an aim of immunochemical characterization of E/S proteins of *H. contortus* isolated from goats. The E/S proteins were isolated by *in vitro* cultivation of *H. contortus* in RPMI 1640 medium. Fractionation of whole E/S antigen was done by SDS-PAGE (12.5%) which revealed the presence of five (24, 55, 62, 66 and 93 kDa) polypeptide bands. The purification of whole E/S antigen was done by gel filtration chromatography using Sephacryl S-200. Purified fraction of E/S antigen revealed two (24 and 66 kDa) polypeptide bands in SDS-PAGE (12.5%). Both these polypeptides were found immunodominant with hyperimmune sera (raised in rabbit) on western blot analysis. Antigenicity of purified E/S antigen was determined by indirect ELISA and the titer was 800-1600. It suggested that the purified E/S proteins have retained enough antigenicity during purification process which may be used as potential vaccine candidates as well as for development of user-friendly, cost effective diagnostic technique like dip-stick ELISA for serodiagnosis of haemonchosis in goats.

Keywords: *Haemonchus contortus*, excretory secretory proteins, gel filtration chromatography, western blot, Indirect ELISA

Introduction
India possesses 135.17 million goats which contributes around 26.40% of total livestock population (Livestock census, 2012) [10]. Goats are vulnerable to various parasitic diseases that not only undermine their health but also play a role in lowering the overall production and are responsible for causing heavy losses due to reduce production, morbidity and mortality in animals especially in developing countries (Singh *et al*., 2015) [15]. *Haemonchus contortus* penetrates the abomasal mucosa to feed on the blood of the host, resulting in anaemia and low total plasma protein (Gadahi *et al*., 2016) [4]. Owing to multiple drug resistant *Haemonchus contortus* strains, producers and veterinarians are now faced with seeking alternative methods of treatment and prevention (Tak *et al.,* 2015) [16]. Excretory and secretory products (ESPs) are produced and released by parasites during *in vivo* (Schallig *et al.,* 1997) [13] and *in vitro* cultivation (Rathore *et al.,* 2006) [12]. *Haemonchus contortus* excretory and secretory products (HcESPs) contain many proteins that can perform various functions including the tissue penetration and host protein degradation (Gadahi *et al.,* 2016) [4].
Excretory-secretory (E/S) products of helminthes have received more attention of researchers to identify specific antigens as it shows a comparatively simple antigenic composition than somatic worm antigens (Arunkumar, 2012) [1]. The aim of the present investigation was to isolate and purify E/S antigenic protein that may be used for serodiagnosis of *Haemonchus contortus*.

Materials and Methods
Collection and morphological identification of *Haemonchus contortus*
Abomasum was collected from goat on the day of slaughter from the slaughter house located at Cantonment Board, Mhow, Madhya Pradesh, India and then it was transported to the laboratory. Abomasum was examined thoroughly and the worms particularly *Haemonchus* were collected in petridish containing PBS (pH 7.4) for washing several times to remove host

material. The worms were identified on the basis of morphological features using standard keys (Keith, 1953) [8].

Preparation of Excretory secretory (E/S) proteins

To harvest E/S products from *Haemonchus contortus*, the standard procedure was used as described by Yatsuda *et al*. (2003) [19] with some modifications. The fresh and highly motile worms were incubated for 4hr in RPMI 1640 medium (100/ml) containing antibiotics (100 IU of penicillin, 0.1mg/ml streptomycin, and 5mg/ml gentamicin) at 37˚C under 5% carbon dioxide and then the medium was removed, and the parasites were incubated in new medium containing 2% glucose for overnight. After that the medium was collected, pooled and centrifuged and the supernatant was filtered through 0.22 micrometer filter unit. The obtained antigen was concentrated by dialysis (membrane cut off, 12 kDa) against polyethylene glycol (PEG 6000) and stored at -20˚C with 0.02% sodium azide.

The concentration of protein in whole E/S products was estimated by Lowry's method (Lowry *et al*., 1951) [11] with some modifications.

Ethical approval

The present investigation has been conducted with the approval of Institutional Animal Ethical Committee, College of Veterinary Science and Animal Husbandry, Mhow, Madhya Pradesh.

Preparation of hyper-immune sera (HIS)

Immunization against antigenic protein of E/S products was done intramuscularly (I/M) and subcutaneously (S/C) into the rabbit at various intervals and at multiple sites as per the method of Hay and Westwood (2002) [5] with some modifications.

E/S products was thoroughly mixed with Freund's complete adjuvant (FCA) (1:1) and a total of 1 ml was injected intramuscularly at 3 sites into the thigh muscle and subcutaneously in the scapular region in a New Zealand white rabbit (weight 1.5 kg). Three booster doses of the same antigen emulsified with Freund's incomplete adjuvant (FIA) (1:1) were given on 14th, 21st and 28th day of first injection. On 34th day, blood was collected and serum was separated by centrifugation at 5000 rpm for 20 minutes. Antibody titer of serum sample was determined by indirect ELISA and was stored at -20 ºC for further use.

Purification of antigenic proteins by gel filtration chromatography

The crude extract of E/S products was subjected to gel permeation chromatography on Sephacryl S-200 and the proteins were fractionated as per the method adopted by (Kandil *et al*., 2015) [7] with some modifications. The filtration material, Sephacryl S-200 was equilibrated with the 0.15 M PBS (pH 7.2) containing 0.04% sodium azide. The sample was loaded directly on Sephacryl S-200 column (50x1.8 cm) and the flow rate was adjusted to 3ml/4min and the eluted fraction (3ml) was collected in each tube. All the tubes were analyzed spectrophotometrically at 280nm for the presence of protein. The purified antigen was concentrated by dialysis against PEG 6000 and the protein content was measured as per the method by Lowry *et al*., 1951 [11].

Double immunodiffusion (DID)

Ouchterlony double immunodiffusion is based on the ability of antibodies to form precipitation lines specifically with antigen. Free diffusion of both antigen and antibody takes place in agarose gel resulting in precipitation lines, which are visible to naked eye (Bailey, 1996) [3].

1.0% agarose was prepared in PBS (0.15 M, pH 7.2) and 0.1% sodium azide was added in it. After boiling, about 5ml of molten agarose was poured onto clean, grease free microscopic slide and allowed to solidify. Wells were then punched by gel puncher. The bottom of the wells was again sealed with molten agarose solution to prevent the leakage of antigen and antibody.

In one slide, wells were charged with 20 µl of whole E/S antigen and 20 µl of hyper-immune serum raised against E/S antigen. In other slide, wells were charged with 20 µl of purified E/S antigen, 20 µl of hyper-immune serum raised against E/S antigen as neat and 1:2 dilution. The loaded gel slides were kept in a humid petridish at 37 ˚C for about 24hr for immunodiffusion. Then precipitation lines between wells were observed after 24hr onwards.

SDS-PAGE and western blotting

Characterization of whole as well as purified E/S antigen was done by SDS-PAGE (12.5% resolving gel) (Laemmli, 1970) [9] and then the resultant proteins were electroblotted to nitrocellulose filter paper (NCP) (Himedia) from gel according to the method of Towbin *et al.* (1979) [18] with some modifications.

Detection of immuno-dominant peptides by Western blotting

After electro-transfer, NCP was kept in blocking buffer (5% skimmed milk powder in PBS, pH 7.4) for two hr at 37˚C in incubator. The NCP was washed for four times with washing buffer (0.05% Tween-20 in PBS, pH 7.4). After that, the NCP was incubated for two hr with hyperimmune serum (1:40 dilutions in blocking buffer) and then washed for four times. Then the NCP was incubated for two hr with goat anti-rabbit horse radish peroxidase conjugate (1:500 dilutions in PBS, pH 7.4) and then washed for four times. The NCP was rinsed with substrate solution [Diaminobenzidine (DAB), H_20_2 and dilution buffer] (DAB system, Bangalore GeNei). After few min the appearing protein band was observed and the reaction was stopped by dipping the NCP into the distilled water.

Indirect Enzyme Linked Immunosorbent Assay (Indirect ELISA)

Hudson and Hay (1989) [6] protocol with some modification was followed for this assay. Purified E/S proteins were used as coating antigens (5 µg protein in each well/100µl, after diluting with coating buffer, pH 9.6). The plate was kept at 4˚C for overnight. The plate was blocked with blocking buffer (5% skimmed milk in PBS) at the amount of 200 µl per well. The plate was kept at 37 ˚C in incubator for two hours. Four times washing was done with washing buffer. 100µl two fold serial dilution (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800 and 1:25600) of hyper immune sera (HIS) raised against E/S antigen and same serial dilution of normal rabbit sera (NS) were added accordingly and kept for 2 hr in incubator at 37˚C. 100µl/well of goat anti-rabbit horseradish peroxidase conjugate (Bangalore GeNei) solution was added in each well. The plate was again incubated at 37 ˚C for two hr. Substrate buffer as 100 µl/well (TMB/H_2O_2 in 1:20 dilution with distilled water) (Bangalore GeNei) was added in each well and kept for 20 min in dark at room temperature.

The absorbance was taken at 450 nm in an ELISA reader (Aspen Diagnostics Pvt. Ltd.).

Determination of titer

Inverse of the dilution of hyperimmune sera in the well at which 50% of λ_{max} exist was considered as titer of the serum.

Results

The protein concentration of whole E/S antigen was 1.5 mg / ml and of purified E/S antigen was 1.8 mg / ml.

Fractionation of E/S antigen

The SDS-PAGE (12.5% resolving gel) profile of whole E/S antigen revealed five polypeptide bands at 24, 55, 62, 66 and 93 kDa molecular weights (Fig. 1). The purified E/S antigenic protein indicated two bands at 24kDa and 66kDa molecular weights (Fig. 2) when resolved by SDS-PAGE (12.5% resolving gel).

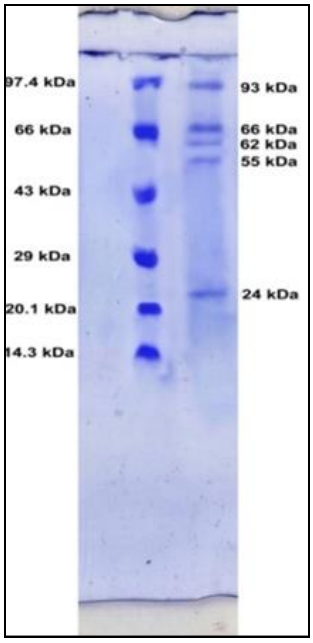


Fig 1: Fractionation of whole excretory-secretory (E/S) antigen by SDS-PAGE (12.5% gel)

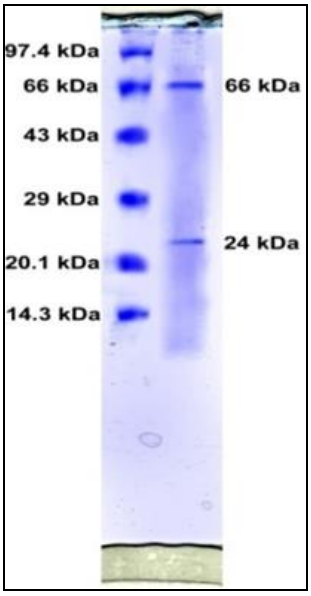
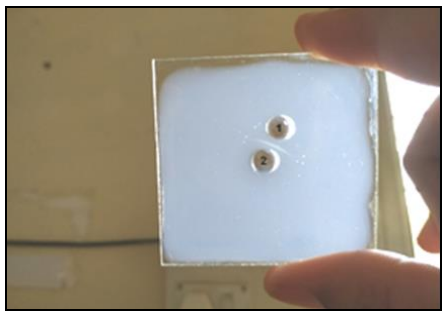


Fig 2: Fractionation of purified excretory-secretory (E/S) antigen by SDS-PAGE (12.5% gel)

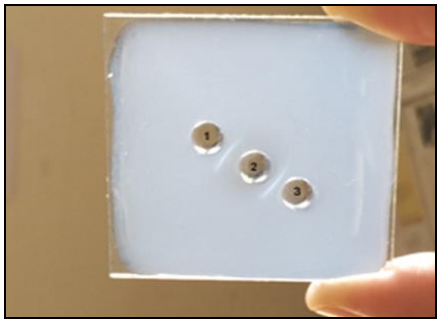
Double Immuno Diffusion (DID)

In double immuno diffusion (DID) assay, specific precipitation bands were observed in between whole E/S antigen with its hyperimmune sera (Fig. 3) and in between purified E/S antigen with hyperimmune sera (raised against whole E/S antigen) as neat and 1:2 dilution (Fig. 4).



Well-1: Whole E/S antigen
Well-2: Hyper immune sera (HIS)

Fig 3: Double Immuno Diffusion test showing the precipitin line of whole excretory-secretory (E/S) antigen and its antibodies



Well-1: Hyper immune sera (neat)
Well-2: Purified E/S antigen
Well-3: Hyper immune sera (1:2 dilution)

Fig 4: Double Immuno Diffusion test showing the precipitin line of purified excretory-secretory (E/S) antigen and its antibodies

Western blotting

The purified E/S antigen when transferred onto nitrocellulose membrane from gel showed strong reactive bands at 24 kDa and 66 kDa (Fig. 5). It indicates the hyperimmune sera could recognize well its homologous antigen.

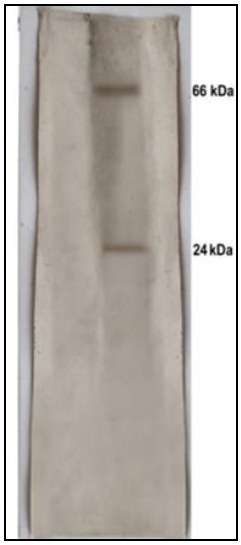


Fig. 5. Immunoblot of 12.5% SDS-PAGE run gel of purified excretory-secretory (E/S) antigen

Indirect Enzyme Linked Immunosorbent Assay (Indirect ELISA)

The titer of rabbit hyperimmune serum was 800-1600 (Table 1 and Fig. 6).

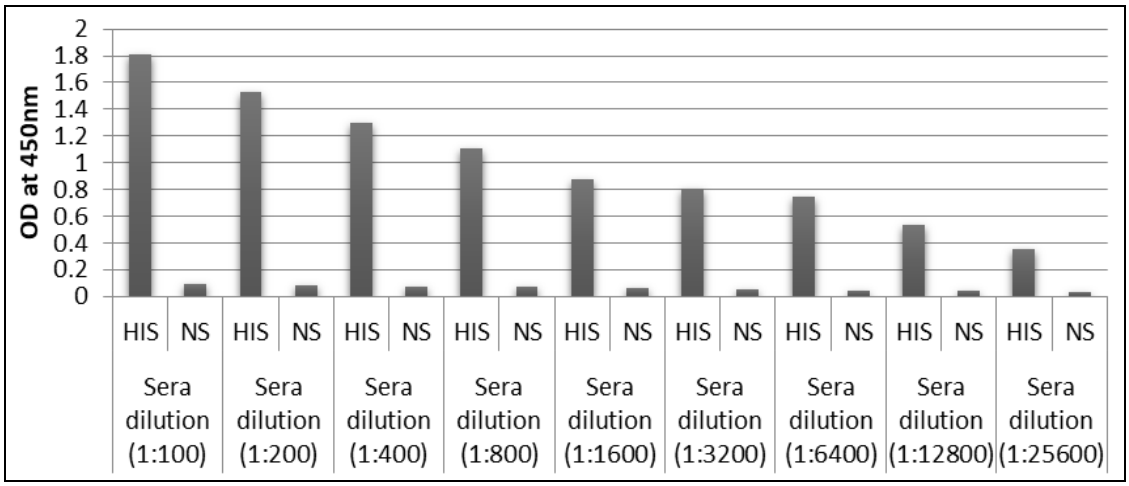
Table 1: Assessment of seroreactivity of purified *Haemonchus contortus* excretory- secretory (E/S) antigen by indirect ELISA using hyperimmune sera raised in rabbit

Types of Antigen	Sera dilution (1:100)		Sera dilution (1:200)		Sera dilution (1:400)		Sera dilution (1:800)	
	HIS	NS	HIS	NS	HIS	NS	HIS	NS
Purified *Haemonchus contortus* E/S antigen	1.806	0.088	1.531	0.081	1.300	0.074	1.105	0.068

Sera dilution (1:1600)		Sera dilution (1:3200)		Sera dilution (1:6400)		Sera dilution (1:12800)		Sera dilution (1:25600)	
HIS	NS	HIS	NS	HIS	NS	HIS	NS	HIS	NS
0.878	0.060	0.802	0.055	0.745	0.046	0.531	0.039	0.352	0.036

HIS= Hyperimmune sera NS= Normal sera

Values are expressed in terms of O.D. at 450 nm



X axis: OD at 450nm; Y axis: Sera dilution

Fig 6. Assessment of Seroreactivity of purified excretory-secretory (E/S) antigen by indirect ELISA

Discussion

Several previous workers have characterized and identified the immunogenic polypeptide bands in the E/S antigens by SDS-PAGE analysis. Schallig *et al*. (1994) [14] fractionated E/S proteins from sheep by SDS-PAGE analysis and revealed at least 15 polypeptides with molecular weights ranging from 10 to 100 kDa (24, 30, 40, 46, 52, 62, 66, 86 and 93) and on western blot analysis, recognized 24, 40, 52, 62, 66, 86 and 93 kDa with sera of primary *Haemonchus contortus* infected sheep. Rathore *et al*. (2006) [12] characterized the E/S antigen of *Haemonchus contortus* from goats and identified the size of polypeptides ranging from 15 to 150 kDa with a 66 kDa protein strongly reacting with sera from goats with *Haemonchus contortus* infection. Arunkumar *et al*. (2012) [2] characterized the E/S antigen of *Haemonchus contortus* from sheep and revealed five polypeptide bands at 24, 29, 46, 66 and 93 kDa molecular weights and on western blot analysis, the whole E/S antigen probed with serum from sheep infected with *Haemonchus contortus* showed five reactive bands at 24, 29, 46, 66 and 93kDa. Kandil *et al.* (2015) [7] identified 7 protein bands with molecular weight ranging from 14 to 93 kDa from E/S antigen of *Haemonchus contortus* isolated from sheep. Tak *et al*. (2016) [17] revealed the presence of 13 prominent protein bands with molecular weight ranging from 15 to 70 kDa (66, 62, 60, 55, 50, 40, 38, 32, 29, 24, 20, 18, 15 kDa) from SDS-PAGE analysis of E/S antigen of *Haemonchus contortus* from sheep. The present findings are in partial agreement with the reports of Schallig *et al*. (1994) [14], Rathore *et al*. (2006) [12], Arunkumar *et al*. (2012) [2], Kandil *et al*. (2015) [7] and Tak *et al*. (2016) [17]. The difference observed in the present polypeptide profiles of E/S antigen might be due to host species variation, difference in the method of antigen preparation and protein concentration of E/S antigen. Indirect ELISA suggests that purified E/S antigen has retained enough antigenicity during purification process. Moreover, after assessing antigenicity of purified E/S antigen, it has been observed that this antigen may be used for serodiagnosis of *Haemonchus contortus* using rapid, user-friendly Serodiagnosis technique like dip-stick ELISA.

Conclusions

The present investigation generates a precise knowledge on the antigenic proteins associated with E/S products of *Haemonchus contortus*. It may be concluded that the purified E/S antigenic proteins have retained enough antigenicity that may be used for serodiagnosis of *Haemonchus contortus* using rapid, user-friendly, cost effective serodiagnostic technique like dip-stick ELISA. E/S antigenic proteins of 24 kDa and 66 kDa can also be explored as vaccine candidates upon assessing their chemical and functional nature.

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