Research Square

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Development and Evaluation of an Indirect ELISA for Detection of Infection with *Teladorsagia Circumcincta* in Sheep

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Abstract

Background

The gastrointestinal helminth, *Teladorsagia circumcincta*, is one of the major health risk and production-limiting disease in small ruminant populations, particularly in temperate regions. With the increasing importance of the disease management and recruited anthelminic resistant types, accurate approaches are needed for diagnosis of the infection in the host. Because of uncertain results using faecal examinations, the ELISA method was indicated for detection of the nematode antigenic materials. Despite some promising results, problems were described in terms of the test specificity and the cross-reactions. The aim of this study was therefore to evaluate worm somatic and excretory/secretory (ES) products using western blot analysis and an indirect ELISA for detecting *T. circumcincta* infection in sheep.

Results

Based on the immuno-reactivity analysis, immunogenic fractions with molecular weights of approximately 60, 75 and 100 kDa were detected in somatic content and two antigens of about 63 and 75 kDa in ES material. Accordingly, a specific product at 75 kDa had the strongest reaction and appeared as the most common antigenic protein. In ELISA, all sera from the infected sheep revealed the OD rates above the calculated cut-off value with about two-fold greater in average. Negative control samples were also specifically recognized with the mean OD rate of about 1/3 of the estimated cut-off value. The cross-reaction test, using rabbit hyperimmune sera, did not show reactivity with ES antigens of other prevalent nematodes include *Haemonchus contortus*, *Protostrongylus rufescens* and *Marshallagia marshalli*. In contrast, a strong positive reaction was found with the somatic antigens of *M. marshalli*.

Conclusions

The results obtained here indicates the indirect ELISA method using the ES content enables distinguishing the *T. circumcincta* infected sheep with high specificity. Those antigenic ES peptides with 63 and particularly 75 kDa molecular weights should be more investigated due to the potential for serological diagnostic methods and immunoprotective targets in the host.

Background

Teladorsagia circumcincta is one of the most prevalent gastrointestinal nematode (GIN) which infects sheep and goats worldwide. The infection coincides with inflammation in the abomasal tissue leading to changes in the gastric physiological functions [1] and contributes to reduced weight gain and productive indicators [2].

Detection of the GIN has been traditionally depend on tracing the eggs in faecal samples by microscopy. Several egg count procedures with subsequent modifications have reported to estimate the level of infection. However, microscopic assessments have some disadvantages and often accompanied with unreliable results [3]. Therefore, alternative diagnostic approaches were developed to identify the present GIN infections in the host. Among the evaluated methods, the response of the immune system have been mostly investigated [4]. In sheep infected with *T. circumcincta*, protective immunity was associated with the parasite-specific antibodies against adult worms [5] and the established larvae [6, 7]. Isotypes of antibodies play important roles in GIN resistance in sheep, including IgA, IgG1 and IgE [8].

In the past, potential of the ELISA technique was assessed to find out the antibody response to *Ostertagia* species in field applications. The IgA reaction comparatively investigated in larval somatic antigen and a fragment of a recombinant protein, disulphide isomerase, in blood, nasal secretions and saliva of the infected ewes [9]. This method was also developed for detection of IgG antibodies against copro-antigens in faecal preparations [10]. Additionally, the level of IgG specific to *T. circumcincta* crude antigens described in milk and blood of goats [11] and experimentally infected lactating ewes [12]. In cattle, ELISA was hopefully performed for diagnosis of gut-associated nematode infections using recombinant *Cooperia oncophora* protein [13], crude *Ostertagia ostertagi* whole-antigen [14] and *Ostertagia ostertagi* copro-antigens containing excretory secretory (ES) products of the worm [15]. Alongside with the successful or relatively promising results, some studies reported the strong cross-reactions with other nematode antigens among the trichostrongyloid members [16] and the difficulty to obtain crude [13] or somatic antigens [9] with highly standardized preparations. More importantly, improvements may also need to enhance the test sensivity for the parasite detection [10].

Because of variations in or lack of data on test specificity, more investigations are needed to enhance diagnosis based on tracing antigens of *T. circumcincta* in the host. Therefore, the objective of this study was first to detect and characterize the somatic and ES antigens of *T. circumcincta* in sheep. In addition, those antigens were used to develop a specific ELISA method with high sensivity or specificity rates. In parallel, the possibility of cross-reactions was evaluated with some prevalent nematodes that are usually found in abomasal and lung tissues.

Results

Species confirmation

In this study, data from molecular evaluations corroborated morphologic diagnosis. The BLAST search indicated major similarities between the sequence data obtained (MN888739) and available reports for *T. circumcincta* (The phylogenetic relations was depicted in Additional file 1).

SDS-PAGE and western blot Analysis

Total proteins of somatic and ES antigens were determined as 22.1 mg/ml and 15 mg/ml, respectively. The SDS-PAGE analysis for somatic antigens revealed 15 protein fractions ranging in size from 20 to 245

kDa, with molecular weights (MW) of 20, 25, 30, 38, 42 (weak band), 45, 47, 60, 63, 65, 75, 80, 100, 180, 245 kDa (Fig. 2). In the pattern of ES antigens, proteins with MWs of 20, 25 (weak band), 28, 35, 48, 50, 63, 68, 75, 80, 100, 135, 180 kDa could be detected (Fig. 2).

The immuno-reactivity of the somatic antigens with sera from the infected sheep revealed three proteins of about 60, 75 and 100 kDa (Fig. 3A). However, with the hyper immune serum of rabbits, the result was fractions with MWs of about 30, 60 and 100 kDa (Fig. 4). The immunoblotting analysis with ES antigens and positive sera of sheep demonstrated only two bands of 63 and 75 kDa (Fig. 3A). One specific band at 75 kDa had the strongest reaction (Fig. 3B). In comparison, fractions with 50, 75 and 135 kDa were obtained using sera of the immunized rabbits (Fig. 3B). Based on the present electrophoretic patterns, the immune response to somatic antigens of 60 and 100 kDa and ES antigen with 75kDa were commonly found in both animal types. In addition, among the somatic and ES protein profiles, the product with 75 kDa MW was the most common antigenic protein. Immunoblotting analysis gave negative reactions with sera from non-infected sheep.

On Western blot analysis, the specificity of rabbit sera against *T. circumcincta* ES antigens was confirmed by lack of any cross reaction with *H. contortus*, *P. rufescens* and *M. marshalli* ES materials. In contrast, positive anti-somatic sera revealed strong reactivities with the somatic antigens of *M. marshalli* at 35, 63, 75 and 100 kDa. A slight reaction was also found against somatic antigens of *H. contortus* (Fig. 4).

ELISA

The ODR values obtained with serum samples of the worm-free (negative) and positive samples against the somatic and ES antigens are plotted in Fig. 5. The Mean±SEM of ODR values were 0.708±0.03 for positive samples against somatic and 0.674±0.02 for ES antigens. The respective cut-off values were calculated as 0.348 and 0.328. All the samples from the infected sheep indicated ODR values above the cut-off thresholds. In addition, negative samples registered ODRs with overall Mean±SEM of 0.12±0.014 ranged 0.038 to 0.215. This means that the present antigens gave a 100% sensivity and 100% specificity for diagnosis of the infection.

According to observed reactions and the ODR values obtained, no cross-reactions were detected between *T. circumcincta* somatic and ES antigens against the same for *H. contortus*, *P. rufescens* and ES from *M. marshalli*. As expected before, the relatively high ODR value confirmed a significant reaction with somatic antigens of *M. marshalli* (Fig. 6).

Discussion

Based on the results obtained in this study, the ELISA test using the somatic and ES antigens is a sensitive approach for detection of the infection with *T. circumcincta* in sheep. Compared with somatic antigens, the ES products was more specific with no cross reactivity with some other prevalent GIN nematode. Among ES antigens, a peptide with 75 kDa molecular weight was commonly identified in

different specimens with negative cross reactions indicating the potential for serological diagnostic methods and immunoprotective studies in future.

The role of somatic and ES materials has been defined according to the host immune response and protection against GIN infections in ruminants. Many of secreted proteins, particularly from the ES components, have been characterized in larval and adult stages of trichostrongylid worms (reviewed in [24]). Studies are more directed on the larval secreted proteins with protective potential and applied them for vaccine development [25]. A previous research on adult *T. circumcincta* ES products reported peptides varying from 18 to 115 kDa with the most prominent proteins at 20, 29 and 63 kDa molecular weights [26]. These proteins, which were also indicated in our results, are homologues of enzymes involved in energy metabolism reported in *Ostertagia ostertagi* and *C. elegans*. In line with our data, a major protein with 35 kDa MW and a relatively weak 25 kDa MW protein band were described in the ES products of fourth stage larva with Cathepsin F function (Tci-CF-1) [27]. The corresponding CF was also explained previously as the most abundant protein with about 27 to 30 kDa molecule in larval ES from *T. circumcincta* [28]. Despite some similarities, other proteins in the adult stage ES with MWs of about 75 kDa and higher and the whole somatic extract of *T. circumcincta* were not described earlier. Nevertheless, according to the immunoblotting data here, ES proteins with MWs of 63 and 75 kDa and somatic antigens with 60, 75 and 100 kDa MW could be considered for immunization against and detection of the infection with *T. circumcincta* in sheep.

In this study, the explained indirect ES ELISA was a promising specific method to distinguish between *T. circumcincta* and some other prevalent nematode infections. The cross-reaction among trichostrongylid nematodes has been a limiting factor for test specificity. Levels of reactivities were reported between somatic [11, 16] and ES [10, 29, 30] products derived from *T. circumcincta*, *H. contortus*, *Trichostrongylys colubriformis* and a trematode, *Fasciola hepatica*. Therefore, improvements in the method applied [10], application of purified somatic antigens [31] and using specific recombinant antigens [24] were suggested to overcome this major problem. However, because of some partial reactivities, the differentiation between *T. circumcincta* and *H. contortus* was remained to be solved. An improved copro-antigen ES ELISA was evaluated for detection of *T. circumcincta* infection in faecal samples in sheep [10]. A great accuracy was obtained and the optimized period of exposure to an optimum temperature largely reduced the cross-reactive signals from all but two cases of *H. contortus*. In another research, the hyperimmune sera against soluble extract of *T. circumcincta* recognized some protein fractions of *H. contortus* except for a 26 kDa peptide, which was exclusively reactive with homologus antiserum [31]. Such common antigens have more reported for somatic antigens [30]. In addition to more sensitive results, the ES content acts more specifically to detect *H. contortus* infections [16]. In our study, a faint reaction was recognized in immunoblotting assay between somatic (but not ES) content of *H. contortus* and *T. circumcincta*. Fortunately, this observation did not confirmed in the ELISA method. In comparison, the significant reactivity was only found against somatic antigens of *M. marshalli*. This result could be expected mainly due to the close phylogenetic relation between *Marshallagia* and *Ostertagia*, both belonged to Ostertagid nematodes. This is worthy of note that we did not access to sera from sheep (experimentally or naturally) mono-infected with prevalent GIN and thereby the immunized rabbit sera

was substituted. Thus, the assay needs more evaluated using sera samples from the infected sheep from different regions.

The primary aim for the ELISA test was developing a feasible and specific assay. The ODR value seems to be a reliable index to for animals with moderate infections (with about 1000 or more adult worms) with need to the anthelmintic treatments. However, those animals with low or subacute chronic infections should be of concern. This issue was previously considered on ES materials in faecal samples from sheep infected with fewer than 1000 *T. circumcincta*, which was coincided with variable and overlapped OD values relative to the calculated threshold [10]. Similar unsatisfied results was also reported by ELISA on faecal *O. ostertagi* ES content in cattle with natural or experimental infections [15]. In this issue, even if it is postulated that the ELISA would not discriminate sera of sheep with low infection intensities and negative controls, our study contributes to show the practical advantage of this method in line with targeted (selective) treatment [32] to detect and treat those animals harboring much heavier infections.

Conclusions

This study concluded the functional value of the ELISA test with ES products for screening of infection with *T. circumcincta* in sheep. A strong cross-reaction was observed with somatic antigens of *M. marshalli*, while this reaction was not the case for ES products. According to immunoblot analysis, purified antigenic fractions (like those with MWs of about 63 and 75 kDa) is suggested to be investigated in a broader number of infected and non-infected sheep and goats.

Methods

Collection of worm and blood samples

A total of forty sheep (between 1-2 years old), with relatively high abomasal infections (harbored about 1000 to 2000 worms), were selected randomly from different flocks referred to the local industrial abattoirs in Shiraz (29.5926° N, 52.5836° E), Fars province, South of Iran. The abomasa cut and placed in warm 37 °C phosphate-buffered saline (PBS). Worms were then recovered, washed three times in PBS, pH 7.2, containing penicillin (10000 IU/ml) and streptomycin (10 mg/ml) and used for antigen extraction. A number of worms were morphologically identified according to previous descriptions for *T. circumcincta* [17]. Regarding the prevalence of other trichostrongylid nematodes in the sampled area, efforts were made to ensure full recovery of worm burden to exclude abomasa with mixed infection. Prior to the slaughtering process, peripheral whole blood samples were collected from the jugular vein of animals. Sera were separated from the samples collected from the infected sheep and maintained at -20$^{\circ C}$ until use. In addition, blood samples from inspected 10 non-infected animals (under 6 months of age) with no nematode infection were sampled as negative controls. Sample collections were performed after the consent to participate was obtained from farm owners.

Molecular diagnosis

Adult *T. circumcincta* worms were randomly selected from at least 2 farms and subjected to genomic extraction using a DNA extraction Kit (MBST, Iran). A primer set was used: F (5'-GCAGACGCTTAGAGTGGTGA-3') and R (5'-TCCTTGTTAGTTTCTTTTCCTCCG-3'), as described previously to identify the complete rDNA ITS-2 region in ostertagiine nematodes [18]. The PCR reaction mix included 12.5 µl of PCR premix (Ampliqon, Denmark, Cat. No. A180301), 1 µl of each primer, 6.5 µl H_2O and 4 µl of DNA as template. The cycling program consisted of an initial denaturation at 95°C for 5 min, 94°C for 30 sec, followed by 35 cycles of 60°C for 30 sec, the extension at 72°C for 30 sec and the final extension at 72°C for 10 min. The products were sequenced (ABI 3730 DNA analyzer; Bioneer, Korea) and compared with other available sequences in NCBI using BLAST search. The sequence data was aligned with homologous sequences existing in the GenBank using Clustal W program by M EGA 6 software [19]. The phylogenetic tree was constructed by maximum likelihood (ML) method and analyses was carried out using the Kimura 2-parameter distance estimate [20].

Preparation of *T. circumcincta* somatic and ES antigens

Somatic antigens were extracted from washed adult specimens. A total of (about) 1000 worms were separately homogenized by tissue grinding, sonicated in 10 mL PBS pH 7.2 and centrifuged at 12000 rpm for 15 min at 4 ˚C. The extract was filtered through 0.45 mm filters and finally stored at –20 ˚C until use.

In order to prepare the ES products, freshly isolated adult worms (n~1000) were washed four times in normal saline and subsequently in PBS, pH 7.2, containing penicillin (1000 IU/ml) and streptomycin (1 mg/ml). Worms with high rates of motility were maintained in a sterile culture flask at a density of approximately 100-200 worms/mL of the culture medium (RPMI-1640; Gibco, ThermoFisher Scientific, USA) with penicillin G potassium and streptomycin (1000 IU/ml and 1 mg/ml) and incubated for 24 hours in 5.0% CO2 at 37 ˚C. Supernatants were collected and centrifuged at 12000 rpm for 15 min at 4 ˚C. The supernatant was then dialysed against PBS over a period of 24 h, and concentrated using freeze-drying process (Lyophilisation) (Zirbus, Netherlands). Protein content was estimated by the Bradford method [21] and stored at –20 ˚C until use.

Preparation of rabbit polyclonal antisera

Ten adult male rabbits of a commercial New Zealand White strain with average weight of 2 ± 0.2 Kg were maintained under constant conditions with access to water and food in the animal house, School Veterinary Medicine, Shiraz University. Rabbits were adapted to new conditions for at least two weeks prior to immunization and divided in to 5 groups. Groups 1 to 4 received somatic and ES antigens of *T. circumcincta*, *H. contortus*, *M.marshalli* and *P. rufescens* and group 5 was defined as control. Blood samples (of about 5 ml) were collected from each rabbit before first injection (day zero) and used as negative controls. According to vaccination protocol (Fig. 1), 300 mg antigen, in a volume of 1 ml of PBS, emulsified with 1 ml of Freund's complete adjuvant (Sigma, USA) was first injected subcutaneously at day zero. This followed by four boosters comprised of 150 mg antigen in a volume of 1 ml of PBS emulsified with 1 ml of Freund's incomplete adjuvant (Sigma, USA). Boosters were given at one week

intervals. The control group (group 5) was administered with 1 mL of sterile PBS plus 1 mL adjuvant. Rabbits were bled one week after final booster (at day 35), sera were collected and stored at –20 ˚C until use. The polyclonal hyper-immune serum was evaluated against somatic and ES antigens by indirect ELISA and Western blotting.

Electrophoretic analysis

Somatic and ES antigens were separated by sodium-dodecyl-sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) based on Laemmeli's method [22]. The samples were mixed with an equal volume of a sample buffer and boiled for 5 min. They then were added to each well of a 5% stacking gel and 12% separating gel. Electrophoresis was run at 100 V for 4 hr under reducing conditions using electrophoresis apparatus (Paya Pajoohesh Pars, Tehran, Iran). Stained molecular mass standards (Cinnagen PR911654 [SL7012]) ranging from 11 to 180 kDa were used. SDS-polyacrylamide gel was stained for protein visualization with 0.05% Coomassie brilliant blue (Sigma, USA.).

Western blotting

For immunoblotting, proteins were first electrophoresed on 12% SDS-polyacrylamide gel. Western blotting was carried out as described previously [22] with modifications. Proteins were transferred onto a nitrocellulose membrane for 1.5 hr. After blocking overnight at room temperature (RT) with 5% skimmed milk in PBS, nitrocellulose membrane stripes were cut, washed with PBST and incubated with serum sample at RT for 1 hr. Sera samples from the immunised rabbits and the infected sheep were diluted as 1:50 and 1:10, respectively. After washing, anti-rabbit and anti-sheep conjugate peroxidase (Sigma, USA) diluted in PBS-T (1:2000 - 1:1000) was added and incubated with shaking for 1 hr at RT. Finally, the membrane stripes was washed and placed into a substrate solution 0.05% diaminobenzidine in 50mM Tris pH 7.4 containing 0.05% H_2O_2. (DAB/ H_2O_2) (Sigma, USA).

The reactivity of sera from sheep and immunized rabbit against *T. circumcincta* somatic and ES antigens was tested against the same products prepared from prevalent field isolates of *H. contortus*, *M. marshalli* and a lungworm species, *P. rufescens*.

Enzyme Linked Immunosorbent Assay (ELISA)

The checkerboard titration for determination of different dilutions of antigen, sera, and conjugate was done [23]. An indirect ELISA (iELISA) was carried out and optimized with serum samples. 96-well microplates were incubated with 100 µl/well of antigen at 1.1 mg/ml for somatic and 7.5 mg/ml for ES proteins in 50 mM carbonate bicarbonate buffer (pH 9.6) at 4ºC overnight. After washing three times with PBS containing 0.05% (v/v) Tween 20 (washing buffer), plates were blocked with 200 µl/well of the blocking buffer (PBS at pH 7.2 with 1% Bovine serum albumin) at RT for 2h. Follow 3 times washings, 100 µl of diluted sheep and rabbit sera 1:2 in 1% BSA were incubated at RT for 1 h. The plates were washed as described above and 100 µl/ well of horseradish peroxidase anti-sheep and anti-rabbit IgG conjugate (Sigma, USA) diluted at 1:5000 were added and incubated for 1h at RT. The plates were

washed three times and 100 µl of the substrate buffer contains (0.02 g Ortho-Phenylenediamine) (Sigma, USA) in citrate buffer and 30% H_2O_2 were added to the plate wells. Finally, the optical density (OD) were obtained from an ELISA reader (Immunoskan BDSL, Thermo Lab. Systems, Finland) at 450 nm. All samples were run in duplicate. A pool containing sera of 10 naturally infected sheep with immune-reactivity against *T. circumcincta* (somatic and ES) antigens in western blot test was used as the positive control. Because no serum samples were available from parasite naïve sheep mono-infected with other GINs, the specificity of the method was checked by cross reactivity test using rabbit hyperimmune sera as described earlier.

Statistical analysis

In order to normalizing the OD estimates in the ELISA, values were quantified as the relative ODR according to the formula: ODR= (OD–N)/(Ps–N), where N and Ps are the mean absorbance values for negative and positive controls. The rates of sensitivity and specificity were evaluated as sensitivity = (true Ps)/(true Ps + false N) × 100 and specificity = (true N)/(true N + false Ps) × 100. In order to determine the best cut-off values, receiver operating characteristics (ROC) analysis was performed and the point showing maximum percents of the sensivity and specificity were considered. The SPSS software (Version 16.0) was used for the statistical analyses and the GraphPad Prism 8 for drawing graphs.

Declarations

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Authors' contributions

ER and AY contributed to generate the study plan, data analysis and the manuscript preparation. JA carried out the sample collections and lab work. All authors approved the final draft of the manuscript.

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Availability of data and materials

The nucleotide sequence obtained for the ribosomal ITS region of *T. circumcincta* was deposited in the GenBank under the Accession number: MN888739 (*http*://www.*ncbi*.nlm.nih.gov/nuccore/1955290121). The datasets used/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This manuscript is part of a Ph.D. thesis performed by Mr. J. Aliabadi in Shiraz University, Iran. The animal ethics and all protocols were approved by the animal welfare and ethics committee in Faculty of Veterinary Medicine, Shiraz University, Iran (letter No. 983/46/22). All methods were carried out in accordance with Shiraz University animal welfare guidelines and policies. In addition, all the samplings was done when the informed consent was obtained from farm owners.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

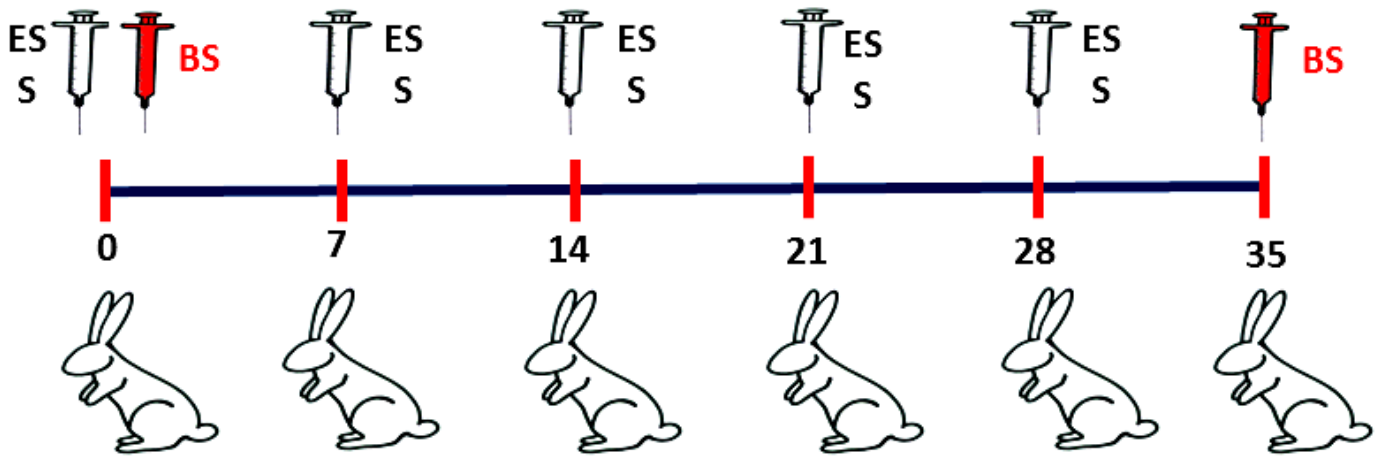

Figure 1

Vaccination protocol for immunization of rabbits against different (somatic(S) and ES) antigenic materials and the respective times for blood sampling (BS).

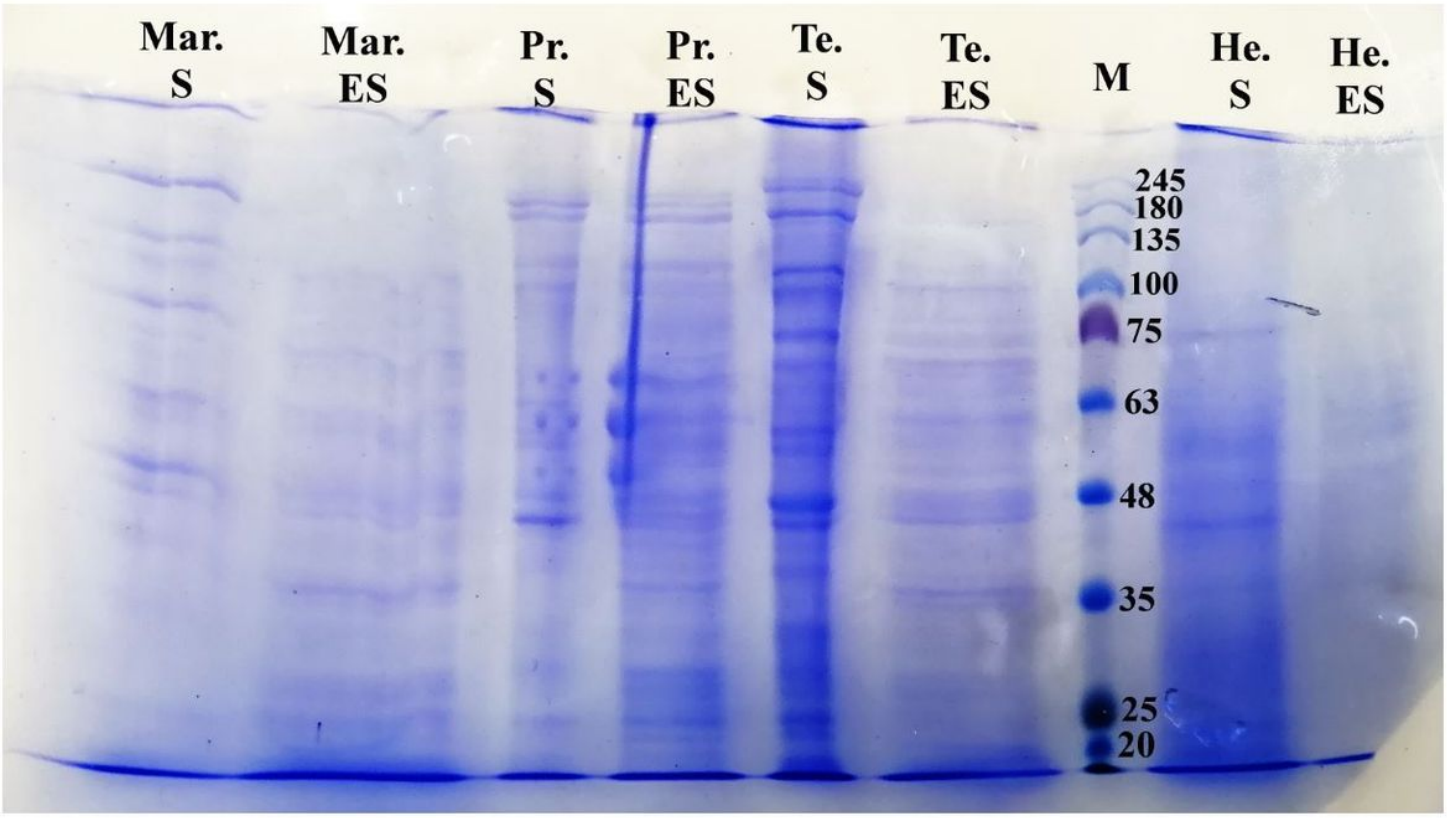

Figure 2

SDS-PAGE analysis of somatic and ES antigens for adult stages of T. circumcincta, H. contortus, P. rufescens and M. marshalli; M: Protein molecular weight marker.

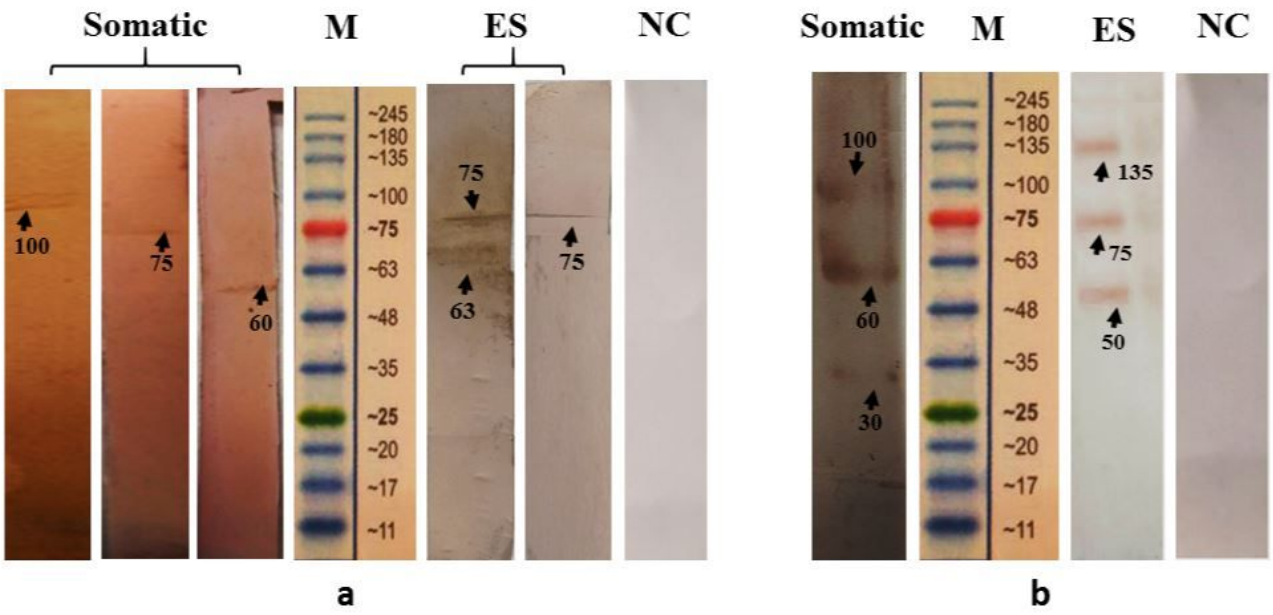

Figure 3

Comparative western blot analysis of immunodominant proteins in somatic and ES content reactive to sera from the T. circumcincta infected sheep (A) and from hyper immune sera raised in rabbits (B). NC: non- immunized rabbit as negative control; M: Protein molecular weight marker.

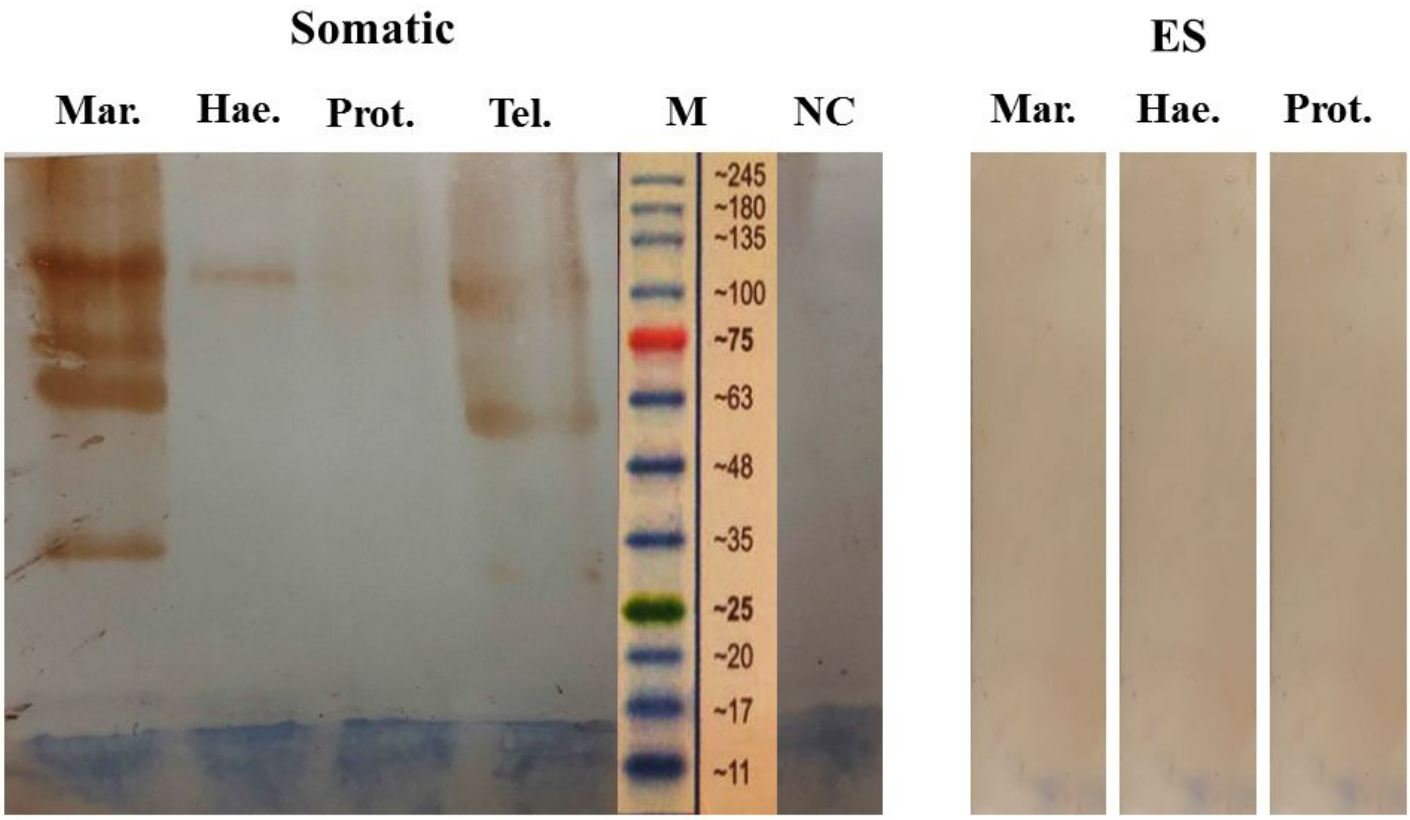

Figure 4

Western blot analysis. Cross reactivity of rabbit hyper immune sera against somatic and ES antigens of P. rufescens, H. contortus and M. marshalli. NC: non- immunized rabbit as negative control; M: Protein molecular weight marker.

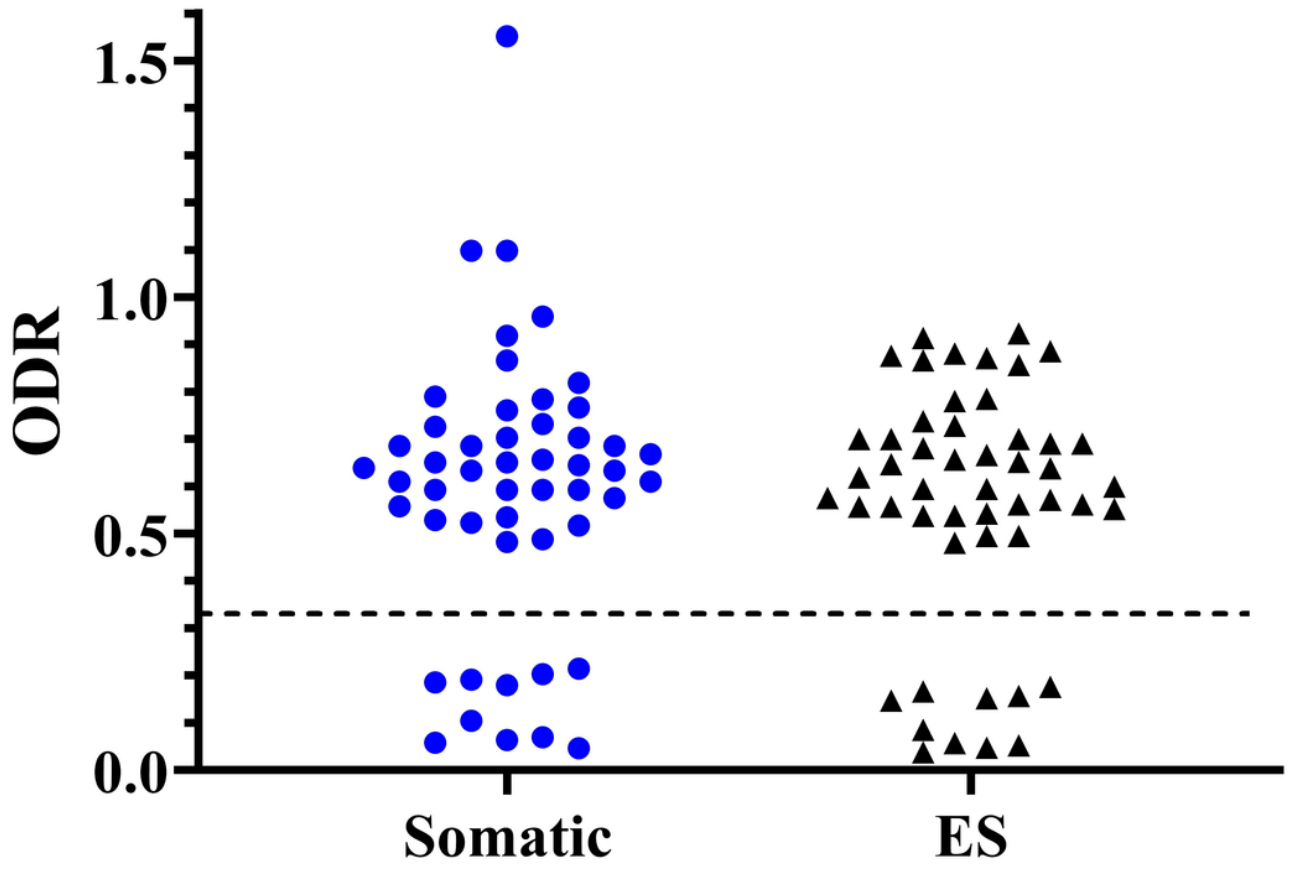

Figure 5

Optical density ratio (ODR) values obtained from sera samples of positive (T. circumcincta infected) and negative (non-infected) controls (NC) against somatic and ES products. The dashed line shows the approximate value of estimated cut-off value. Samples under the line are representative of negative controls and those above the line relates to positive ones.

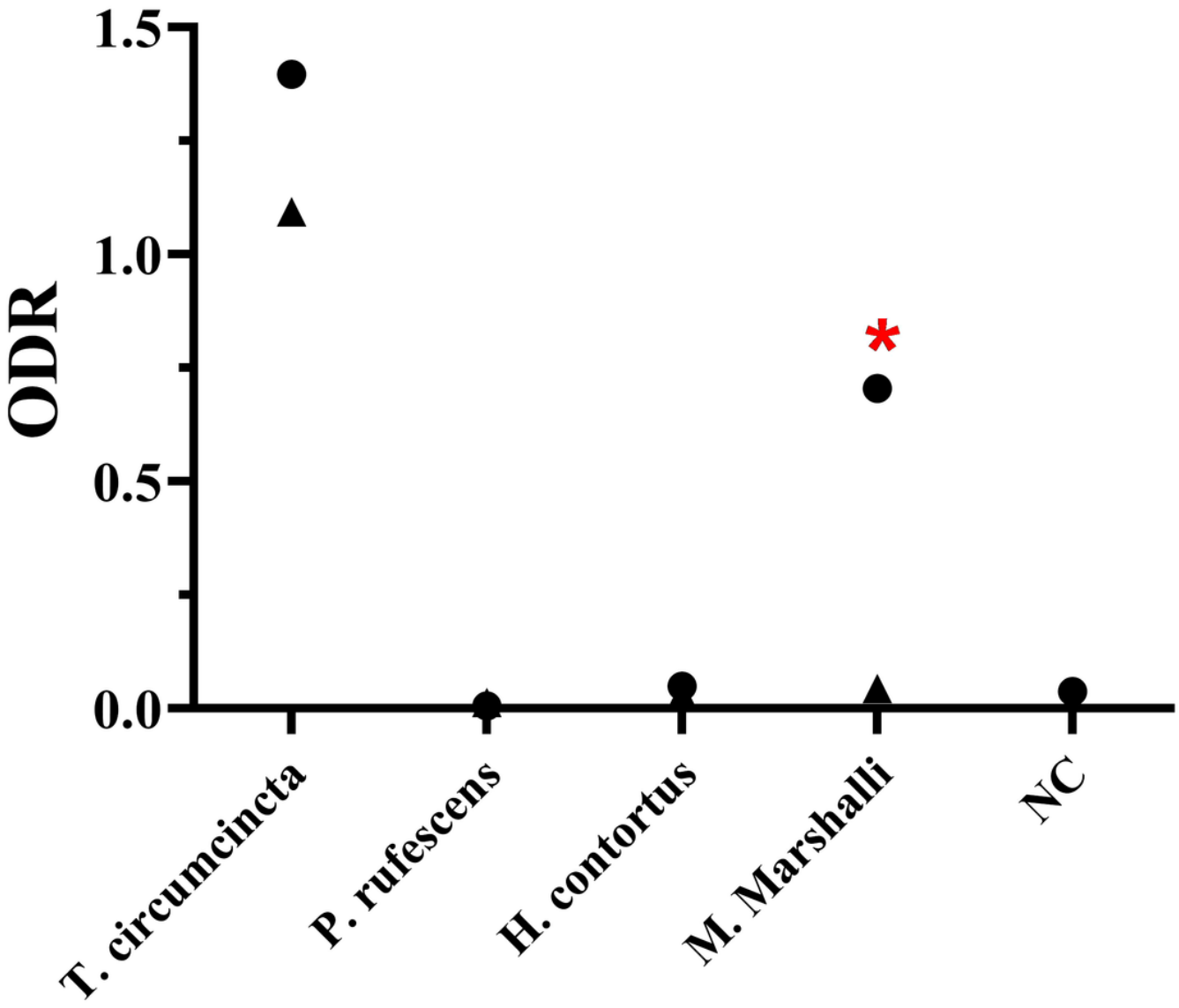

Figure 6

Cross-reactivity by ELISA method. The optical density ratio (ODR) values obtained from rabbit hyperimmune sera against somatic and ES of different prevalent worms.