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ORIGINAL RESEARCH ARTICLE

A preliminary study on the protein profile of *Marshallagia marshalli*

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Abstract. Trichostrongyloid nematodes are common and pathogenic in small ruminants. *Marshallagia marshalli,* belonging to sub-family Ostertagiinae, is currently one of the major cause of parasitic gastroenteritis in Iran. This nematode may be a circumscription on the growth and productivity of sheep. Drug resistance in this species is rising and alternative methods are essential for its control. While the helminths products are important in term of evasion strategies, penetration into host, feeding and reproduction, little is known about protein profiles of *M. marshalli.* This study was designed to characterize protein profiles of *M. marshalli* by SDS-PAGE and to find a condition for maintenance of adult worms for culture and preparation of excretory/secretory (E/S) products.

Adult worms of *M. marshalli* were collected from the abomasum and were washed several times in PBS. Then living and highly motile worms were incubated in medium culture under 37°C, 5% co_2 and humid air. The medium supernatants were collected and used as ES products. Preparation of somatic products were performed by homogenizing of worms in PBS. Proteins of ES and somatic products of *M. marshalli* were separated by a well-defined SDS-PAGE.

SDS-PAGE profile of ES and somatic products of *M. marshalli* revealed several protein bands from 20 to 120 kDa. Bands at 20 and 28 were prominent proteins in electrophoretic patterns of both ES and somatic products. Survival and motility of *M. marshalli* were maintained for 2 weeks, whereas adult female worms were able to lay eggs for only 72 hours after incubation.

This study provide a basic information about protein profiles and maintenance of *M. marshalli.* Further studies are needed to identify biological function of proteins and usage of them in control programs, serologic monitoring of infected sheep and therapeutic targets for immune-mediated diseases.

Keywords: Excretory/secretory (E/S) products; *Marshallagia marshalli;* Somatic Products; SDS-PAGE.

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Introduction

Nematodes species of the super-family Trichostrongyloidea are known to be important pathogens of domestic ruminants. Classification of the Ostertagiinae at the genus level recommended numerous genera for the ten species known sofar. The most commonly known of them are *Ostertagia, Teladorsagia* and *Marshallagia*. A diagnostic key for identification of subfamily Ostertagiinae is presented according to bursa formula. Species characterization by bursa formula describes nematodes that are characterized by a bursa formula of 2-2-1 *(Teladorsagia),* and those which are described by a 2-1-2 pattern of lateral rays *(Marshallagia, Ostertagia). M. marshalli* (major species) and *Ostertagia occidentalis* (minor species) should be included in the genus *Marshallagia* (Lichtenfels et al., 1988).

M. marshalli is a pathogenic parasitic nematode that inhabits in the abomasum of small ruminants. This nematode has a direct life cycle that is similar to other species of Ostertagiinae nematodes. The infective L3 larvae are ingested by feco-oral transmission and penetrate into the abomasal glands. They undergo two developmental molts before they emerge back into the lumen as adult male and female worms. This parasite is currently one of the major cause of parasitic gastroenteritis in Iran (Borji et al., 2010; Eslami et al., 1979; Nabavi et al., 2011). Although the economic loss caused by *M. marshalli* infection has not been estimated, this nematode may be a constraint on the growth and productivity of sheep. *M. marshalli* reduces the abomasum acidity and increases pH and serum pepsinogen that causes pathological change (Moradpour et al., 2013). Similar results are reported with other abomasal nematodes such as *Teladorsagia circumcincta* (Scott et al., 2000), *Ostertagia ostertagi* (Fox et al., 1993) and *Heamonchus contortus* (Simpson et al., 1997).

During each stage of the developmental cycle in the super-family Trichostrongyloidea, a variety of molecules are released into host that have been known as Excretory-Secretory (ES) products.

With respect to the various biological functions of helminth products such as immune evasion (Hewitson et al., 2009) penetration into host (De Cock et al., 1993; Geldhof et al., 2000), reproduction (Haffner et al., 1998) and parasite feeding (Karanu et al., 1993), characterization of these products can provide new insights into the development of control programs with a focus on vaccination. Furthermore, recent studies have revealed the benefits of helminth products in realizing of host-parasite interaction and treatment of immune-mediated diseases (McSorley et al., 2015; Rosche et al., 2013).

Based on our knowledge there is little information about protein profile of ES and somatic products of *M. marshalli*. This study was conducted to determine protein profiling of *M. marshalli* and to design a condition for *in vitro* culture of this nematode. The identification of ES and somatic products of *M. marshalli* will provide a basis for further studies on the recognition of immunogenic molecules for use in serological monitoring, molecules that are involved in helminth immune evasion strategies and identification of biological function of ES and somatic products. These products could be a source of therapeutic targets and potential vaccine components.

Material and methods

Parasites

Adult *M. marshalli* worms were obtained from abomasa of necropsied sheep in the slaughter house.

Isolated worms were placed in a petri dish with PBS and were identified according to morphological characteristics under a light microscope (Lichtenfels et al., 1988). The worms were washed several times in PBS and those who did not show any kind of external or internal damage were isolated.

Preparation of ES products

ES products were prepared by incubating of living and highly motile worms in serum free DMEM medium supplemented with 4.5g/l D-

glucose, 2mML-glutamine, 25mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml gentamicin (Bio-Idea, Iran). The worms were cultured at a concentration of approximately 1000 worms per 5 ml in a culture flask at 37°C, 5%CO2 and 95% humidity. The medium was changed every 48 h after incubation and fresh medium was added. Worm viability and contamination were monitored through the incubation period. After incubation, the medium supernatants were collected and centrifuged at low speed to discard larger debris and eggs. The supernatants were sterilized by passing through 0.2 µm filter. Pool *M. marshalli* ES were concentrated over a 3,000 MWCO Amicon membrane.

Preparation of somatic products

In a similar way to ES preparation, adult worms were collected from abomasum and strongly washed in PBS. Worms were homogenized in PBS with a sonicatore while on ice, then were centrifuged at 4000g for 10 min at 4°C. Sterilization of the supernatants was performed by 0.2 µm filter.

Protein determination

Protein concentration was measured by Bradford assay as previously described by Harlow and lane (Harlow and Lane, 2005) (Bio-Rad, Hercules, CA, USA) and aliquots were stored in -80°C until use.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Both ES and somatic products of *M. marshalli* were analyzed by 12.5 % SDS-PAGE according to the method of Laemmli (Laemmli, 1970). Each sample was mixed with loading buffer (0.05 M Tris, pH 6.8, containing 5% (w/v) SDS, 20% (v/v) glycerol, 0·01% (w/v) bromophenol blue and 10 mM DTT) and was heated in boiling water for 5-10 min. Prepared samples were loaded into wells and the gel was run at an appropriate voltage. The separated proteins by electrophoretic technique were visualized with silver staining. For this purpose, the gel was placed in a Petri dish containing staining solution and the staining was done at room temperature for 2 hours. De-staining was carried out to remove the excess stain. The molecule weight of protein bands of parasite were compared with known molecular weight marker.

Results

Survival and motility maintenance of M. marshalli

Survival and motility of *M. marshalli* were monitored daily and dying parasite were removed to prevent of ES contamination by proteins released from dying worms. The medium supernatants were collected to estimate viability and protein concentration of ES products in different weeks of culture. Survival of parasites under 5% CO_2, humid air and 37°C was maintained for 3 weeks. In the first culture all worms displayed motility similar to that observed at collection. Although the worms were highly motile in 4, 6 and 8 days after incubation, they were less active than their first culture. In the other days worms were motile but sluggish. At the end of second week some worms moved when stimulated by touching with pipette and some worms did not response when touched with a pipette. Adult female worms were able to lay eggs for only 72 hours after incubation in culture media. In this condition no eggs hatched to L1 in the culture medium. The presence of CO_2 was critical to survival, as when placed in a 0% CO_2 worms did not survive longer than 24 h.

Protein concentration of ES and somatic

The culture media supernatants were collected at 2 day interval. At the end of each week protein concentration was calculated by Bradford assay. Protein concentration of adult worms cultured in the first week was similar to that cultured in subsequent week.

Characteristics of ES and somatic products

The protein profiles of ES and somatic extracts of *M. marshalli* are shown in figure 1. ES products were containing a less number of proteins compared to somatic extract. SDS-PAGE analysis of somatic extracts of

M. marshalli revealed a pattern of >17 distinct bands with molecular weights ranging from 20 to 120 kDa, while the ES profile had fewer bands. The prominent somatic protein bands were presented at 20, 22, 25, 27, 28, 35 and 37 kDa. The electrophoretic pattern of ES products showed one prominent band at 50 kDa.

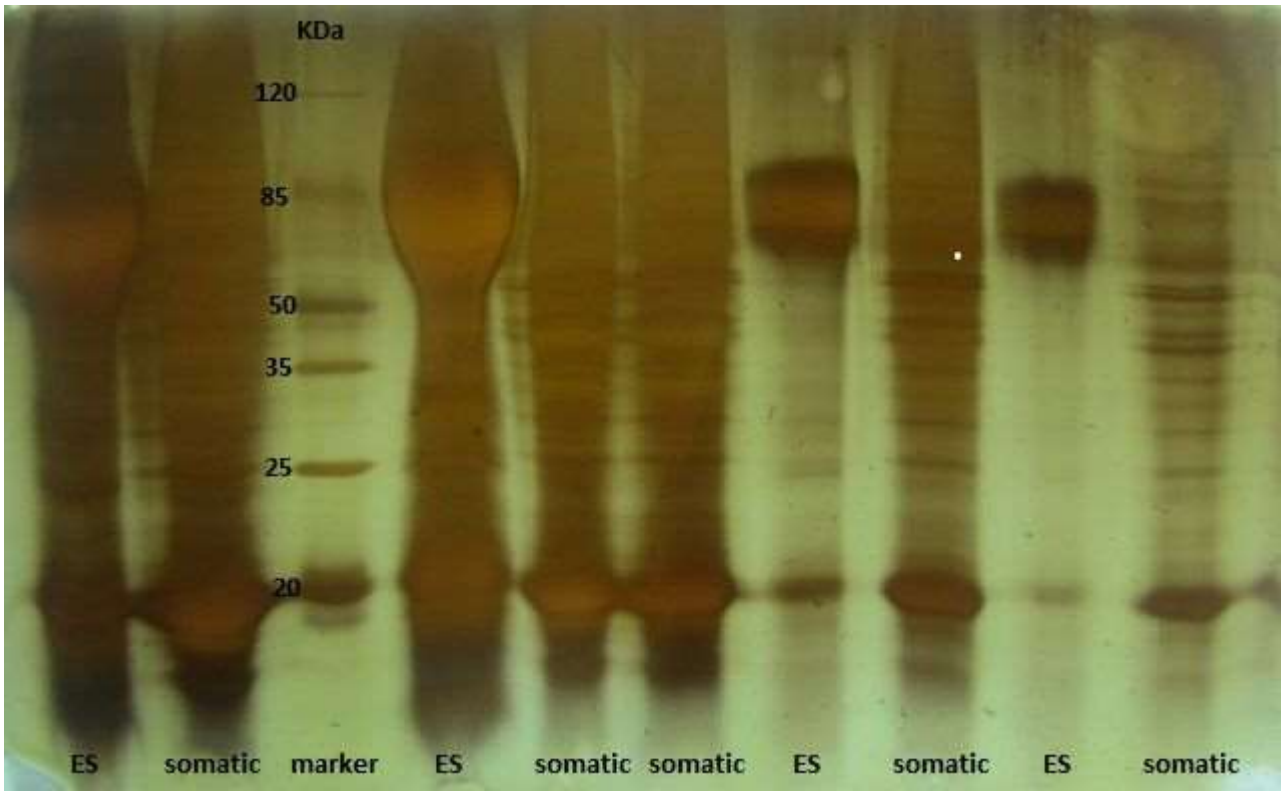

Figure 1. Protein profiles of ES and somatic extracts of *M. marshalli*

Discussion

This study was carried out to identify the protein profile of somatic and ES products of *M.marshalli.* With respect to the nature of helminth product effects on the host immune system, defining of the protein presented in helminth products by SDS-PAGE can provide a basic information for further investigation in term of identification of novel targets for control infection with a focus on vaccination and biological function of ES and somatic products. It is established that helminth products possess immunomodulatory molecules that could be effective in treatment of immune-mediated diseases such as allergic asthma (McSorley et al., 2015) and autoimmune diseases (Correale and Farez, 2013; Ruyssers et al., 2009). Maintenance of adult worms *in vitro* for a long period of time provides an available source for ES and adult worms. In this study, a condition for maintenance of adult *M. marshalli* in culture media was illustrated to provide an available source for ES products and adult worms.

This study showed that DMEM media under 5% CO_2, 37°C and 90% humidity could be used to preserve *Marshallagia marshalli* for a long term peroid. Although a number of *Marshallagia marshalli* maintained for 21 days, the majority of them survived for 14 days. Luque and coworkers evaluated different conditions for maintaining adult *T. circumcincta* in culture and the optimum condition was obtained by co-culture of the HeLa cell line with adult worms and the longest period of *T. circumcincta* survival was reported 14 days in these conditions (Luque et al., 2010). However, in this study the effects of different media and co-culture with cell line were not evaluated. Further investigations are needed to find optimum condition for

maintaining adult *M.marshalli* more than 14 days in culture.

The protein electrophoresis of *M. marshalli* ES products yielded protein bands with molecular weights ranging from 20 to 120 kDa.

Somatic extract showed a greater number of protein bands as compared to ES proteins. Similar pattern has been observed in the adult worms of *O. ostertagi,* another nematode belonging to Ostertaginae subfamily (Vercauteren et al., 2003).

M. marshalli ES proteins showed a prominent band at 50 kDa. Proteomics analysis should be performed to determine biological function of this protein. This protein may be similar to band seen in adult *T. circumcincta.* The protein at 50 kDa in *T. circumcincta* was homologues of *C. elegans* enolase, a metabolic enzyme that is involved in glycolysis (Craig et al., 2006). In contrast, *O. ostertagi* revealed a prominent band around 35 kDa that strongly reacted with the rabbit serum antibodies. Cross-reactivity of the anti-ES sera with somatic products of adult *O. ostertagi* showed a similar recognition patterns of ES products (Vercauteren et al., 2003). ES products of *M. marshalli* also revealed a weak protein band around 35 kDa.

Another protein isolated from *M. marshalli* ES at 20 kDa was also identified in *T. circumcincta* ES. This protein was the most prominent protein in *T. circumcincta* ES and was a homologue of a globin-like ES protein from *O. ostertagi* (Craig et al., 2006). Globin-like proteins are commonly associated with oxygen transport (Vinogradov et al., 2007). Furthermore, it has been known that parasitic nematodes contain globin proteins (Blaxter, 1993).

Fractionation by SDS-PAGE of somatic proteins of *M. marshalli* yielded a more complex profile varying from 20 to 120 kDa. The protein bands of 20, 22, 25, 27, 28, 35 and 37 seem to be prominent proteins. In our study, bands at 20 and 28 kDa were common proteins in electrophoretic patterns of both ES and somatic products. Protein bands at 29, 25 and 22 were also prominent in PAGE analysis of crude Extract of *Heamonchus longistipes,* a nematode belonging to Trichostrongyloidea from abomasum of camels (Hassan and El-Bahar, 2012). Protein bands of around 20, 28 and 35 kDa were also present in the somatic extract from third-stage infective larvae of *O. ostertagi* (Baker and Gershwin, 1993). However, it should be noted that, the size similarities of protein bands between different nematodes does not correlate with their biological functions. Detailed proteomic analysis of somatic and ES proteins of *M.marshalli* will shed more light on their biological functions.

In conclusion, in the present study for the first time, we identified the protein patterns of *M.marshalli* ES and somatic products by SDS-PAGE. Further studies should be performed to identify homologous sequence of *M. marshalli* proteins by proteomic analysis.

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