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Identification of antigenic proteins of *Setaria labiatopapillosa* in cattle (*Bos taurus*)

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Abstract

Setaria labiatopapillosa is one of the parasitic nematodes found in bovines, which causes verminous encephalitis in non-specific hosts. This study aimed to determine the protein and antigenic profiles of *S. labiatopapillosa* in cattle using immunoblotting. The samples were taken from cattle at Ahvaz slaughterhouse. Somatic crude extracts of adult and microfilariae of the nematode (as S and MF proteins or antigens) were prepared separately by homogenization. Excretory secretory products (ES) were prepared by incubating the adult nematode in RPMI medium. Protein profiles and antigenic properties of ES, S, and MF samples of *S. labiatopapillosa* were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunogenic properties of the proteins were determined using immunoblotting and positive sera from cattle infected with the adult nematode. More than two, 8, and 15 predominant protein bands at 200 to 22 kDa were demonstrated in SDS-PAGE gels of ES, MF, and S samples. Consequently, in immunoblotting of ES, MF, and S, two, 14, and 4 predominant ES antigenic bands were demonstrated; among them, the 68- and 64-kDa proteins were the most common antigenic components. The results of immunoblotting of ES, MF, and S of *S. labiatopapillosa* showed that the 68- and 64-kDa proteins were the most common antigenic components, and can be potential candidates for future studies.

Key words: Antigens, Bovine, Immunoblotting, *Setaria labiatopapillosa,*

Introduction

The parasitic nematode *Setaria labiatopapillosa* is a causative agent of verminous encephalitis and an important member of the Onchocercidae family that inhabits the peritoneal cavity of bovids (Kuts et al, 2012; Singh et al, 2015).

Setaria worms have indirect life cycles that have not yet been fully elucidated. Various mosquito species (*Aedes* spp, *Anopheles* spp, *Culex* spp and *Armigeres* spp) are the main intermediate hosts. The adults are commonly found in the abdominal cavity of definitive hosts, domestic and wild animals (Anderson, 2000; Cancrini et al, 1997).

Generally, the presence of *Setaria* adult worms in the peritoneal cavity of infected hosts is not considered pathogenic, but their

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migration to various organs, particularly the eyes, liver, brain, heart, etc., can cause significant harm (Shin et al, 2017; Sunder et al, 2005).

Ocular Setariosis can cause blurred vision and even blindness in the host. The particular form of cerebrospinal infection is caused by migration of parasitic infective microfilariae (third stage larvae) in the nervous system of non-specific hosts (such as sheep, goats and horses) as well as non-infective microfilariae in specific hosts.

Due to serious threat of setariasis to livestock in endemic areas, it is of particular importance in veterinary medicine (Bazargani et al, 2008; Mahmoud et al, 2004; Tung et al, 2003; Wang et al, 1986).

Detection of the parasite infection is only concluded by microscopic observation of the parasite microfilariae on blood smears of infected animals.

However, most reports on the infection in animals have come from postmortem examinations or abattoir inspections, which have mostly isolated adult nematodes from thoracic or abdominal cavities of the host animals (Khedri et al, 2014; Kim et al, 2010; Mrifag et al, 2021; Nakano et al, 2007; Rodrigues et al, 2021).

The control of vector mosquitoes is an important part of preventing or at least reducing the infection of the hosts (cattle) although it is difficult due to the behavior and extent of their intermediate mosquito hosts (Cancrini et al, 1997; Laaksonen et al, 2009; Karunamoorthi and Sabesan, 2013).

A thorough understanding of pathogenic agents in the region is essential for diagnosis, control, and prevention.

A more practical and important measure for controlling the disease is to identify and treat infected animals.

In our previous study, using molecular and scanning electron microscopy techniques, *S. labiatopapillosa* was the only species of *Setaria* isolated from calves in southwestern Iran (Alborzi et al, 2019).

Identifying and assessing the antigenic properties of somatic and excretory-secretory proteins of parasites exposed to the host's immune response is crucial for serological diagnostic studies and the development of preventive vaccines.

Moreover, excretory-secretory products can be one of the targets of immune control against parasites due to their important role in parasite biology (Richer et al, 1992; Ring et al, 1993).

Considerable studies have been conducted on excretory-secretory and somatic antigens of various parasites (Alborzi et al, 2006; Alborzi et al, 2014; Alborzi et al, 2016; Alborzi et al, 2022; Razi Jalali et al, 2014; Rashidi et al, 2018; Virginio et al, 2012)

A greater number of studies have identified antigens in excretory-secretory products and somatic extracts of some *Setaria* species to detect human filariasis, but fewer have focused on the diagnosis of animal setariasis (Raj et al, 1997; Mohanty et al, 2006; Kaushal et al, 2009).

The present study aimed to characterize the antigens of *S. labiatopapillosa* so that its results could provide a clearer background for future research on the specific immunological diagnosis of setariasis in cattle.

Materials and Methods

Samplings

The samples were taken from animals at the Ahvaz slaughterhouse. The slaughterhouse of Ahvaz is a center for receiving animals from different areas of Khuzestan province. Following the marking of the cattle, two blood samples were collected from each animal, one with an anticoagulant (EDTA) for microfilaria observation and another for separating serum. Modified Knott's technique was used to detect microfilariae of the nematode in the blood (Watanabe et al, 2004).

The abdominal cavity of the slaughtered animal was carefully checked for worms; those that were found were transferred into containers containing phosphate buffered saline (PBS, pH=7.2). After three washes

with serum saline solution, the collected worms were used for culture, antigen preparation, and morphological identification.

Preparation of protein products/ extracts (antigens)

In order to prepare the excretory-secretory products (ES), *S.labiatopapillosa* adults collected from the peritoneal cavity of infected cattle were washed with physiological saline three times.

The nematodes were cultured in a 50 ml flask containing Roswell Park Memorial Institute medium (RPMI-1640, Bahar Afshan, Tehran, Iran) supplemented with penicillin (100 U/ml) and streptomycin (100 mcg/ml).

The culture medium (3 mL/nematode) was changed every 12 hours, and the survival of the worms was observed by examining and observing their movement.

The flask was incubated for 24 hours in 5.0% CO2 at 37°C. Media were collected, centrifuged at 4000g for 4 min, filtered through 0.22m filters, the filtrate (soluble proteins) was then added to sterile falcons as ES, and kept at -20°C for further analyses.

In order to prepare somatic crude extracts of adult and microfilariae collected from the uterine of adult females (as S and MF proteins or antigens), some nematode parts (without uterus) and microfilariae were homogenized separately. The homogenization was performed with a Bandelin homogenizer (Bandelin, Berlin, Germany) using 10 cycles lasting 15 seconds each at a voltage of 80 in 0.2 ml PBS (pH = 7.2). Homogenized solutions were centrifuged at 4000 rpm for 4 minutes at 4 °C, and the supernatants were filtered through 0.22 m filters (Biofil Syringe Filter, Shanghai, China). The refined liquids were collected as S and MF separately and stored at -20 ° C until use. The concentration of proteins in ES, S, and MF solutions was determined by the Bradford method (Bradford, 1976). Positive serum samples were obtained from animals with both adult worms in their peritoneal cavity and microfilariae in their blood.

Electrophoretic analysis

Proteins in ES, S, and MF samples of *S. labiatopapillosa* were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Based on the Laemmeli's method (Laemmeli,1970), it was performed in an electrophoresis apparatus (Paya Pajoohesh Pars, Tehran, Iran) with 12.0% separating gels (6 ml of 30% acryl amide + bis, 3.8 ml of 1M Tris-HCl pH 8.8, 150 µl of 10% (w/v) SDS, 150 µl of 10% Ammonium persulfate, 6 µl of TEMED [(Tetramethylethylenediamine) and 4.9 ml deionized water (DH2O)] and 5% stacking gels (1.3 ml of 30% acryl amide + bis, 1ml of 1M Tris-HCl pH 6.8, 80 µl of 10% SDS, 80 µl of 10% Ammonium persulfate, 8 µl of TEMED and 5.5 ml DH2O.

Before electrophoresis, 60 µl of each sample (ES, S, and MF) were mixed with 30 µl of non-reducing buffer (1 ml 1 M Tris-HCl (pH 6.8), 5 ml of ddH20, 2ml 10% SDS, 5 mg Bromophenol Blue, 1 ml 100% glycerol, 1 ml β-mercaptoethanol) and boiled for 5 min then centrifuged for 2 min. Unstained Protein Markers (AbCam) ranging from 254 to 17 kDa were used in 5 µl in 10 µl sample buffers as molecular weight standards.

Electrophoresis was run at 100 V for 5 hr. under reducing conditions. The gels were stained with Coomassie Brillant Blue R-250 according to the manufacturer's instructions and silver staining as follows: After electrophoresis, gels were fixed for 1 hr. in an appropriate volume of fixing solution (50 ml ethanol, 2 ml acetic acid and 448 ml DH2O).

After discarding the fixing solution, the gel was stained in silver staining solution (0.1% (w/v) silver nitrate) for 15 min. The gels were rinsed in deionized water twice for 30 sec. and then in the developing

solution (3% (w/v) sodium carbonate and 0.04% formalin).

Next, the gels were washed in deionized water. In the final step, the reactions terminated by adding 5% acetic acid.

Immunoblotting

For immunoblotting, proteins of ES, S, and MF samples of *S. labiatopapillosa* were first electrophoresed on 12 % SDS-polyacrylamide gel. Western blotting was carried out following established protocols (Towbin et al, 1979). Proteins were transferred onto a nitrocellulose membrane using Tris-glycine-methanol buffer for 3 hr. at 60 V.

The membrane was blocked in 5% skim milk in PBS for overnight at -4 ºC temperature (refrigerator).

After rinsing 3 times (each 5 min) in the washing buffer (PBS-T: PBS,0.05% Tween20) and once in PBS.

Sera (10 positive and 10 negative) were diluted (1:4, 1:5, 1:8, 1:10, 1:16, 1:20, 1:32, 1:40 and 1:80) in PBS-T buffer and the membranes incubated with stirring at room temperature (RT) for 1 hr. and again washed as above.

After 4 repetitions, 1:8 dilution of S, 1:10 dilution of MF, and 1:4 dilutions of ES were determined as the most proper dilutions.

the membrane was cut in strips and incubated with positive and negative sera and with stirring, for 1 hr. at 37 ºC and then washed as above.

The strips were then incubated with secondary antibody (anti-Bovine Anti-IgG horseradish peroxidase conjugated antibody Sigma- Aldrich, USA) diluted with PBS-T (1:1000) with stirring, for 1 hr. at 37 ºC, after washing, developed in a mixture of 4-cloronapthol and hydrogen peroxide for 15 min.

Results

Protein profiles of ES, S, and MF samples of *S. labiatopapillosa*

Excretory- secretory products of cultured adults of *S. labiatopapillosa* (ES), somatic extracts of adults (S) and microfilariae (MF) were analyzed by SDS-PAGE and the gels were stained with Coomassie Brillant Blue R-250 and a silver staining method. As shown in Figure 1, silver-stained gel of ES revealed the presence of more than six protein bands ranging in molecular weight from 250 to 17 kilodaltons (KDa). However, the predominant ES protein bands were at 74, 68, 64, 51, 38, and 22 KDa. In Coomassie –stained gel of S, there were more than 15 protein bands with weights of approximately 200, 133, 107, 91, 80, 74, 68, 64, 60, 51, 48, 40, 38, 22 and several bands above 245 kDa were more predominant (Figure 2). Protein dominants in gel of MF were 8 bands including 124, 107, 96, 68, 64, 48, 31 and 22 kDa (Figure 3).

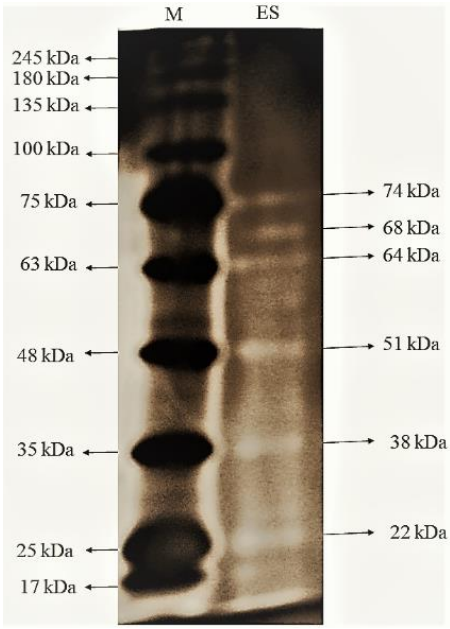

Figure 1: Protein profile on a silver-stained SDS-PAGE gel of excretory-secretory-products (ES) from *S. labiatopapillosa*, Molecular weight marker (M).

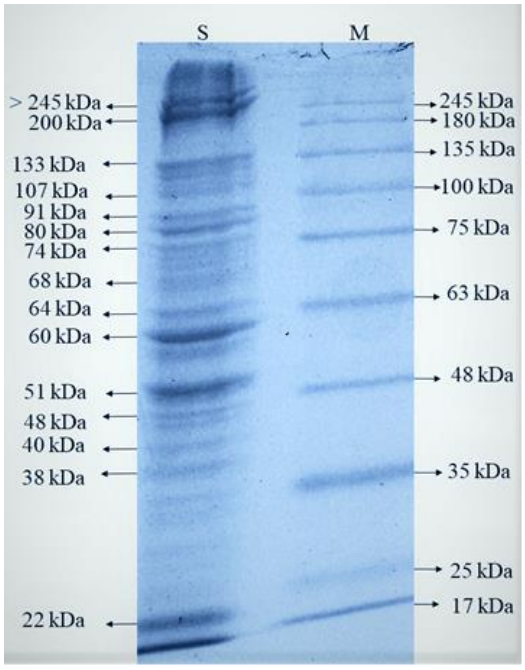

Figure 2: Protein profile on a Coomassie-stained SDS-PAGE gel of somatic extract (S) of *S. labiatopapillosa***, Molecular weight marker (M).**

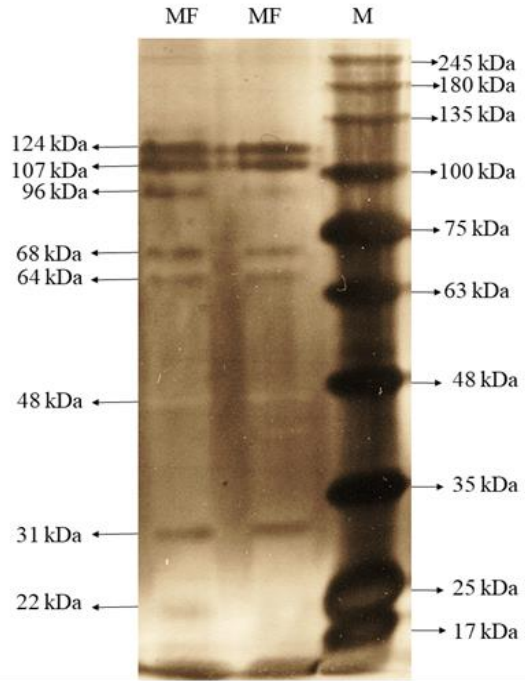

Figure 3: Protein profile on a silver-stained SDS-PAGE gel of somatic extract of microfilariae of *S. labiatopapillosa* **(MF), Molecular weight marker (M).**

Immunoblotting

Analysis of electrophoretic patterns in immunoblotting of *S. labiatopapillosa* ES, S, and MF with dilutions: 1:5, 1:8, and 1:10 of sera respectively of naturally infected and 1:1000 dilution of anti-bovine IgG conjugate, positive reactions (as visible bands) were obtained.

The results demonstrated that the predominant ES antigenic bands were two bands at 68 and 64 kDa (Figure 4). The somatic extracts (S) of 4 antigenic bands were recognized, including bands of 68, 64, 60, and 56 kDa (Figure 5). In somatic extracts of microfilariae(MF) 14 predominant antigenic bands including bands of 182, 157, 133, 124, 107, 102, 88, 74, 68, 64, 60, 51, 41, and 38 (Figure 6) were observed. Among the protein profiles, the 68 and 64 kDa protein was the most common antigenic component.

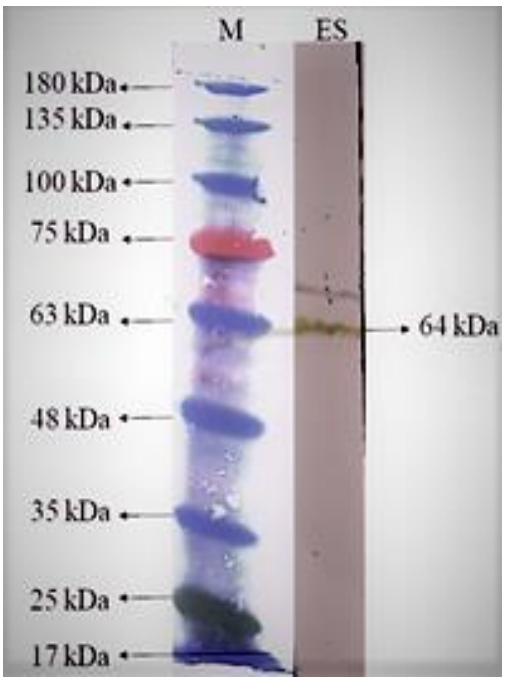

Figure 4: Immunoblotting of excretory-secretory antigens of *S. labiatopapillosa* **(ES), with sera of naturally infected cattle. Molecular weight marker (M).**

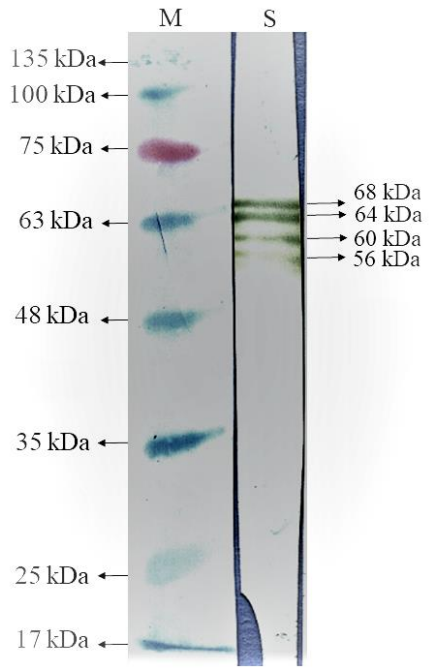

Figure 5: Immunoblotting of somatic antigens of *S. labiatopapillosa* (S), with sera of naturally infected cattle. Molecular weight marker (M).

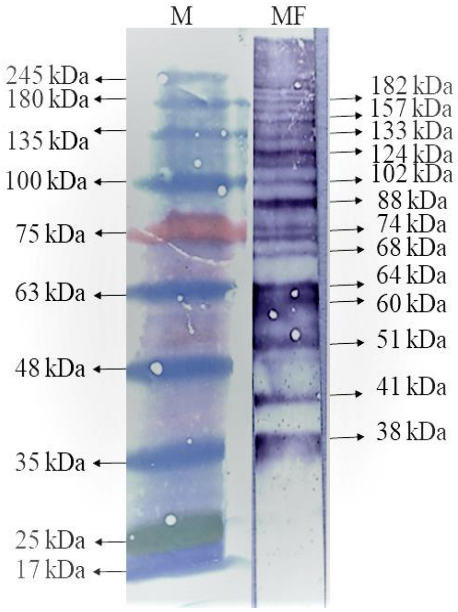

Figure 6: Immunoblotting of somatic antigens of *S. labiatopapillosa* microfilariae (MF), with sera of naturally infected cattle. Molecular weight marker (M).

Discussion

In this study, preparations of excretory products (ES) and crude somatic extracts from *S. labiatopapillosa* adults and microfilariae (as S and MF proteins), and positive serum samples from the nematode-infected animals were obtained.

The antigenic profile was determined in excretory-secretory, somatic extracts of adult and microfilariae of *S. labiatopapillosa* females using SDS-PAGE and immunoblot methods.

Immunoblotting of ES, MF and S proteins demonstrated two, 14 and 4 predominant ES antigenic bands.

The presence of immunogenic proteins indicates stimulation of the host immune system at different stages of the parasite infection and the production of humoral antibodies, especially systemic IgG.

Among the protein profiles, the 68 and 64 kDa protein was the most common antigenic component.

It appears that these proteins are expressed at all stages of the parasite development in the host.

Although, in the protein profiles, the number of adult *S. labiatopapillosa* proteins (bands) was much higher than that of microfilariae, in the immunoblotting results, more immunogenic proteins were observed with microfilaria extract (MF) than with adult ES and somatic extract(S).

It appears that the high number of immunogenic proteins found in microfilaria extracts results from their entry into the blood stream, possible death, and exposure of the released proteins to the immune system of the host, as well as their secretory excretory proteins.

While the mature worms are primarily present in the abdominal (peritoneal) cavity of their hosts, adult female worms release microfilariae in the abdominal cavity. These microfilariae get into the blood stream. Most studies on the protein and immunogenicity characteristics of some *Setaria* species, including *S. digitata*, have been conducted on its both specific host (cattle) and non-specific host (horse). These studies have shown that antigens from these animal nematodes can be used in the diagnosis of filarial nematode infections in animals and even in humans. However,

there are very few reports on *S. labiatopapillosa* (Mohanty et al, 2006; Takesue, et al, 2016).

Berezhko et al, (2016) demonstrated that *S. labiatopapillosa* can be used as an antigenic source for diagnosing dirofilariasis (*Dirofilaria immitis*) in dogs. According to Bright and Raj, (1991), SDS-PAGE analysis of adult *S digitata* surface antigen preparations (SAPs) revealed three major proteins with molecular weights 17, 29 and 36 KD, and all SAPs were antigenic. The release of 29 KD surface protein from in vitro culture of the adult and cross-reactivity between it and antisera to surface antigens revealed the possibility of natural surface shedding (Bright et al, 1991). The cross-reactivity of filarial species has made access to heteroantigens to detect infection in humans. Dissanayake and Ismail (1980) isolated several antigenic components from *Setaria digitata* extract, and then showed that they cross-reacted with surface antigens from microfilarae of *Wuchereria bancrofti*, a filarial nematode that is the main cause of lymphatic filariasis in human. Immunoelectrophoretic analysis of ES products of *Setaria cervi*, a bovine filarial parasite, in RPMI-1640 medium which revealed 11-15 antigens most of were common to somatic antigenic preparations from both adult worms and microfilariae (Malhotra et al, 1987).

Beuria and Das, (1995) isolated a serine protease of 30 kDa from the extract of *S. digitata* and investigated the immediate hypersensitivity reaction in lymphatic filariasis patients. Dalai, et al, (1998) showed that *S. digitata* adult 14- to 20-kDa antigens induce differential Th1 and Th2 cytokine responses in the lymphocytes of endemic normals and asymptomatic microfilariae carriers in bancroftian filariasis. Interestingly, Sharma, et al, (1998) found that *S. cervi* microfilariae secrete acetyl cholinesterase, which has antigenic cross-reactivity with *Wuchereria bancrofti*. A study by Subhachalat et al, (1999a) differentiated *S. digitata* and *Setaria marshali* based on their antigenic profiles and found that *S. marshali* contained a 69 kDa protein different from *S. digitata* (Subhachalat et al, 1999a). Further investigation of the protein by two-dimensional gel electrophoresis revealed a spot ranging from 64 to 73 kDa in *S. marshalli* (Subhachalat et al, 1997b). There is a high level of anti-*Setaria* antibodies in cattle infected with microfilariae or adult worms as well as apparently uninfected cattle. It may be indicative of the similarity between human and bovine anti-microfilaria immune responses.

Sasisekhar et al, (2005) identified two proteins of 34 and 64 kDa in the crude protein extract of *S. digitata*. It was found that these proteins were sheath proteins located in the parasite's hypodermal region. As a result of the investigation by Pokharel et al, (2006a, b) a collagenase and leucine aminopeptidase of 175 kDa have been isolated from *Setaria cervi* showing their potential to be used as vaccines or diagnostic markers for *Brugia malayi* infection, human lymphatic filariasis. Madathiparambil et al, (2009) isolated a 200 kDa glycoprotein from the surface pores of *S. digitata* male and female worms. In SDS-PAGE analysis, several proteins ranging from 11 to 66 kDa were identified in this glycoprotein. According to the findings of this study, the glycoprotein (200kDa) can be a strong candidate for antigen-based immunodiagnosis of filariasis in humans.

Due to the stimulation of the host immune system by different stages of the parasite and the presence of anti-nematode antibodies, especially IgG, in the serum of infected animals, antibody detection immunological methods can be used to detect infected animals.

Results of immunoblotting of ES, MF and S of *S. labiatopapillosa*, the 68 and 64 kDa proteins were the most common antigenic components that can be candidate to future studies.

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Conflict of interest

The authors confirm that there is no conflict of interest.

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شناسایی پروتئین‌های آنتی ژنی *ستاریا لابیاتوپاپیلوزا* در گاو (*باس تورس*)

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چکیده

ستاریا لابیاتوپاپیلوزا یکی از نماتدهای انگلی موجود در گاو است که باعث آنسفالیت کرمی در میزبان‌های غیر اختصاصی می‌شود. پروتئین‌های آنتی‌ژنی می‌توانند برای توسعه آزمایش‌های تشخیصی و واکسن‌ها مورد استفاده قرار گیرند. این مطالعه با هدف تعیین پروفایل پروتئینی و آنتی‌ژنی *ستاریا لابیاتوپاپیلوزا* در گاو با استفاده از ایمونوبلاتینگ انجام شد. نمونه‌ها از گاوها در کشتارگاه اهواز گرفته شدند. عصاره‌های خام سوماتیک نماتد بالغ و میکروفیلاریا (به عنوان پروتئین‌ها یا آنتی‌ژن‌های S و MF به طور جداگانه با همگن‌سازی تهیه شدند. مواد دفعی ترشحی (ES) با انکوباسیون ۵ نماتد بالغ در محیط RPMI، (۳ میلی‌لیتر برای هر نماتد) حاوی پنی‌سیلین (۱۰۰ واحد در میلی‌لیتر) و استرپتومایسین (۱۰۰ میکروگرم در میلی‌لیتر) تهیه شدند. پروفایل پروتئینی و خواص آنتی‌ژنی نمونه‌های ES، S و MF نماتد *ستاریا لابیاتوپاپیلوزا* با الکتروفورز ژل پلی‌اکریل‌آمید سدیم دودسیل سولفات (SDS-PAGE) تعیین شد. خواص ایمنی‌زایی پروتئین‌ها با استفاده از ایمونوبلاتینگ و سرم‌های مثبت از گاوهای آلوده به نماتد بالغ تعیین شد. بیش از دو، ۸ و ۱۵ باند پروتئینی غالب در وزن مولکولی ۲۰۰ تا ۲۲ کیلو دالتون در ژل‌های SDS-PAGE نمونه‌های ES، MF و S نشان داده شد. در نتیجه، در ایمونوبلاتینگ ES، MF و S به ترتیب دو، ۱۴ و ۴ باند آنتی‌ژنی غالب مشاهده شد. نتایج ایمونوبلاتینگ ES، MF و S در *ستاریا لابیاتوپاپیلوزا* نشان داد که پروتئین‌های ۶۸ و ۶۴ کیلو دالتونی رایج‌ترین اجزای آنتی‌ژنی هستند که می‌توانند کاندیداهای بالقوه‌ای برای تحقیقات آینده در مورد تشخیص ایمونولوژیکی اختصاصی ستاریازیس گاوی باشند.

کلمات کلیدی: آنتی‌ژن‌ها، نمایه‌های آنتی‌ژنی، گاو، ایمونوبلاتینگ، *ستاریا لابیاتوپاپیلوزا*

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