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Original article

CYTOKINES AND IMMUNOGLOBULIN G RESPONSE IN DONKEYS WITH SPONTANEOUS *SETARIA EQUINA* INFECTION

A. H. EL NAMAKY, S. H. HENDAWY, F. A. ABO-AZIZA & H. M. ASHRY

Department of Parasitology and Animal Diseases, National Research Centre, Giza, Egypt

Summary

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Setaria equina (*S. equina*) is a filarial worm that exists in peritoneal cavity of equines. This study aimed to evaluate cytokine mediators tumour necrosis factor alpha (TNF-α), interleukin-4 (IL-4) and immunoglobulin G (IgG) responses in spontaneously *S. equina* infected and non-infected donkeys with emphasis on choosing the best antigen that could be used in diagnosis of such filarial infection. A total of 87 donkeys were examined. Two *S. equin*a antigens: crude somatic *S. equin*a antigen (CSS) and excretory secretory *S. equina* antigen (ESS) were prepared. They were evaluated in diagnosis of the infection using indirect ELISA and electrophoretically characterised through sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) and western blotting technique. The results indicated that both TNF-α and IL-4 in the serum of infected donkeys were significantly higher compared with the non-infected group at $P<0.05$ and $P<0.01$, respectively. However, the IL-4 level of infected donkeys was significantly higher than that of TNF-α ($P<0.01$). Apparent prevalence, specificity and positive predictive values (96.55%, 100%, and 100% each) of CSS showed higher diagnostic accuracy than that of ESS. In addition, electrophoretic protein profile and IgG reactivity of CSS antigen via western blot presented a prominent reactive protein band at 28 kDa. It was concluded that the CSS antigen was the best antigen that could be used in serodiagnosis of *S. equina* infection. The cytokine responses were explored in order to differentiate infected from non-infected donkeys.

Key words: cytokines, diagnosis, immunoglobulin G, *Setaria equina*

INTRODUCTION

S. equina is one of the several filarial nematodes affecting equines worldwide (Bahgat *et al.,* 2011). A high incidence of infection is recorded by several surveys all over the world including Egypt (Mahmoud, 1998; Hornok *et al.,* 2008; Suleiman *et al.,* 2012). The incidence of this filarial nematode is revealed in both the definitive (equines) and the intermediate (mosquitoes) hosts (Bahgat *et al.,* 2011). The adult forms usually reside floating freely in the peritoneal cavity and occa-

sionally lodged in erratic habitat such as the pleural cavity, eye, brain, spinal medulla and testicles of the horse (Solusby, 1982; Marzok & Desouky, 2009). Larvae produced by adult worms in the peritoneal cavity reach the circulation and are taken up by mosquito species. Infective larvae develop in the mosquito flight muscle and are reinjected into hosts when the mosquitoes feed. In most cases, adult stages are considered non-pathogenic but may cause various degrees of fibrinous peritonitis (Rhee *et al.,* 1994; Mahmoud, 1998), whereas the serious pathogenic effect of *S. equina* occurs when the microfilariae migrate erratically into unusual habitats in the host such as the ocular globe or central nervous system (Hillyer *et al.,* 2001).

Traditionally, the Knott technique is a common diagnostic procedure that depends on detection of microfilaria in peripheral blood of infected equines (Suleiman *et al.,* 2012). Although identification of filarial parasite in blood is acknowledged, today accurate diagnosis is crucial for the effective treatment and successful eradication of the disease. Accurate diagnosis is dependent on the clinical condition of the host to distinguish between active and past infection. Recently, early diagnosis of such infection has been associated with concurrent release of cytokine mediators. Since filarial infections are chronic, many research studies have focused on the T and B cell adaptive immune response (Kwarteng *et al.,* 2016). T helper (Th) cells from the majority of T lymphocyte responses and following activation differentiate into effector Th1 and Th2 which are associated with the development of type-2 cytokines immune response and impairment of type-1 cytokine production (Wammes *et al.,* 2012; Kwarteng *et al.,* 2016). Increased levels of IL-4, IL-5 and IL-10 were reported in all chronic, microfilaraemic and endemic control cases while a significant decrease was observed in IL-2 and IFNγ levels in microfilaraemic patients as compared to chronic and endemic control cases (Sharma *et al.,* 2005; Wammes *et al.,* 2012). In addition, detection of anti-filarial antibodies against purified and crude antigens in the sera of infected equines is also a tool for diagnosis. Hence, detection of total anti-filarial IgG antibody is effective in the diagnosis of latent infections (Njenga *et al.,* 2007; Simonsen *et al.,* 2008). The release of macromolecules such as excretory-secretory antigens and somatic sheath cells by living adult filarial nematodes into their surroundings induce higher antibody titres that are capable of modulating the immune responses (Kaushal *et al.,* 1984; Tizard, 2000). An accurate diagnostic technique has to be reliable and reproducible thus focusing on characterisation and determination of potential immunodiagnostic antigens using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting techniques along with a reliable immunoassay technique such as indirect enzyme linked immunosorbent assay (ELISA) (Mohanty *et al.*, 2006; Kaushal *et al.*, 2009; Abdel-Latif & Sakran, 2016).

Our research goal was to investigate the significance of cytokines and total IgG response in *S. equina* infected donkeys with emphasis on most immunogenic antigen chosen by SDS-PAGE and immunoblotting techniques that could be used in sero-diagnosis of such infection.

MATERIALS AND METHODS

Animals and samples collection

A total of 87 donkeys were slaughtered at Giza zoo's abattoir between November

2015 and March 2016. Blood samples were collected from the animals, sera were separated by centrifugation for 10 min at 3000 rpm (Sigma-202c, Germany) and stored at –20 °C for cytokines and IgG analysis. All institutional and national guidelines for the care and use of animals were followed.

Parasite and antigen preparation

At necropsy, the peritoneal cavity and its fluid from all slaughtered donkeys were parasitologically examined for the existence of *S. equina*. Twenty-nine donkeys were infected with *S. equina* and 58 donkeys were non-infected. Collected worms were classified by morphological criteria according to Solusby (1982). The identified worms were individually washed several times in phosphate buffered saline (PBS), pH 7.2 then divided into 2 batches, one for preparation of crude somatic antigen and the other for the excretory-secretory antigen. CSS antigen was prepared according to Theodore & Kaliraj (1990). Briefly, washed worms were homogenised in PBS pH 7.2 and centrifuged at 13000 rpm/30 min in a refrigerated micro-centrifuge. The supernatant was aspirated off and aliquots were stored at –20 °C until used. Also, ESS antigen was obtained using the method of Thilagavathy *et al.* (1990) with some modifications. Living washed adult worms were incubated in 4 mL PBS pH 7.2 with penicillin (100 IU/mL) and streptomycin (100 IU/mL) at 37 °C for 24 hours in a 5% CO_2 incubator. The buffer was collected then centrifuged at 10000 rpm for 20 min in a refrigerated micro-centrifuge. The ESS antigen was dialysed. The obtained ESS antigen was frozen at –20 °C until used. Total protein content of all prepared antigens (CSS and ESS) was estimated according to Lowry *et al.* (1951).

Immunological assays

TNF-α and IL-4 concentrations in serum samples were measured by an equine-specific ELISA kit (WKEA MED Supplies). The concentrations of serum TNF-α and IL-4 were determined by comparing the samples OD to the standard curves.

IgG analysis was done by indirect ELISA optimised by serial checker board titration to the following setup according to Kaushal *et al.* (2009) with a slight modification. Ninety–six well micro-titre plates (Grienger, Germany) were individually coated with 100 µL per well of each diluted antigen at concentrations of 4 and 6 µg per well for CSS and ESS, respectively, in carbonate-bicarbonate buffer, pH 9.6 and incubated at 37 °C for 1 h, then stored overnight at 4 °C. The coated plates were blocked with 200 µL per well of blocking solution (2% dry skimmed milk in PBS – 0.05% Tween 20) and incubated at 37 °C for 1 h. Then, 100 µL per well of serum sample diluted 1:200 was added to individual wells in duplicates and incubated for 2 h at 37 °C. Positive, negative and blank controls were included on each plate in duplicates. One hundred µL of HRP-conjugated goat anti-horse IgG conjugate (Sigma, Aldrich, USA) diluted 1:2500 was added to each well and the plates were incubated for 1 h at 37 °C. After that, the wells were incubated with 100 µL of substrate solution 20 mg o-phenylenediamine dissolved in 50 mL substrate buffer, pH 5 and 25 µL 30% H_2O_2) for 10 minutes at 37 °C. The reactions were stopped with 100 µL of stopping solution (5% SDS) to each well and the optical densities (OD) were determined at 450 nm using an ELISA reader (BIO-TEK, INC., ELx, 800UV). A cut off value based upon 20% of the positive control serum was selected to discriminate between positive and negative tested sam-

ples (Bauer *et al.,* 2002). Antibodies percentage was calculated as OD(%)=100× (Mean OD_{sample}–$OD_{negative\ control}$) / ($OD_{positive\ control}$–$OD_{negative\ control}$).

Sensitivity, specificity, positive predictive value, negative predictive value and apparent prevalence were calculated for each antigen according to Tabouret *et al.* (2001) and Bauer *et al.* (2002).

SDS-PAGE and western blotting

Both CSS and ESS were resolved on three separate 10% polyacrylamide gels under reducing conditions according to Laemmli (1970). Pre-stained molecular weights protein markers (Fermentas and Genedirex BLUltra, USA) were included on each gel. After electrophoresis, one gel was stained with coomassie brilliant blue R-250 dye and the other two were transferred to 0.45 nitrocellulose membranes according to Towbin *et al.* (1979). Membranes were blocked for 1 hour in 1% dry skimmed milk dissolved in PBS pH 7.2, then probed overnight with control positive naturally infected and control negative sera at 1:100 in Tris-buffered saline (TBS) with 0.5% bovine serum albumin (BSA) against both antigens. The nitrocellulose strips were incubated with HRP-conjugated goat anti-horse IgG conjugate at 1:2500 in 0.5% BSA/TBS buffer for 1 h. The immunoreactive bands were developed by incubation of the blot in the substrate solution (1-chloronaphthol, Sigma-Aldrich, USA – one tablet 30 mg/1 mL methanol added to 10 mL methanol, 39 mL TBS and 30 μL 30% H_2O_2) for 5 min.

Statistical analysis

Analysis of variance (ANOVA) was used to compare blood levels of TNF-α and IL-4 in *S. equina* infected and non-infected donkeys. OD data were expressed as mean with standard error (mean±SEM) and analysed using chi-square test by Graph Pad Prism version 7. Differences were considered significant at $P<0.05$ level.

RESULTS

Immunological assays

Current data revealed that both TNF-α and IL-4 levels in the blood of *S. equina* infected donkeys were significantly higher compared to the non-infected group ($P<0.05$ and $P<0.01$, respectively; Table 1). In the infected donkeys, blood level of IL-4 was significantly higher than TNF-α ($P<0.01$).

Necropsies results of slaughtered donkeys revealed that the prevalence of *S. equin*a infection was 33.76%. Results showed that CSS immunodiagnostic values of IgG were significantly higher by using CSS antigen (369.70%) than with the ESS antigen (175.97%, $P<0.05$) (Ta-

Table 1. Blood levels (ng/mL) of tumor necrosis factor alpha and interleukin-4 in *S. equin*a infected and non-infected donkeys. Data are presented as mean± SEM

Parameter	*S. equina* infected donkeys (n=29)	Non-infected donkeys (n=59)
Tumor necrosis factor-alpha	10.184 ± 1.09* ^{A}	7.294 ± 0.41
Interleukin-4	17.292 ± 1.68** ^{A}	9.156 ± 1.35

* $P<0.05$; ** $P<0.01$vs non-infected animals; ^{A} Values in the same column are significantly different at $P<0.01$.

Table 2. Antibodies' optical density and diagnostic accuracy (%) using different prepared *S. equina* antigens by ELISA

Diagnostic accuracy %	*S. equina* antigens		Chi square P value
	Crude somatic *S. equina*	Excretory secretory *S. equina*	
Antibodies OD	369.70±14.6	175.97±8.98	<0.05
Apparent prevalence	96.55	79.31	<0.05
Sensitivity	35.10	32.70	–
Specificity	100	41.10	<0.05
Positive predictive value	100	65.50	<0.05
Negative predictive value	4	2.17	–

ble 2). The ELISA results showed that the apparent prevalence of *S. equina* infection was significantly higher using CSS antigen (96.55%) than ESS antigen (79.31%) ($P<0.05$). However, there was no significant difference in sensitivity between CSS and ESS, 35.1% and 32.7%, respectively, while the specificity was significantly higher using CSS (100%) vs ESS (41.1%) ($P<0.05$). In addition to these findings, higher positive predictive value percentages were achieved using CSS antigen (100%) than with the ESS antigen (65.5%) ($P<0.05$). The negative predictive values were 4% and 2.17% for CSS and ESS antigens, respectively (Table 2).

SDS-PAGE and western blotting profiles

The coomassie stained SDS-PAGE and western blotting profiles of ESS and CSS antigens were resolved. The obtained results showed variations in protein bands between the prepared *S. equina* antigens. The ESS antigen showed 8 protein bands with molecular weights ranging from 180 to 17 kDa; however, the CSS exhibited 17 protein bands with molecular weights ranged from 273 to 17 kDa (Fig. 1). On the other hand, immunogenic bands were detected from pooled sera of infected and non-infected donkeys against the *S. equina* antigens via western blotting (Fig. 2 and 3). The ESS antigen presented 6

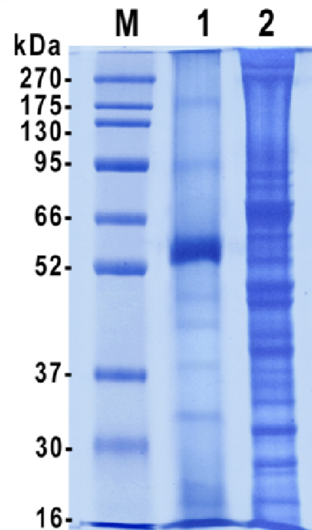

Fig. 1. Electophoretic protein profile of *S. equina* antigens: ESS (lane 1), CSS (lane 2), prestained molecular weight protein ladder (M).

immunoreactive bands at molecular weights ranging from 55 to 15 kDa, whereas the CSS antigen – 17 immunoreactive bands from 86 to 15 kDa using infected pooled sera (Fig. 2). When pooled non-infected sera were used, only 6 immunoreactive bands from 262 to 65 kDa were recognised at higher molecular weights and 4 immunoreactive bands from

260 to 102 kDa with ESS and CSS antigens, respectively (Fig. 3).

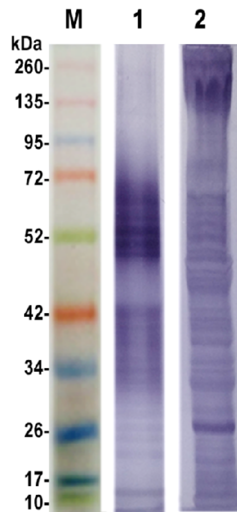

Fig. 2. Western blotting analysis of *S. equina* antigens: ESS (lane 1), CSS (lane 2) and prestained molecular weight protein ladder (M) against infected serum.

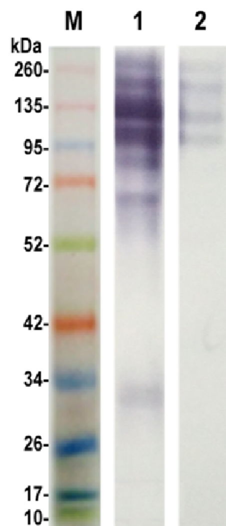

Fig. 3. Western blotting analysis of *S. equina* antigens: ESS (lane 1), CSS (lane 2) and prestained molecular weight protein ladder (M) against non-infected serum.

DISCUSSION

S. equina infection has been reported worldwide (Hornok *et al.,* 2008; Suleiman *et al.,* 2012). Our study revealed that 33.76% of the examined donkeys were infected with adult *S. equina*. This result differed from the stated percentage of 15% recorded by Oge *et al.* (2003) when looking for the existence of adult *S. equina* in slaughtered donkeys and horses in Turkey, while it was lower when blood samples were checked for microfilaria. However, the percentage was 4% in Turkey (Oge *et al.,* 2003) and 9.2 % in Hungary (Hornok *et al.,* 2008) and in Iraq it reached 30.76% (Suleiman *et al.,* 2012).

Direct measurement of cytokine responses (TNF-α as Th1 and IL-4 as Th2 indicators) to *S. equina* infection was carried out initially to clarify immunological differences to obtain the most immunogenic agents responsible for the provoked immune response. In this study blood levels of TNF-α (Th1) and IL-4 (Th2) in *S. equina* infected donkeys were significantly higher compared to the non-infected group ($P<0.05$ and $P<0.01$ respectively). These results were in agreement with the studies of Wammes *et al.* (2012) and Kwarteng *et al.* (2016) concluding that T helper cells from the majority of T lymphocyte responses, following activation, differentiate into effector Th1 and Th2 phenotypes associated with the development of type-2 cytokine immune responses and impairment of type-1 cytokine production. However, in infected donkeys blood level of IL-4 was significantly higher than TNF-α ($P<0.01$). The elevation in IL-4 levels might indicate that the animals were chronically infected. This result was in agreement with the previous studies of Sharma *et al.* (2005) and Wammes *et al.* (2012) in which the levels

of IL-4, IL-5 and IL-10 increased in all chronic, microfilaraemic and endemic control cases. T-cells play a key role in regulating the balance between infection and disease, with Th1 and Th2 phenotypes being predominantly related to susceptibility and protection respectively (Carvalho *et al.,* 2009). In contrast, in the early filarial infection there was an elevation in the rate of T cells expressing TNF-α rather than IL-4 (Subash & Nutman, 2003). Thus, filarial parasites could provoke early activation of Th1 cells which is important to understand the infection pathogenesis and the host-parasite relationships. In addition, the early T cell response to this parasite could show evidence about the host immune response manipulation to produce resistance against infection. The early immune response to the filarial parasite was predominated by early stimulation and production of T cells pro-inflammatory cytokines. This response could be the beginning of the acute filariasis and the formation of host resistance to the helminth infection (Subash & Nutman, 2003). Monocytes and macrophages performed a major role in antigen processing and presentation by cytokines releasing such as IL-1 and TNF-α. These cytokines activated T cells to stimulate clonal-proliferation induction. Although filarial-specific proteins have been produced, the host immune responses to these antigens and their interaction with the monocytes were not well defined yet (Uma *et al.,* 1999).

Furthermore, the IgG concentrations in collected sera were measured with a focus on the diagnostic accuracy of the antigens used along with electrophoretic protein profile and IgG reactivity via western blotting. Our results revealed that immunodiagnostic values of IgG were significantly higher using CSS antigen (369.70%) than ESS antigen (175.97%, $P<0.05$). Also, the prevalence of 96.55% was combined with highest specificity and positive predictive value – 100% each achieved with the CSS antigen. This finding could be returned to the immune reactive band at 28 kDa which was the most prominent in its binding reactivity. In addition, a higher number of reactive CSS antigen protein bands binding to IgG were detected by western blot than those presented by the ESS antigen. This finding might be explained by the complex protein nature of crude antigenic materials of adult *S. equina,* as well as the persistence of the adult form, and its capability of immune evasion may provide a good chance for the production of IgG against the epitopes of this parasitic macromolecule (Tizard, 2000; Kaushal *et al.,* 2009). Additionally, host immunity is raised against a common variant, one or more newly expressed variants can arise so the host must then build another specific immune response of IgG and increase its titre against the new variant form (Frank, 2002). This suggestion comes along with the previously mentioned data that excretory secretory antigens of *Setaria* spp. are formed in the uterus during the embryonic development and released during hatching, thus the antigenic material of ESS products during the total time of infection is less (Decruse & Kaleysaraj, 1988; Bahgat *et al.,* 2011). The current data of ESS and CSS immunogenic reactive bands appeared against infected donkeys' sera agreed with the findings of Bahgat *et al.* (2011) in which reactive bands were detected at molecular weights 200, 100.5, 93.3, 66.2, 60, 33.6, 27.8 and 17 kDa after using crude male and female *S. equina* antigens and their excretory-secretory antigens against clinically different human sera. Also, Maizels *et al.* (1995) and Oge

et al. (2005) reported that the IgG4 antibodies present in filarial sera reacted preferentially with parasite antigen of low molecular weight pattern on using *S. digitata* and *Dirofilaria immitis* antigens, respectively. However, Dalai *et al.* (1998) and Mohanty *et al.* (2006) indicated that endemic and chronic subjects do not make detectable IgG4 antibodies against the 14- to 20-kDa antigens. Hence, the CSS antigen could be used as a promising antigen in serological diagnosis of *S. equina* infection by assaying the total circulating IgG in the collected sera as part of the performed immunological studies. Our current study explored the impact of cytokine responses (TNF-α and IL-4) in *S. equina* infection where both TNF-α and IL-4 found in the serum levels of infected donkeys were significantly higher than the non-infected group. Further studies were recommended to investigate the differences between *S. equina* acute and chronic infection and to elucidate their effect on the immune response modulation.

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Correspondence:

Amira H. El Namaky, PhD
Department of Parasitology and
Animal Diseases,
National Research Centre,
El-Bohouth Street, Dokki,
Postal Box 12622, Giza, Egypt.
tel: +2 0100 634 0534
fax: 002 33370931
e-mail: amiraelnamaky@gmail.com