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CYTOKINES AND IMMUNOGLOBULIN G RESPONSE IN CATTLE WITH ACCORDED *SETARIA DIGITATA* INFECTION: A RESEARCH PROSPECTIVE STUDY

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Abstract:
Background: *Setaria digitata* is a filarial parasite that exists in peritoneal cavity of cattle. This study aims to evaluate cytokine mediators like Tumor Necrosis Factor-Alpha (TNF-α), Interleukin-4 (IL-4) and Immunoglobulin G (IgG) responses in spontaneously *S. digitata* infected and non-infected cattle, with emphasis on choosing the best antigen that could be used in diagnosis of such filarial infection.
Materials and Methods: A total of 95 cattle were included in this study. Two *S. digitata* antigens: Crude Somatic Antigen (CSS) and Excretory Secretory antigen (ESS) of *S. digitata* were prepared. They were evaluated in diagnosis of the infection using indirect ELISA and electrophoretically characterized through sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) and western blotting technique.
Results: The results indicated that both TNF-α and IL-4 in the serum of infected cattle 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 cattle 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.
Conclusion: It was concluded that the CSS antigen was the best antigen that could be used in sero diagnosis of *S. digitata* infection. The cytokine responses were explored in order to differentiate infected from non-infected cattle.
Keywords: Cytokines, Diagnosis, Immunoglobulin ,*Setaria digitata,* Markers, Interleukins

Introduction:

Animal models of filariasis have been used widely for understanding the pathogenesis of the disease, protective immunity and for screening potential anti-filarial drugs. Although Brugia malayi, a human filarial parasite has been adopted in small laboratory animals such as Gerbils and Mastomys coucha, these animals do not display clinical features associated with the human disease[1]. *S. digitata* is a common filarial parasite of cattle. Due to the easy availability of large quantities of parasites from slaughtered animals, the parasite has been increasingly used in recent years for immunological, chemotherapeutic and other biological studies[2-6]. A high incidence of infection is recorded by several surveys all over the world including India. The incidence of this filarial nematode is revealed in both the definitive (cattle) and the intermediate (mosquitoes) hosts[7]. The adult forms usually reside floating freely in the peritoneal cavity and occasionally lodged in erratic habitat such as the pleural cavity, eye, brain, spinal medulla and testicles of the cattle[8]. 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 rejected into hosts when the mosquitoes feed. In most cases, adult stages are considered non-pathogenic but may cause various degrees of fibrinous peritonitis[9], whereas the serious pathogenic effect of *S. digitata* occurs when the microfilariae migrate erratically into unusual habitats in the host such as the ocular globe or central nervous system. Traditionally, the Knott technique is a common diagnostic procedure that depends on detection of microfilaria in peripheral blood of infected cattle[10]. 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[11] 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[11,12]. 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 micro filaraemic patients as compared to chronic and endemic control cases[13].

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[14]. The release of macromolecules such as excretory-secretory antigens and somatic sheath cells by living adult filarial nematodes into their surroundings induce higher antibody titers that are capable of modulating the immune responses[15,16]. An accurate diagnostic technique has to be reliable and reproducible thus focusing on characterization 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 immune sorbent assay (ELISA)[17,18]. Our research goal was to investigate the significance of cytokines and total IgG response in *S. digitata* infected cattle 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 98 cattle were slaughtered at animal house of abattoir from Trivandrum Corporation between July 2016 and February 2017. The worms, located in the peritoneal cavity of the cattle, were collected, immediately after opening the abdomen, in modified Tyrode solution (NaCl, 0.8 %; KCI, 0.02 %; $CaCl_2$, 0.02%; $MgCI_2$, 0.01%; $NaHCO_3$, 0.015%; Na2HPO4,0.0500; glucose, 0.1%; pH adjusted to 7.4), brought to the laboratory and washed free of extraneous materials using the same salt solution. Worms of average length of about 5 cm and width of about 1 mm were selected in these experiments. Blood samples were also collected from the animals; sera were separated by centrifugation for 10min 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 cattle were parasitologically examined for the existence of *S. digitata*. 30 cattle were infected with *S. digitata* and 58 cattle were non-infected. Collected worms were classified by morphological criteria according to Solusby et.al., study[8].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[19]. Briefly, washed worms were homogenized 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 by using the standard method[20] 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 minutes in are frigerated micro-centrifuge. The ESS antigen was dialyzed. 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 standard methods[21].

Immunological assays

TNF-α and IL-4 concentrations in serum samples were measured by a *S. digitata* specific ELISA kit. 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 optimized by serial checker board titration to the following setup according to standard procedures[15] with a slight modification. 96 well micro-titre plates 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 hour, 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 hour. Then 100 µL per well of serum sample diluted 1:200 was added to individual wells in duplicates and incubated for 2 hours 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 diluted 1:2500 was added to each well and the plates were incubated for 1 hour at 37 °C. After that, the wells were incubated with 100 µL of substrate solution 20mg o-phenylene diamine dissolved in 50mL substrate buffer, pH 5 and 25uL 30%H2O2) 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[22]

Antibodies percentage was calculated as

$$\text{OD (\%)} = \frac{100\times \text{(Mean OD sample–OD negative control)}}{\text{(OD positive control–OD negative control)}}$$

SDS-PAGE and western blotting

Both CSS and ESS were resolved on three separate 10% polyacrylamide gels under reducing conditions according to standard method[23]. Pre-stained molecular weights protein markers 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 various protocols[24]. 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 hour. The immune reactive bands were developed by incubation of the blot in the substrate solution (1-chloronaphthol Sigma-Aldrich, USA – one tablet 30 mg/1mL methanol added to 10 mL methanol, 39 mL TBS and 30 µL 30% H_2O_2).

Statistical analysis

The data was expressed in mean and standard deviation. One was ANOVA (Post hoc) followed by Dunnet t test applied to find the statistical significant. p value less than 0.05 ($p<0.05$) considered statically significant at 95% confidence interval.

Results

Necropsies results of slaughtered cattle revealed that the prevalence of *S. digitata* 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 %, $p<0.05$). The ELISA results showed that the apparent prevalence of *S. digitata* 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. The ELISA results showed that the apparent prevalence of *S. digitata* 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-1, 2). 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. digitata* 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. On the other hand, immunogenic bands were detected from pooled sera of infected and non-infected cattle against the *S. digitata* antigens via western blotting. The ESS antigen presented 6 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. When pooled non-infected sera were used, only 6 immunoreactive bands from 262 to 65 kDa were recognized at higher molecular weights and 4 immunoreactive bands from 260 to 102 kDa with ESS and CSS antigens, respectively

Table 1: In the infected cattle blood level of IL-4 was significantly higher than TNF-α (P<0.01)

Parameter	*Setaria digitata* infected cattle (MEAN±SD)	Non Infected cattle (MEAN±SD)
Tumor necrosis factor- α	10.184±1.09	17.292 ±1.68*
Interleukin-4	7.29.156 ±1.35	94.00±0.41*

(*p<0.05 significant compared infected cattle to non-infected cattle)

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

Diagnostic accuracy %	Crude somatic (MEAN±SD)	Excretory secretory (MEAN±SD)	p value
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	--

(*p<0.05 significant cure somatic and excretory secretory)

Discussion

S. digitata infection has been reported worldwide[25]. Our study revealed that 33.76% of the examined cattle were infected with adult *S. digitata* This result differed from the stated percentage of15% recorded[26] when looking for the existence of adult *S.digitata* in slaughtered cattle and buffalos, while it was lower when blood samples were checked for microfilaria. Direct measurement of cytokine responses to *S. digitata* infection was carried out initially to clarify immunological differences to obtain the most immunogenic agents responsible for the provoked immune response. In these study blood levels of TNF-α (Th1) and IL-4 (Th2) in *S. digitata* infected cattle were significantly higher compared to the non-infected group. These results were in agreement with the other studies 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-1cytokine production. However, in infected cattle, blood level of IL-4 was significantly higher than TNF-α. The elevation in IL-4 levels might indicate that the animals were chronically infected. This result was in agreement with the previous studies[28] 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[29]. In contrast, in the early filarial infection there was an elevation in the rate of T cells expressing TNF-α rather than IL-4[30]. 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. 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. 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 than ESS antigen. 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. digitata*, 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. 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 titer against the new variant form. This suggestion comes along with the previously mentioned data those 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.

Conclusion

The cytokine responses were explored in order to differentiate infected from non-infected cattle. The presser study results concluded that the CSS antigen was the best antigen that could be used in sero diagnosis of *S. digitata* infection.

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