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Research Article
Diagnosis of Sheep Trichostrongylosis Based on Imune Response Profile in Experimental Rabbit Infection

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Abstract

Background and Objective: *Trichostrongylus colubriformis* is a common nematode which infect livestock and can cause accidental infection in humans. In the present study, affinity purification process succeeded in the isolation of a single specific fraction identified by experimentally infected rabbit sera. **Methodology:** The isolated fraction detected *T. colubriformis* antibodies in 68 (77.3%) out of 88 examined sheep serum samples. By SDS-PAGE, the isolated fraction showed simple electrophoretic profile and resolved into three bands of molecular weights 74, 85 and 97 kDa. While eight bands of molecular weights ranged from 25.7-189 kDa were detected in crude adult worm extract. In immunoblot assay, the three bands of isolated fraction were reacted with infected rabbit serum compared with four bands of molecular weights 41, 74, 85 and 97 kDa in crude extract. **Results:** Concerning, humeral immune responses of infected rabbits, the level of IgG antibodies increased to reach (0.526 OD±0.0027) in the first-week post infection. Then decreased to reach (0.34 OD±0.02) at the end of the experiment using ELISA. A significant expression of IL4 was observed and reached the highest level (225±0.012) at second-week post infection. While, IFN-ɣ showed less and nearly constant expression through out the time of the experiment. **Conclusion:** The partially immuno-affinity purified fraction of *T. colubriformis* adult antigen proved high diagnostic efficacy in diagnosis of trichostrongylosis in sheep. In addition, *T. colubriformis* infection stimulates rabbits cellular and humeral immune responses IL4, IFN-γ and IgG which, possibly create some level of protection to re infection.

Key words: *Trichostrongylus colubriformis*, affinity purified fraction, diagnosis, cellular and humeral immune response, SDS, immunoblot

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Gastrointestinal nematodes are one of the most restrictions on ruminant production in the world. It causes a reduction in nutrient availability to the host through both reducing optional feed intake and/or reductions in the efficiency of utilization of absorbed nutrients (Dynes *et al*., 1998). This lead to weight loss, anemia, edema and reduction in the resistance to other diseases. Annual treatment costs due to these parasites have been reached to $46 and $103 million in South Africa (Waller and Chandrawathani, 2005) and in India (McLeod, 2004), respectively. *Haemonchus contortus* and *Trichostrongylus colubriformis* are the most important gastrointestinal nematodes of sheep (Rocha *et al*., 2008). Because, *H. contortus* is the most pathogenic parasite it had been focused in scientific research in tropical and sub tropical countries. In contrast little attention has been concerned *T. colubriformis* which proved a reduction in animal performance caused by the damages in the intestinal mucosa, and by immunopathological changes associated with the infection (Cardia *et al*., 2011). This loss associated with *T. colubriformis* infection must be oriented the scientific study to including this neglected nematode. The diagnosis of this gastrointestinal nematode mostly depends on the detection of eggs which observed in feces after the prepatent period. So, a trustworthy serological assays such as ELISA which enables the detection of host immune responses at early phase of infection is very important (Lone *et al*., 2012).

Hosts have developed a large variety of immune components and functions to recognize and target different parasite life stages and their products (Murphy *et al*., 2011). In turn, parasites display different mechanisms to evade or modify the immune response of the host to persevere and survive in the host (Schmid-Hempel, 2009). Generally, helminth infections lead to polarization of the immune system towards a Th2 response which is responsible for the production of IL-4, IL-5, IL-13 in addition to IgE and eosinophilia (Anthony *et al*., 2007; Allen and Maizels, 2011). In a few exceptions, infection with some tissues helminths directed the immune system towards a Th1 response in particular during specific tissue phase of the infection process (Mulcahy *et al*., 2005; Zhang *et al*., 2005; Fabre *et al*., 2009). These cells are responsible for the production of IFN-γ and TNF-α and enhance the activation of macrophages which are responsible for the production of antibodies (Abou-El-Dobal *et al*., 2015). Mean while, mixed Th1/Th2 response was found toward *Trichostrongylus retortacformis* in experimetally infected rabbits (Murphy *et al*., 2013). So, infected rabbits proved increase in the level of IFN-γ, IL-4, IgG, IgA and eosinophilia.

The current study aims to evaluate *T. colubriformis* partially purified fraction in the diagnosis of natural sheep trichostrongylosis after following up its effect on the level of IgG in experimentally infected rabbit sera at different weeks post infection. Rabbit IL-4 and IFN-γ levels will also be determined.

MATERIALS AND METHODS

Animals: Adult 10 male New Zealand parasite-free rabbits weighing 3-5 kg were fed *ad libitum* and maintained at 25°C in a conditioned vivarium in the animal house of the Veterinary Division, National Research Centre. Adult 88 males and females out-bred sheep weighing 40-50 kg were supplied by local slaughterhouse. All procedures related to animal experimentation met the International Guiding Principles for Biomedical Research involving animals as issued by the International Organizations of Medical Sciences.

Parasites: Infective larvae of *T. colubriformis* field strain were obtained from fecal cultures of sheep harboring *T. colubriformis* infection (Langrova and Jankovska, 2004). A single dose of 5000 freshly cultured infective larvae was administered orally for each rabbit. Twenty days after infection, infected rabbits were slaughtered and adult worms of *T. colubriformis* were harvested from small intestine using Baerman technique (Baerman and Wetzal, 1953). Larvae and worms of *T. colubriformis* were identified according to Soulsby (1986).

Preparation of somatic worm antigen: Adult worms of *T. colubriformis* were washed several times in saline chopped and homogenized in PBS pH 7.2. Particulate materials were removed by centrifugation at 14000 rpm for 30 min the supernatant was decanted and used as somatic adult antigen according to the method of Murphy *et al.* (2011). Protein concentration was determined by the method of Lowry *et al.* (1951).

Antibody-sepharose 4B affinity column chromatography: Rabbits experimentally infected sera were dialyzed against 100 mM $NaHCO_3$ buffer pH 8.3 containing 500 mM NaCl and 0.02% $NaNO_3$ and coupled to cyanogen bromide-activated sepharose-4B (CNBr-sepharose 4B) at the ratio of 2 mg mL^{-1} swollen beads by strictly following the manufacturer instructions. *Trichostrongylus colubriformis* mature worm extract was applied to the column. The column was washed with 0.015 M PBS containing 0.02% $NaNO_3$ pH 8 and bound materials were eluted with 50 mM glycine-500 Mm

NaCl 0.02% w/v $NaNO_3$ pH 2.3. The protein content of isolated fraction was measured according to the method described by Lowry *et al.* (1951).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE): The SDS-PAGE was performed in polyacrylamide gels according to Laemmli (1970). Both crude antigen and bound fraction were separately mixed with sample buffer containing 5% 2-mercaptoethanol before loading to the gel. After separation, the gel was fixed in 50% methanol and stained with silver stain according to the method of Wray *et al.* (1981). Relative molecular weights of bands were calculated using marker supplied by Fermentas International INC, Canada.

Immunoblot: The assay was utilized to identify the immunoreactive components recognized in the crude and purified antigens using *T. colubriformis* rabbits experimentally infected sera. After electrophoresis, protein components of the two antigens were immunoblotted onto nitrocellulose membrane according to the method of Towbin *et al.* (1979). The nitrocellulose membrane was incubated with positive rabbit sera (diluted at 1:50) and anti-rabbits IgG (whole molecule) peroxidase conjugate (diluted 1:3000) as a second antibody. The ECL Western blotting detection reagents (Amersham, UK) were utilized to visualize the immunoreactive bands.

Enzyme linked immunosorbent assay: The ELISA was adopted to evaluate the success of the purification process by determination of the antigenic activities of the eluted fraction compared with crude extract in a detection of IgG level in experimentally infected rabbits at different weeks post infection according to Engvall and Perlmann (1971). In brief, plates were coated, separately with 5 μg mL^{-1} isolated fraction and crude extract in carbonate buffer. Positive and negative rabbit serum samples diluted at 1:100 was added to the coated plates separately. Anti-rabbit IgG horse radish peroxidase labeled-conjugate (1:1000) and ortho-phenylenediamine (OPD) 1 mg mL^{-1} substrate buffer (Sigma) were used. Also, ELISA was adopted to the diagnosis of trichostrongylosis among sheep using *Trichostrongylus colubriformis* affinity purified fraction. The optimum antigen concentration, antibody and conjugate dilutions were determined by checker board titration. Plates were read spectrophotometrically at 450 nm and the cut off values of Optical Densities (OD) were calculated according to the method of Woo *et al.* (2001).

Evaluation of cytokines level: Serum levels of IL-4 and IFN-γ were measured with double-antibody sandwich ELISA kit (Glory Science Co., Ltd, Del, Rio, Tx 78840, USA). The concentration was calculated from the standard curve that was performed in the same assay.

Statistical analysis: Data are expressed as Mean±SD. Comparison between the mean values of different parameters in the studied groups was performed using 1-way ANOVA test.

RESULTS

Electrophoretic profile of *Trichostrongylus colubriformis* extracts: By SDS-PAGE 10% slab gel under reducing condition, the isolated fraction proved simple electrophoretic profile and resolved into three bands of molecular weights 74, 85 and 97 kDa. While the crude extract resolved into 8 bands of molecular weights ranged from 25.7-189 kDa (Fig. 1).

Immunogenic components of *Trichostrongylus colubriformis* extracts: By immunoblot assay and rabbit positive sera the isolated fraction proved three immunogenic bands of molecular weights 74, 85 and 97 kDa, while four bands of molecular weights 41, 74, 85 and 97 kDa were detected in crude extract (Fig. 2).

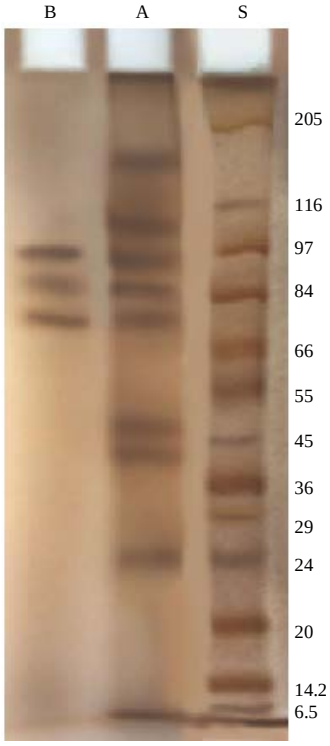

Fig. 1: ELectrophoretic profile of *T. colubriformis* extract. Lane A: Crude antigen, lane B: Isolated fraction and lane S: Molecular weight standards

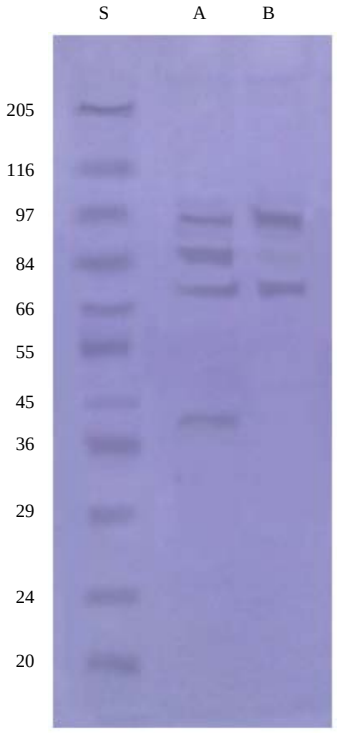

Fig. 2: Immunoblotting assay showing reactivity of *T. colubriformis* extract with positive rabbit serum. Lane A: Crude antigen, lane B: Isolated fraction and lane S: Molecular weight standards

Immune response of rabbits experimentally infected with *Trichostrongylus colubriformis*

Humoral response: The IgG response due to *T. colubriformis* infection was detected by ELISA using isolated fraction compared with crude extract. As depicted in Fig. 3, the isolated fraction has the most diagnostic potential. The level of antibodies increased to reach (0.526 OD±0.0027) in the 1st week post infection. Following this activity period, there is a little or no change in immune response from the 2nd week to 5th week post infection. The response decreased in 6 week post infection and reached a plateau until 8 week then decreased to reach (0.34 OD±0.02) at the end of the experiment.

Cellular response: Level of IL-4 increased in rabbit sera with the infection course and reached the highest level (225±0.012) at 2nd week post infection. Then slightly decreased to reach (128.75±0.013) at the end of the experiment. While IFN-γ showed constant expression over the time of the experiment although there was significant higher level in infected animals compared to control ones (Fig. 4).

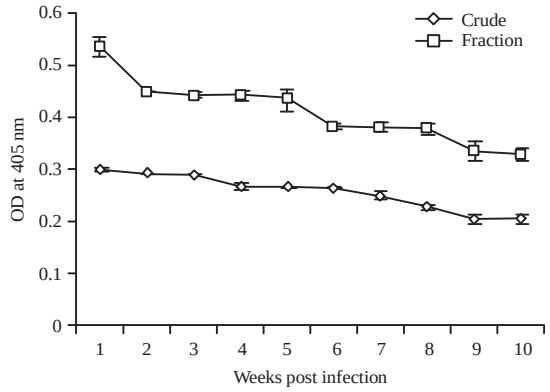

Fig. 3: Mean level of IgG antibodies in experimentally infected rabbit sera at different weeks post infection

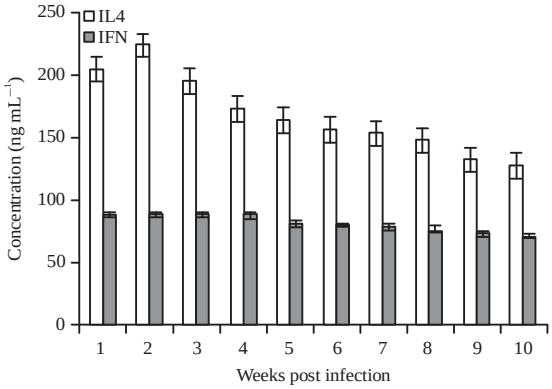

Fig. 4: Mean level of IL-4 and IFN-γ in experimentally infected rabbit sera at different weeks post infection

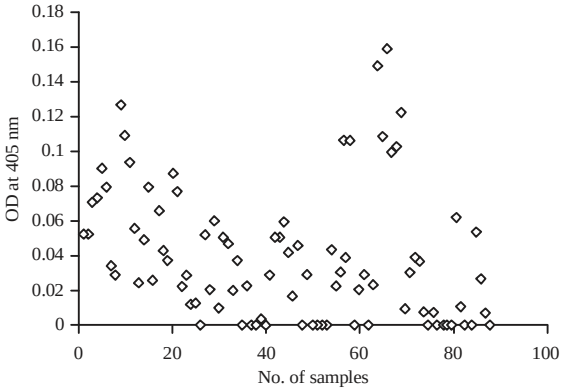

Fig. 5: Scatter graph represents the efficacy of *T. colubriformis* isolated fraction in the diagnosis of Trichostrongylosis in sheep

Diagnostic potential of *Trichostrongylus colubriformis* isolated fraction: As shown in Fig. 5, the isolated fraction succeeded in detection of *T. colubriformis* antibodies in 68 (77.3%) out of 88 examined sheep serum samples.

DISCUSSION

In the present study, rabbits were selected as lab animals based on the previous study suggested similarity between rabbits and ruminants immune response toward *T. colubriformis* infection (Bezubik *et al*., 1988; Langrova and Jankovska, 2004). These in addition to low price and easy manipulation in experimental infection. In current study examined immune response of rabbits to experimental infection with gastrointestinal *T. colubriformis*. The infection stimulated rabbits immune system and directed it toward Th2 activation more than Th1. So, infected animals exhibited strong anti-inflammatory cytokine (IL-4) which reached its highest level at secondary week post infection, while IFN-γ proved less and nearly constant expression level during the experiment. The current result was coincided with Anthony *et al.* (2007) and Allen and Maizels (2011), who proved that helminths infection mainly directed immune system toward a Th2 response. These cells characterized by high production of IL-4, IL-5, IL-13 in addition to IgE and eosinophilia. Also, these results were confirmed by Abou-El-Dobal *et al*. (2015) in experimental infection of rabbits with *Fasciola gigantica*, where IL-4 showed higher level compared to IFN-γ.

In previous study to follow up the immune response of rabbits experimentally infected with *T. retortaeformis*, Th1 was firstly activated and animal proved an initial strong inflammatory response (IFN-γ) followed by increase in IL-4 expression (Murphy *et al*., 2011, 2013). They contributed this firstly strong inflammatory response to bacterial invasion into the small intestine mucosa following movement of larvae into the tissue. Actually, this relative increase in IL-4 in presence of IFN-γ indicated that both immune phenotypes can work and this coincided with that reported in the present study.

In the current study a single dose of 5000 *T. colubriformis* infective larvae not only induced cellular immune response but also humeral immune response represented by high level of IgG antibodies which detected at first week post infection and remained high compared to the control animals throughout the experiment . Comparable humeral immune response was previously reported in rabbits experimentally infected with *T. retortaeformis* (Murphy *et al*., 2011). The high level of IgG in infected rabbits, throughout the experiment, directed toward isolated fraction proved success of both experimental infection in rabbits and purification process of crude extract. So, the isolated fraction was utilized in the diagnosis of trichostrongylosis in sheep, it succeeded in the detection of antibodies in 68 (77.3%) out of 88 examined sheep serum samples. A comparable infection percentage (79.8%) was detected in goats in Malaysia although they used PCR (Tan *et al*., 2014), this confirmed the sensitivity of utilized antigen and technique. While, less prevalence of *Trichostrongylus* (7.33%) was detected in Pakistan by ELISA using somatic crude antigen (Razzaq *et al*., 2013). The difference in infection percentage may be due to difference in utilized antigen, where they used crude antigen but in the current study partially purified isolated fraction was utilized.

By SDS-PAGE, the isolated fraction exhibited simple electrophoretic profile and resolved into 3 bands of molecular weights 74, 85 and 97 kDa compared with 8 bands of molecular weights ranged from 25.5-189 kDa were detected in the crude extract. To our knowledge, there no electrophoretic profile concerned adult *T. colubriformis* but in the related nematode, *Haemonchus contortus*, four bands of different molecular weights 66, 40, 33 and 26 kDa were detected (Tak *et al*., 2015).

Three bands of molecular weights 74, 85 and 97 kDa were detected in the isolated fraction by immunoblot with rabbit sera experimentally infected with *T. colubriformis*, while four bands of molecular weights 41, 74, 85 and 97 kDa were detected in crude extract. In the previous study, a 31 kDa allergen was detected in *T. colubriformis* third larval stage with IgE purified from infected sheep serum (Shaw *et al*., 2003).

CONCLUSION

Trichostrongylus colubriformis infection stimulates rabbits immune system and appear to elicit an unequivocal Th2 based immune response. Also, the present study introduced partially purified fraction that can be successfully utilized in the diagnosis of trichostrongylosis in sheep after proving success in rabbits. It deserves further purification to use in the diagnosis of this parasite in farm animals on large scale.

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