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Research paper

# Immune response against subclinical haemonchosis in Himalayan hill goats

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ABSTRACT

Haemonchosis commonly occurs as chronic and subclinical infection in small ruminants, and understanding of immunological response against subclinical haemonchosis is of paramount importance for designing and implementing effective control strategies. The present study was designed to evaluate immunological response during subclinical haemonchosis, experimentally established in goats. Sixteen 5–6 month-old helminth naive kids were randomly allocated into one of two groups, infected and uninfected; the infected group being infected *per os* with 250 *Haemonchus contortus* larvae per kg body weight. Faecal, blood and serum samples were collected every third day up to 30 days post-infection (DPI), thereafter weekly up to 58 DPI to record changes in faecal egg count (FEC), haemoglobin (Hb), packed cell volume (PCV), peripheral eosinophil percentage and immunological parameters, such as macrophage cytokine interleukin-12 (IL-12), Th1 cytokine (IFN-γ), Th2 cytokines (IL-4, 13, 25, 33) and immunoglobulins (IgG and IgE). Pre-patent period of *H. contortus* in the present study was 18 days and eggs per gram (EPG) peaked on 30 DPI. The total reduction in body weight gain in the infected group was 26 g per day when compared with uninfected animals. Hb (7.35 ± 0.34 g/dL in infected animals compared with 9.76 ± 0.67 in control animals) and PCV levels (22 ± 1.54 g/dL in infected animals compared with 29.2 ± 1.27 in control animals) decreased significantly up to 44 DPI in infected group (P = 0.000). IL-4, IL-13, IL-33, IgG and IgE showed significant increase in infected animals at different periods. IFN-γ, IL-12 and IL-25 did not show any significant changes barring a steep rise of IFN-γ on 27 DPI. A positive correlation was observed between IgE and IL-4 in subclinical haemonchosis. Of particular note was that all the major cytokines, such as IFN-γ (P = 0.000), IL-4 (P = 0.000), IL-13 (P = 0.009), and both IgG (P = 0.000) and IgE (P = 0.003), were observed at the lowest concentration on 24 DPI. The effect of infection was found to be significant on cytokines with a strong interaction with time. Taken together, the data suggest that Th2 immune response is predominating in subclinical haemonchosis. The economic loss in term of body weight gain due to subclinical haemonchosis was considerable.

## 1. Introduction

*Haemonchus contortus* is a ubiquitous and highly pathogenic strongyle of small ruminants. Control of haemonchosis mainly relies on chemotherapy; however, widespread emergence of resistant strains against all the available anthelmintics (Torres and Hoste, 2008) and concern towards the drug residues in food and in the environment necessitates alternate antiparasitic control strategies. Among the proposed strategies, development of specific vaccines and the use of animals resistant to parasite infections are suitable alternatives to chemical control. The effective immune mechanism varies with different helminth parasites, the intensity of infection and different life-cycle stages, which requires detailed understanding. The knowledge related to the dynamics of eliciting immune responses against gastrointestinal strongyles in ruminants is generally well characterized (Jackson and Miller, 2006), but not complete.

Studies have been carried out in sheep related to immune response to clinical haemonchosis (Gill et al., 2000; Shakya et al., 2011). Gastrointestinal nematode (GIN) induces CD4 + T cell proliferation and mucosal mast cell hyperplasia, tissue eosinophilia and polarization of type 2 helper cells (Th2) immune response (Balic et al., 2002; Meeusen et al., 2005; Lacroux et al., 2006; Artis and Grencis, 2008). Th2 cells produce interleukin – 4 (IL-4), IL-5, IL-9, IL-13, IL-25 and IL-33 upon helminth infection, which causes differentiation and maturation of

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intraepithelial mast cells, eosinophilia, and goblet cell development (Artis and Grencis, 2008). Elevated levels of Th2 cytokines is a hallmark of nematode infections and detailed investigation on the role of these cytokines in helminths infections could enlighten the use of alternate control strategies like immunoprophylaxis and breeding for resistance. However, there are few reports characterizing the immune response in goats despite the consequences of GI parasitism being more severe compared with sheep (Hoste et al., 2010). The development and augmentation of immune responses against GIN species are less efficient and delayed in goats than in sheep (Pomroy et al., 1986; Hoste et al., 2008, 2010). In field conditions, intensities of GIN infection varies due to various factors such as repeated exposure to infection, the quantum of larval ingestion, nutritional status of animal and climate. In this scenario, the host may develop a different immune profile in response to these changing parasite challenges (Meeusen et al., 2005). Despite haemonchosis commonly occurring as chronic and subclinical infection in goats and sheep, the majority of earlier studies on immune response are with clinical haemonchosis. An animal suffering from subclinical haemonchosis is not destined to become clinically apparent and symptoms will never become discernible. Subclinical infection reduces productivity and feed conversion (Ndarathi et al., 1989; Cobon and O'Sullivan, 1992). The condition causes substantial economic losses in the small ruminant industry with 25% reduction in the growth rates of goats (Howlader et al., 1997), a significant reduction in weight gain, wool growth, milk yield and lamb survival in sheep (Cobon and O'Sullivan, 1992). A daily reduction in live weight growth of about 30 g per day was observed in both sheep and goats infected with 500 larvae of *H. contortus*thrice a week for 16 weeks (Beriajaya and Copeman, 2006). Understanding of immunological response against subclinical haemonchosis is of paramount importance for designing and implementing effective prevention/control strategies. Therefore, the present study was designed to evaluate Th1 and Th2 immune responses to the experimental subclinical haemonchosis in native Himalayan hill goats.

## 2. Materials and methods

### 2.1. Experimental design

Twenty native Himalayan hill goats' kids were housed in indoor conditions from birth to end of the experimental period at the Small Ruminant Farm, Mukteswar, Nainital, Uttarakhand, India. The farm is located in the temperate Himalayan region of India at 29°28′20″N, 79°38′52″E, has an average elevation of 2,171 m (7123 feet) above the mean sea level (MSL). Animals were considered helminth naive and maintained in concrete floor pens; fed growing ration (crude protein 17% and TDN 68%) and water *ad libitum*. From the kids, sixteen 5–6-month-old animals with body weight ranging from 8.2 to 9.1 kg, were randomly selected and allocated into one of two groups, infected and uninfected control, each containing 8 animals. The experimental protocol was approved by the Institutional Animal Ethics Committee (8–12/2007-Adm (M)/2015/Pt.No.1). The kids were examined for endo- and ectoparasites and also tested for Peste des petits ruminants, capripox, *Brucella* and blue tongue infections. The kids belonging to infected group received 250 *H. contortus* infective larvae/kg body weight per os and uninfected kids served as controls. Body weight of all animals was measured weekly throughout the experimental trial from 12th December to 7th February.

### 2.2. Establishment of H. contortus infection

Adult nematode parasites were collected in PBS (pH 7.4) from the abomasa of goats slaughtered at the local meat shop in Mukteswar, Uttarakhand, India. *H. contortus* females were identified based on morphological keys (Das and Whitlock, 1960; Mckenna, 1971). The female worms were incubated for egg laying in RPMI -1640 media (Sigma Aldrich) with antibiotics penicillin G 10,000 U/ml and streptomycin 10 mg/ml (HiMedia) for 8–12 hrs at 37 °C. The eggs were pelleted by centrifugation at 1000 x g for 5 min, mixed with sterilized bovine faeces and cultured at 26 °C for 7–10 days. Water in the culture was replaced every day until the fifth day of incubation to reduce protozoal contamination. After 10 days of incubation, infective third stage larvae ($L_3$) were harvested, concentrated, counted and given to donors. Two worm-free donor goats were infected once with 10,000 $L_3$ of *H. contortus.*Faecal samples were collected after patent infection and cultured to obtain infective larvae and used to infect experimental goats.

### 2.3. Faecal egg count (FEC)

Faecal samples were collected directly from the rectum on every third day up to 30 days post-infection (DPI), thereafter weekly up to 58 DPI for FEC. Faeces were subjected to modified McMaster technique to determine FEC (Whitlock, 1948) and the faecal egg counts are presented as eggs per gram (EPG).

### 2.4. Blood packed cell volume and haemoglobin

Fresh peripheral blood was collected in EDTA coated vacutainer (BD vacutainer®) on every third day up to 30 DPI, thereafter weekly up to 58 DPI for the determination of haemoglobin (Hb) and packed cell volume (PCV) (Wintrobe, 1975).

### 2.5. Cytokines and immunoglobulins estimation

Blood samples were collected separately in vacutainer (BD vacutainer® without anticoagulant) for serum collection on every third DPI up to 30 days and thereafter weekly up to 58 days. The different cytokine concentrations in the serum were determined by commercially available ELISA kits. The concentrations of macrophage IL-12 (Goat Interleukin-12 (IL-12) ELISA kit, Catalogue no. EO6I0033, BlueGene Biotech, Shanghai), Th1 cytokine, Interferon-γ (Goat Interferon γ ELISA kit, Catalogue no. EO6I0345, BlueGene Biotech, Shanghai) and Th2 cytokines, namely IL-4 (Goat Interleukin 4 (IL-4) ELISA kit, Catalogue no. EO6I0007, BlueGene Biotech, Shanghai) and IL-13 (Goat Interleukin 13 (IL-13) ELISA kit, Catalogue no. EO6I0036, BlueGene Biotech, Shanghai), IL-25 (Goat IL-25 ELISA kit, Catalogue no. BYEK1547, Chongqing Biospes Co. Ltd) and IL-33 (Goat IL-33 ELISA kit, Catalogue no. BYEK1553, Chongqing Biospes Co. Ltd), were determined. The immunoglobulins IgG (E50-104 Goat IgG ELISA kit, Bethyl Laboratories, USA) and IgE (Goat Immunoglobulin E ELISA kit, Catalogue no. EO6I0037, BlueGene Biotech, Shanghai) concentrations were estimated. The serum was diluted to obtain the OD values of different cytokines and immunoglobulins within the range of standard concentrations. The plates were developed and OD was read at 450 nm in a microplate reader (Bio-Rad, USA). All samples were run in duplicate and the standard curve generated from OD of standards was used to interpolate cytokine levels, expressed as protein concentration (pg/ml), and immunoglobulins expressed as (mg/dL) in the samples.

### 2.6. Statistical analysis

SPSS version 16.0 was used for statistical analysis. The body weight, EPG, biochemical and haematological data and also cytokine and immunoglobulin estimates for the experiments were assessed using the repeated measures analysis of variance method (RAMOVA). In the RAMOVA, the effect of time (days) with 15 levels along with the infection (2 levels) was assessed to see if there was any significant association of the time combined with infection on the expression of these parameters.

Separately, the differences for the mean estimates along with standard errors for expression of EPG, biochemical and haematological data

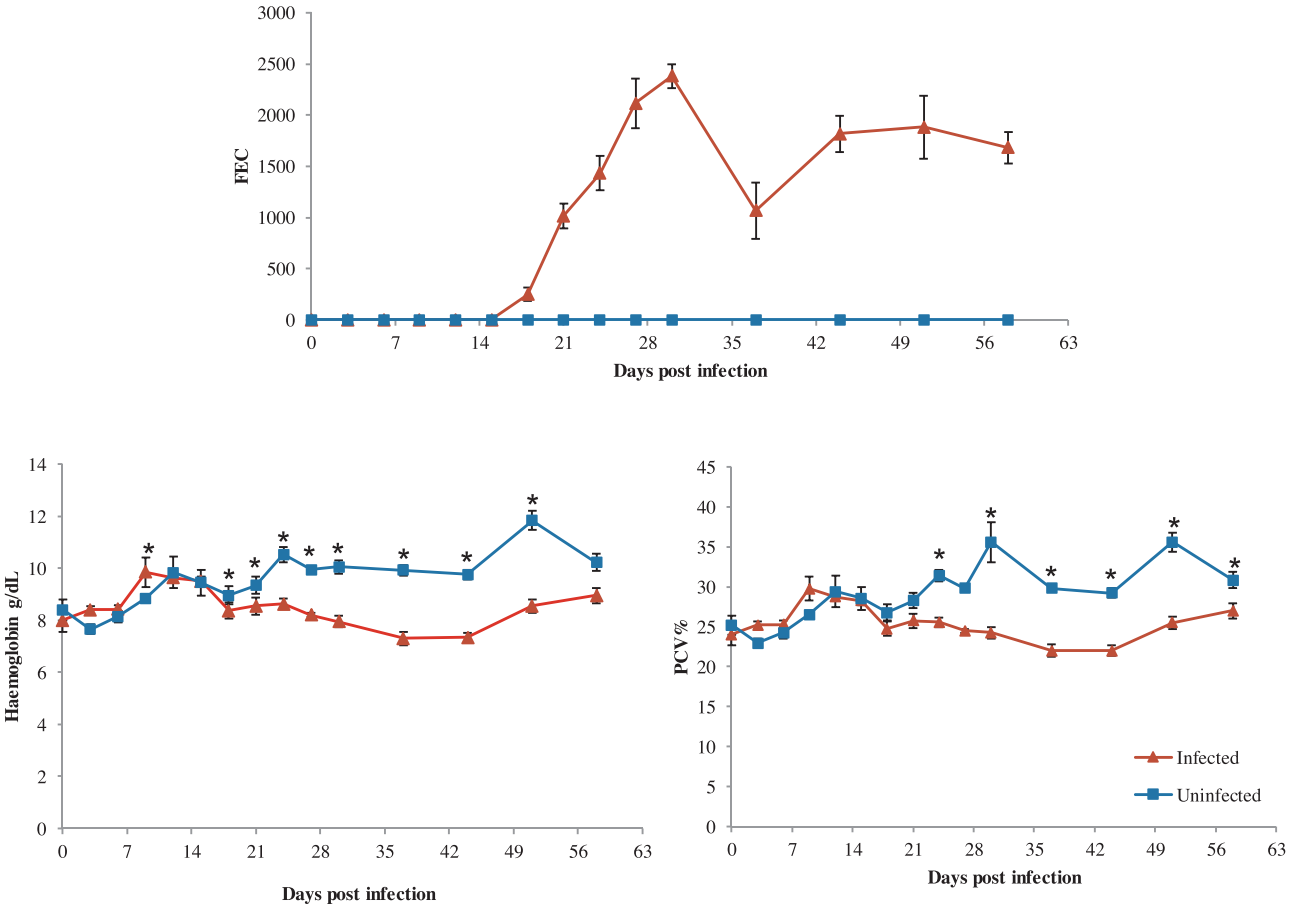


**Fig. 1.** a–c: Mean levels of FEC, haemoglobin and PCV in sub-clinically infected and uninfected goats (* Indicates P < 0.05).

and also cytokine and immunoglobulin estimates were plotted using MS Excel spreadsheet. The differences between control and treatment group at every time point was independently assessed for EPG, biochemical, haematological, cytokine and immunoglobulin data using student's *t*-test (Snedecor and Cochran, 1994). These statistics were required for graphs.

## 3. Results

### 3.1. Faecal egg count

FECs were 'zero' in both groups of animals at the start of the experiment. All the animals were sero-negative for Peste des petits ruminants, capripox, *Brucella* and bluetongue infections. FECs are presented in Fig. 1a. Overall, there was an infection x time interaction (P = 0.000) which reflected increases in the FEC of infected animals only from day 18 to a peak of 2383 ± 288.6 EPG on day 30. On 37 DPI, a sudden decrease in EPG was observed and henceforth maintained at the same level until the end of the experimental trial (Fig.1a). In one goat, faecal egg counts were consistently higher than other animals of the group; it raised up to 6400 EPG on 30 DPI. No eggs were found in the uninfected animals throughout the experimental period.

### 3.2. Haemoglobin and packed cell volume

The mean Hb and PCV values are presented in Fig. 1b and c, respectively. There was an infection x time interaction (P = 0.000) which reflected a decrease in the Hb and PCV values in *H. contortus* infected goats when compared with uninfected animals. Initially, both haemoglobin and PCV levels increased up to 9DPI in infected animals. Thereafter, both values decreased significantly up to 44 DPI (P = 0.000) compared with uninfected animals; however, after 44days, little increase in the mean values was observed till the end of the experimental trial. The mean peripheral eosinophil percentages were comparable between groups up to 15 DPI; thereafter, infected animals showed non-significantly greater percentage than uninfected animals till the end of the experiment.

### 3.3. Body weight

The gain in mean body weight in the infected and uninfected group was 0.4 ± 0.019 kg and 1.96 ± 0.027 kg, respectively, during the trial period. Gain in mean body weight per day in the infected and uninfected group was 6.66 g and 32.66 g, respectively.

### 3.4. Expression of interferon gamma and interleukin-12

The expression level of cytokines, IFN-γ and IL-12 in the serum of all the animals, was determined by ELISA and the results obtained are presented in Fig. 2a, b. There was an infection x time interaction (P = 0.000) for IFN-γ and IL-12 (P = 0.000). However, the concentration of IFN-γ was more or less constant up to 24 DPI in both infected and uninfected animals and a sudden 10.2 fold increase (P = 0.001) was observed in the infected group on 27 DPI. Control group showed more or less constant basal level throughout the experimental trial. We could not see a definite pattern for IL-12 in both the groups.

### 3.5. Expression of interleukin-4, interleukin-13, interleukin-25 and interleukin-33

The study revealed that there was again an infection x time interaction (P = 0.000) for IL-4, IL-13, IL-33 and IL-25 (Fig. 2c–f) using

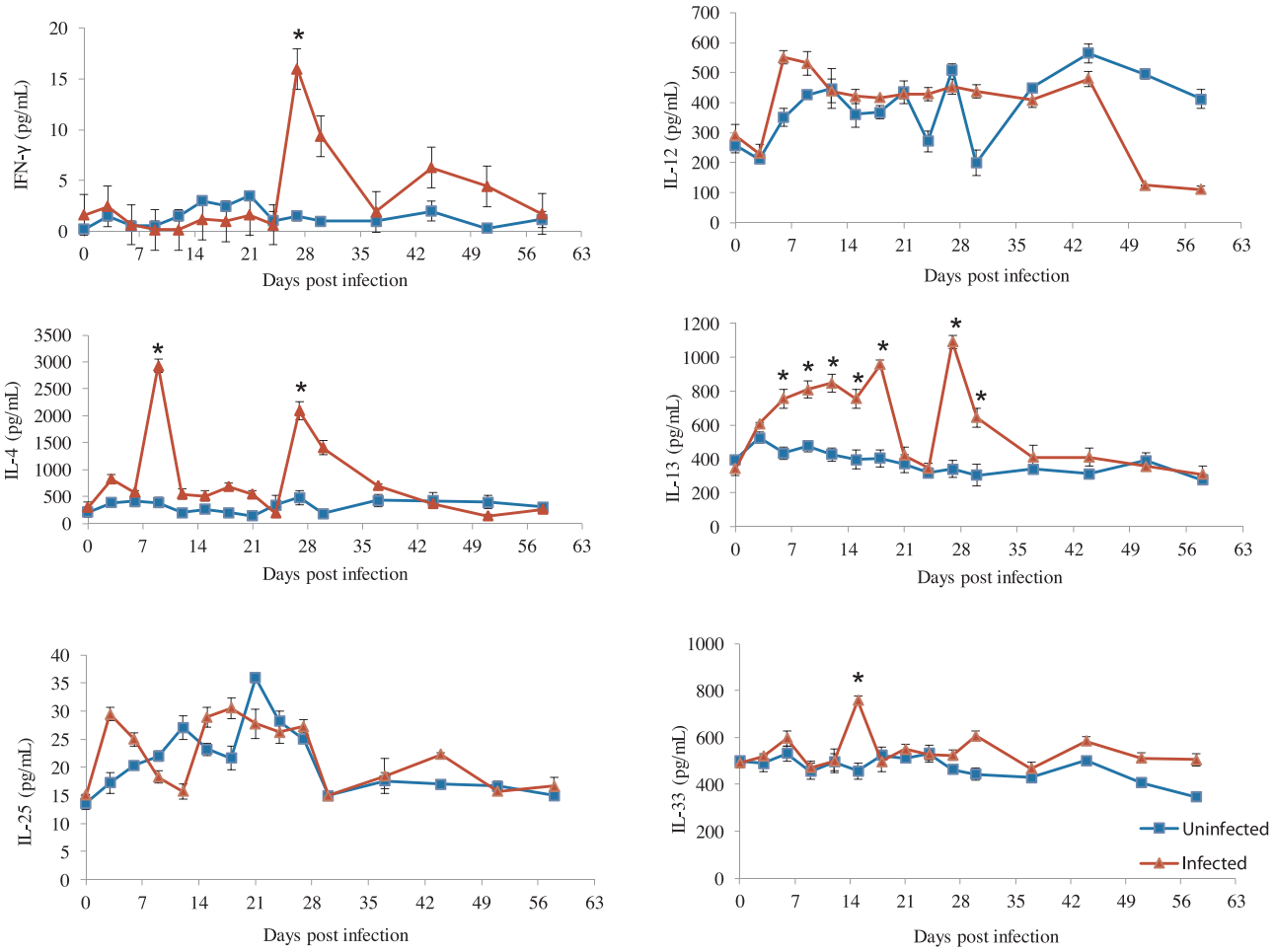


**Fig. 2.** a–f: Mean levels of different cytokines levels in subclinically infected and uninfected goats (* Indicates P < 0.01).

repeated measures ANOVA. Infected goats demonstrated a higher serum IL-4 level on 9 and 27 DPI (P = 0.000 and P = 0.000) in comparison with the corresponding uninfected group. The fold increase was 7 and 5 times in infected group animals on 9 and 27 DPI, respectively. The marker cytokine for haemonchosis *i.e.* IL-13 showed highly increased level of expression in infected animals from 3 to 30 DPI barring a brief period between 21 and 24 DPI (Fig. 2d). The increase was higher on 6, 9, 12, 18, 27 and 30 DPI (P = 0.000). The level was almost same as uninfected animals on 21 and 24 DPI, thereafter, a significant spike on 27 DPI was observed, after which concentration continuously decreased in the infected group till the end of the experiment. There was no sizeable difference observed in the expression of IL-25 cytokine for the infected and uninfected animals (Fig. 2e). Infected goats demonstrated a higher level (P = 0.000) of IL-33 on 15 DPI (Fig. 2f) with the tendency to remain at an increased level in maximum part of experimental trial barring 18 and 24 DPI when compared with uninfected goats.

### 3.6. IgG and IgE expression

For IgG and IgE expression, we observed an infection x time interaction (P = 0.000). Infected group of animals produced relatively higher levels of IgG and IgE compared with uninfected goats throughout the experimental period. IgG level of the infected group increased steadily from 9 DPI (P = 0.000) and maintained during remaining days of the trial with the increase being more prominent on 44 DPI (P = 0.000) compared with the uninfected group (Fig. 3a). IgE level also showed steady increase from 6 DPI (P = 0.003) and peaked on DPI 9 (P = 0.002) in the infected group. The increased level was maintained up to 37 DPI with rise in the expression level on 9, 12, 18 and 30 DPI as compared to un-infected group (Fig. 3b).

## 4. Discussion

In the present study, animals were infected with 250 infective larvae of *H. contortus* /kg BWfor simulating field conditions, as we observed that the majority of the animals in natural conditions were sub-clinically infected, and the EPG noticed was less than 2000 in India (Annual reports of Gastrointestinal Parasitism, 2013; 2014, unpublished data). As reported by various workers (Soulsby, 1982; Wanyangu et al., 1997; Abakar et al., 2000; Shakya et al., 2009), the prepatent period for *H. contortus*in the experiment is 18 days and the peaked on day 30. However, there was a sudden decrease in the EPG on 37 DPI in our study, the reason behind which is not understood; however, the same type of results was also found in earlier studies by Wanyangu et al. (1997) and Shakya et al. (2009). The level of FEC, Hb and PCV is the true phenotypic indicator for the estimation of the severity of haemonchosis (Behnke et al., 2006). In the present study, the elevated level of Hb and PCV up to 9 DPI might be due to development of resilience resulting in endurance in infected animals with the sub-clinical infection and decreased thereafter. Consistent with the present study, a significant reduction was observed in mean Hb and PCV level in haemonchosis till to 56 DPI, thereafter, increased till the end of the trial (Wanyangu et al., 1997). The PCV was positively correlated with body weight and negatively correlated with FEC in haemonchosis in sheep (Vanimisetti et al., 2004; Yadav et al., 2006). We observed that the reduction of weight gain was approximately 26 g per day, if the animal is infected, which reinforces a previous finding of Beriajaya and

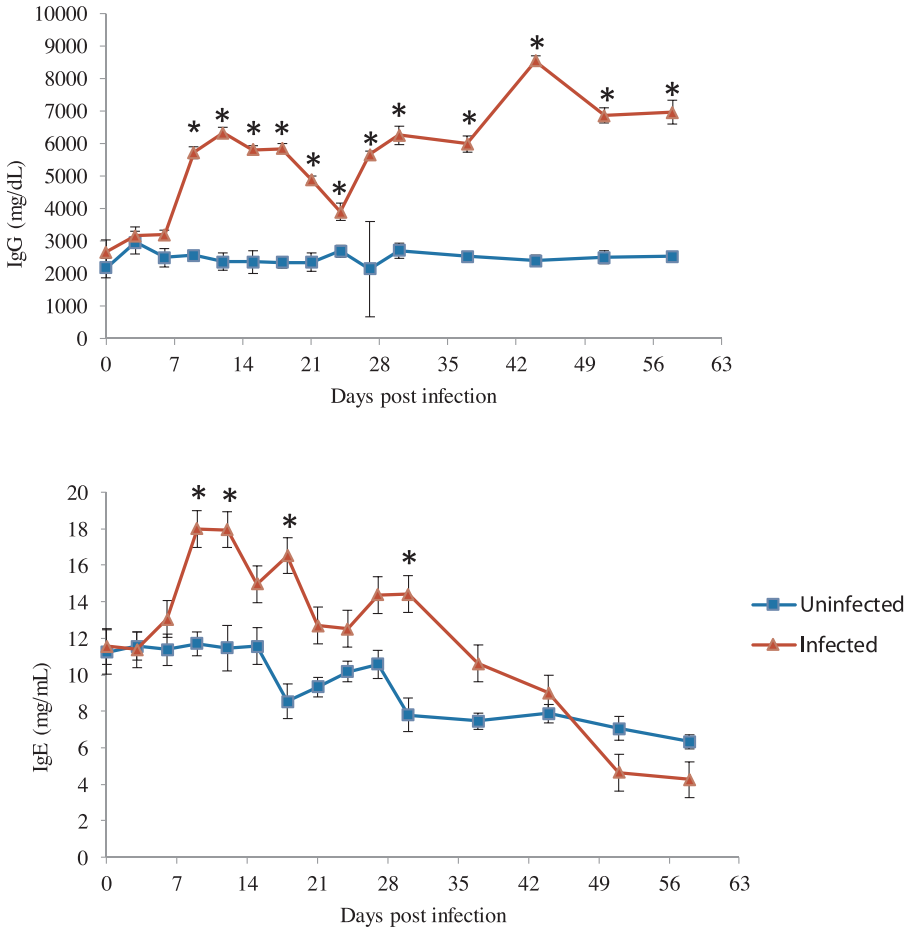


**Fig. 3.** a, b: Mean levels of IgG and IgE in subclinically infected and uninfected goats (* Indicates P < 0.01).

Copeman (2006), where the daily reduction in live weight growth of about 30 g per day in both sheep and goats infected with small burdens of *H. contortus*.

The immune response to *H. contortus* infection is tightly regulated by a complex interplay between various cells, cytokines, immunoglobulins and other immune components of hosts and it varies with different helminth parasites, life-cycle stages and intensities of infection (Meeusen et al., 2005). IFN-γ concentration was more or less constant in the infected group of animals throughout the experiment in comparison with uninfected animals, however, a significant abrupt rise was observed on 27DPI. Similar results were also observed by Shakya et al. (2009) in which, susceptible Suffolk lambs showed non-significantly higher expression of IFN- γ mRNA compared with native lambs. In contrast, decreased the level of IFN-γ was expressed in *in vivo* stimulatedabomasal lymph node and mesenteric lymph node cells from *H.contortus* infected resistant and random-bred lambs (Gill et al., 2000). IFN- γ is one of the core Th1 cytokines, found increased up to 21 fold compared with uninfected and maintained till 28 DPI during *Oestertagia oestertagi* infection in cattle (Almería et al., 1997; Canals et al., 1997; Gasbarre et al., 2001). IL-12 is secreted by macrophages and plays a critical role in Th1 immune response. We observed a little increase at 6DPI in infected group, probably due to larval development in the abomasum and consequent innate immune response by macrophages. The result is corroborated with earlier experiments by Li et al. (2007) and Zaros et al. (2010) where no changes were found in IL-12 level in *H. placei* infected cattle.

IL-4 and IL-13 are known for their role in Th2 immune response and IL-4 along with IL-5 mainly responsible for IgE production during helminth infection (Matsuda et al.,1995; Gill et al., 2000). Up-regulation of IL-4 in haemonchosis has been attributed to increased production of IgE specific to *H.contortus* (Kooyman et al., 1997; Gill et al., 2000) and *T. colubriformis* (Pernthaner et al., 2005). In our experiment, the concentration of IL-4 in the infected group of animals was higher throughout the experimental trial with two highly significant peaks on 9 and 27 DPI compared with the uninfected group. The absolute increase in IL-4 level in the present study suggested an early induction of Th2 immune response. The initial IL-4 expression along with the peak concentration of IgE has indicated a correlation between IL-4 and IgE in helminth infection. An increased early expression of IL-4 gene in abomasum was noticed in resistant sheep breed during haemonchosis (Jacobs et al., 2016). Infected group of animals showed an increased concentration of IgE in the maximum part of the experimental trial compared with the control group and the significant increase was observed on 9, 12, 18 and 30 DPI. In addition, the third integral component of this group, the peripheral eosinophils, whose percentage peak was observed from 15 DPI. These results are consistent with IL-4 up-regulation for IgE production (Kooyman et al., 1997). Shakya et al. (2009) reported a relative increase in IL-4 mRNA expression level in *H. contortus* infected sheep and found significantly higher serum IgE after day 14 and increased expression of IL-4 mRNA on day 10 post-exposure. The similar result was also found in *H. placei* infection in cattle (Zaros et al., 2010). Elevated serum IL-4 concentration was found in *H. contortus* resistant St. Croix sheep after challenge with 10,000 infective larvae (Jacobs et al., 2015), which was associated with lower faecal egg count.

Like IL-4, there was a continuous increase in the concentration of IL-13 up to 18 DPI in the infected group of animals. IL-13 is marker cytokine for helminth infection. The first peak was observed on 18, and second on 27 DPI, after which it continuously decreased in infected group. The role of IL-13 in mediating immunity to nematode infection

was explored in mice (Bancroft et al., 2000; Urban et al., 2000) and sheep (Pernthaner et al., 2005). Pernthaner et al. (2005) found consistently high expression of IL-13 in *T. colubriformis* infected sheep.

Alternatively activated macrophages (AAM) are elicited by Th2 cytokines, including IL-4, IL-13, IL-25, and IL-33, which is highly, produced following helminth infection (Kreider et al., 2007). IL-25 and IL-33 can directly induce AAM gene expression and also activate innate lymphoid cells and CD4 + Th2 cells to promote the Th2 cytokines allowing the control of helminths including GIN *Trichuris muris* (Humphreys et al., 2008). However, in our experiment, no changes were observed in the concentration of IL-25 after infection and maintained a more or less constant level in both groups. A significant IL-33 increase was observed on 15 DPI in the infected group. These two cytokines are important for regulating helminth infections. It was reported that IL-33 is a strong activator of adaptive immunity to intestinal nematodes (Humphreys et al., 2008). To the best of authors' knowledge, this seems to be the first report on the role of IL-25 and IL-33 in haemonchosis.

All of the immunoglobulin isotypes have been reported to be produced after *H. contortus* infection in sheep with variation in chronology, magnitude and time sequence. This variation may be an indication of their importance and role played in different stages of infection. In the present study, the infected group of animals showed a higher concentration of IgG and IgE compared with control throughout the experimental trial. There was a rapid increase in IgG concentration on 9 DPI and thereafter, a steady and significant level was maintained until the end of the experiment. The highest concentration of IgE was observed on 9–37 DPI. Increased level of IgG1 has been reported followed by IgG2, IgM, and IgA in haemonchosis (Schallig et al., 1997). Akin to our results, Kooyman et al. (1997) reported the increased level of IgE specific to *H. contortus* 2–4 weeks post-infection. Similarly, resistant lines of sheep were found to produce a higher level of IgE, IgG1 and mucosal IgA when compared with randomly bred lambs (Gill et al., 2000; Hernandez et al., 2016). On the contrary, all the isotypes were reported to have been produced in sheep; however, they were not found to be significantly correlated with resistance against *H. contortus* (Gomez-Munoz et al., 1999). The interesting and significant note in the present experiment is the immunological numbness observed on 24 DPI. All the major cytokines such as IFN-γ, IL-4, IL-13 and both IgG and IgE were observed at the lowest concentration on 24 DPI. Together, this data speculate that decreased level of major cytokines and immunoglobulins might be due to peak egg production of *H. contortus*. In our study, the peak egg production was observed on 30 DPI and lowest concentration level of different immune mediators was on 24 DPI. However, the precise mechanism behind the diminution of major immune mediators in serum is needed to be investigated. The analysis on the interaction between immunological responses over time points showed a significant impact due to sub clinical haemonchosis. All the parameters except IL-12 were significantly different among sampling time point. Earlier a study showed regulatory T cells (Treg) play a critical role in Th2 immune response post-low-dose infection with the intestinal helminth *Trichuris muris* in mice by time-dependent manner (Sawant et al., 2014). Depletion of Treg reduced worm burden and histopathology in mice during the onset of infection; however Treg depletion later in infection can enhance parasite burden by polarising of Th2 responses.

## Conflict of interest

None of the authors has any conflict with other people or organizations that could unprofessionally influence the content of the paper.

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