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Haemonchus contortus Crude Proteins Produced Partial Protective Immunity against the Experimental Infection

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HMS performed the experiments. JAG and MT planned and supervised the research. BB provided the chemicals and reagents. ZAL and TAK collected the samples. JS analysed the blood samples. JAG and AGA wrote this article.

Key words
***Haemonchus contortus*, Goat, Immunity, Somatic antigen**

ABSTRACT

Haemonchus contortus is a blood sucking abomasal nematode parasite of small ruminants producing the economic losses. The present study was conducted to explore the immunogenic properties of *H. contortus* crude proteins (HcCP). Protein profile of HcCP was checked by SDS PAGE and immunogenic proteins were recognized by the antisera produced by using the HcCP as antigen. Infective stage of the *H. contortus* (L3) was used for the challenge infection. Protein band pattern ranging from 10 to 170 kDa was observed and protein bands at 10-20 kDa, 24, 26 and 40-60 kDa were recognized by the antibodies against HcCP through western blot. Feacal egg count reduction test (FECRT) revealed that egg production was significantly reduced as 75.06%, 76.36% and 57.01 % respectively in vaccinated group as compared to positive control at 3rd, 4th and 5th week of immunization. Impact of the HcCP on haemoglobin level was also evaluated. After second week of infection significant difference ($P<0.05$) was found between the negative and positive control. However no significant difference ($P>0.05$) were found between vaccinated and control groups. Statistically significant difference ($P<0.05$) was observed among positive control group and vaccinated group at 3rd week of post infection. Packed cell volume (PCV) was also recorded and findings indicated that no significant differences ($P>0.05$) was observed among the all groups after the first week of challenge infection. After 2nd week of infection no significant difference ($P>0.05$) was found among the negative control and vaccinated group, however significant differences ($P<0.05$) was found between the positive control and vaccinated group. It is concluded that HcCPs were able to produce the antibodies, that means these proteins are able to produce the immunity and can be used as vaccine against the infection. However the results of FECRT indicated that HcCP produced partial immunity against the infection.

INTRODUCTION

Livestock is considered very important subsector of agriculture, it shares about 58.55% of the agriculture and produced about 12% to gross domestic product (GDP) of Pakistan during 2015-2016. Livestock play important part in the rural economy of Pakistan and it has been reported that nearly 30 to 35 million rural population is engaged in this sector (Jan *et al*., 2015). However, this sector plays a key role in the development of national economy in the form of draught power, animal proteins, hides, wool, bones and manure (Sarwar *et al*., 2002). Small ruminants particularly goat is considered as a cow of poor man and usually considered that goat farming is a major source of income for the rural population (Hasnain and Usmani, 2006). Gastrointestinal (GI) helminthes particularly nematodes cause considerable production decline (Rehman *et al*., 2016; Chartier *et al*., 2000) which negatively affects the farmers of developing countries. Goat and sheep have low ability to support the immune response against nematodes, that's why these animals are more susceptible for gastrointestinal nematodes (GIN) infection (Hoste *et al*., 2008).

Approximately 70 % nematode species have been recovered from small ruminants, among them over 30 % have been isolated from the digestive system worldwide (Taylor *et al*., 2007). GIN infection ranks top on a global index and among them *Haemonchus contortus* has devastating effect

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(Perry and Institute, 2002) in terms of low productivity, decline in weight gain, and heavy infection leading to death (Idris *et al*., 2012). *H. contortus* is one of the very important nematode due to its feeding habit, female of this parasite is very voracious to suck the host blood and causing the anemia, bottle jaw and death if properly not treated in the warm and humid climates (Onyenwe *et al*., 2017).

Various factors have been involved in the genetic variation of *H. contortus* such as geography, population, host movement, different host species being reared together and grazed. In previous studies it was reported that geographical region and host movement was correlated for the gene variation in the *Haemonchus* populations (Brasil *et al*., 2012; Gharamah *et al*., 2012; Yin *et al*., 2013). The load of infection or prevalence of *Haemonchus* significantly varied in different areas depending on many factors like nutrition, pasture management, climatic condition and, host immunity (Radostits *et al*., 2006).

Haemonchus has a direct life cycle lasting approximately four weeks. Three developmental stages of the *H. contortus* larvae (L1, L2 and L3) takes place on the ground. The 3rd stage larvae (L3) are infective stages ingested by the host during grazing. After ingestion, L3 moves to the abomasum and moults into the 4th stage of larvae (L4), this stage is the hematophagous and starts to feed on blood. Approximately 3 weeks after infection the worms become adult and the female starts to release eggs. For the survival of *H. contortus* at different developmental stages it may require rapid transcriptional changes, for that purpose every developmental stage has different motility, sensory and hormonal regulation requirements (Schwarz *et al*., 2013).

Currently haemonchosis has been considered as economic threats on livestock production. The adult female of *H. contortus* is capable to produce over 5000 eggs daily (Hale, 2006) and approximately 10,000 adult worms can kill a sheep (Fitch, 2006). Immune response against GIN, has some unique features particularly in the recruitment and activation of 'allergic' or type-2 effector cells (Anthony *et al*., 2007c; Hein *et al*., 2010; Meeusen *et al*., 2005a).

The sheep acquire resistance to the *H. contortus* infection associated with breed, age and exposure to previous infection, however immunological phenomenon is not very clear (Alba-Hurtado and Muñoz-Guzmán, 2013; Andronicos *et al*., 2010; Saddiqi *et al*., 2011). Several events are required for the clearance of nematode from the immunized host including the activation of nonspecific defense mechanisms, somatic and excretion/secretion antigens recognition by dendritic cells which act as antigen presenting cells (APC) for T cells and the induction of an appropriate acquired response (Meeusen *et al*., 2005b).

From the previous studies, it is generally considered that helminth infection generally leads to type 2 (Th2) responses (Andronicos *et al*., 2010; Kemper *et al*., 2009). The responses are the part of the humoral response associated with the helminths, involving immune system cells and cytokines (Anthony *et al*., 2007b). Th2 responses are stimulated by the secretion of the IL4, IL-5, IL-10 and IL-13 (Munoz-Guzman *et al*., 2012; Shakya *et al*., 2011). IL-4 and IL-13 are key regulators in humoral immunity, induce B-cell class switching to IgE and regulate MHC class II production (Anthony *et al*., 2007a, b; Moreau and Chauvin, 2010).

Anthelmintic resistance of GIN is a global problem and it is very difficult to afford costs associated with repeated medication for many farmers (González-Sánchez *et al*., 2018). The effective control of haemonchosis will require an integrated approach including immunoprophylaxis. Furthermore, under experimental conditions, vaccination with different larval (L3) and adult antigens of *H. contortus* induces significant levels of immune protection (Cachat *et al*., 2010; Newton and Munn, 1999; Schallig and Van Leeuwen, 1997) The present study was conducted to evaluate the immunogenic properties of *H. contortus* crude proteins (HcCPs).

MATERIALS AND METHODS

Collection of adult H. contortus

Abomasums of the goats were collected from the abattoir of Hyderabad-Pakistan and transported to the laboratory of Department of Veterinary Parasitology, Sindh Agriculture University Tandojam. Male and female adult worms were collected from the infected abomasum according to method describe by Souza *et al*. (2015). Female worms were identified by their barber pole appearance and bursa with long spicules of males (Han *et al*., 2011). Worms were placed in petri-dishes containing phosphate buffer saline (BPS). Then adult male and female worms of *H. contortus* were stored at -20° for further process.

Production of H. contortus *crude proteins (HcCP)*

After collection worms were washed with PBS and crude proteins were prepared separately from male and female worms and also from the mixture of male and female worms. For that purpose, worms were homogenized in 5ml PBS, and the homogenate was centrifuged at 13000 rpm for one hour at 4°C and the supernatant was collected as adult crude proteins or antigen. Protein concentration was checked by using Nano Drop Spectrophotometer and stored at -20°C.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

HcCP were diluted with an equal volume of SDS sample buffer and boiled for 5 min. After boiling, sample were cooled at room temperature and centrifuged at 10000 rpm for 5 min and the extract were used for SDS PAGE or aliquoted into 200 µL and stored at -20°C until the time of electrophoresis. After SDS PAGE the gel were stained with coomassie brilliant blue stain for 2 to 4 h. After completing the stain, the gel were destained for overnight on 5% methanol, 7.5% acetic acid, 87.5% distilled water. Protein band pattern were checked by using the gel documentation system by using the proteins marker.

Production of L3 for challenge

Faecal samples were collected from the infected goat and confirmed by parasitological examination for eggs per gram (EPG). The faecal sample containing *H. contortus* egss were cultured for the L3 production at room temperature. During the culture time regularly checked for the desiccation and moistened if needed. After two weeks of incubation L3 were recovered by using the Baermann technique for 24 h. The L3 were washed with PBS and stored at 4°C for further use.

Immunization trail and challenge infection

Nine local goat breed were used for immunization trial. Goats were divided into 3 experimental groups (3 goats in each group). Group 1 was immunized with HcCP antigen, Group 2 received only adjuvant and used as challenged control, while Group 3 were unchallenged and unvaccinated negative control. Goats of group 1 were immunized on days 0, 14, and 28. The first injection (400 µg HcCP) was administered in 1 mL Freund's complete adjuvant; the second and third injections were administered in 1 mL Freund's incomplete adjuvant. On the same days goats from Group 2 received only adjuvant. On day 42, animals from Groups 1–2 were challenged with 16000 L3 of *H. contortus* oraly. Serum containing antibodies against HcCP (IgG_{HcCP}) were collected one week post last injection and stored at -20°C. Blank sera were collected from control group (uninfected) and kept as negative control. The quality of the generated antibodies was checked by western blot technique.

Feacal sample collection

Faecal samples were collected 1st, 2nd, 3rd, 4th and 5th week post infection and faecal egg counts were made by using the McMaster method (Eysker *et al*., 1989) and expressed as eggs per gram faeces (EPG). Feacal egg count reduction test (FECRT) was also conducted by using following formula.

$$\frac{\text{Positive control} - \text{Vaccinated group}}{\text{Positive control}} \times 100$$

Blood sampling for hematological analysis

For hematological analysis blood samples were collected from jugular vein weekly. Haemoglobin concentration and packed cell volume (PCV) were checked by standard laboratory techniques at Laboratory of Veterinary Physiology, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University, Tandojam.

Western blot analysis

Twenty microgram of HcCP were separated by 12% SDS-PAGE gel. Then the protein bands were transferred to nitrocellulose membranes. Membranes were blocked for non-specific binding sites by using 5% (w/v) skim milk powder in Tris-buffered saline (TBS) for 1 h at room temperature. After blocking the membrane were washed 5 times (5min each) with TBST and were incubated with the primary antibodies (IgG_{HcCP}) at 1:100 in blocking buffer for 1 h at room temperature. After being washed as above, the membrane were incubated with horseradish peroxidase (HRP)-conjugated anti-goat IgG as second antibody for 1 h at room temperature. After washing, finally the bound antibody were visualized using freshly prepared 3,3 diaminobenzidine tetrahydrochloride (DAB) as a chromogenic substrate.

Statistical analysis

All statistical analysis were performed by Graphpad Prism 5 software (USA). The difference of the data between all groups were compared by one way ANOVA. The result in comparison between groups were considered different if the p value was less than 0.05.

RESULTS

SDS PAGE analysis of HcCP

Crude proteins were prepared from the homogenate of adult worms of *H. contortus* separately from male and female. Protein concentrations were checked by using Nano Drop Spectrophotometer and results revealed that the concentration of proteins were 5.30 and 3.07 µg/µl from female and male worm, respectively. Protein profile of HcCP were checked by SDS-PAGE and the results showed different band pattern ranging from 10 to 170 kDa (Fig. 1).

Western blot analysis of HcCP

Western blot technique were used to identify the

immunogenic proteins of HcCP and our findings revealed that protein bands at 10-20 kDa, 24, 26 and 40-60 kDa were recognized by the antibodies against HcCP (Fig. 1). Western blot results confirmed that the molecules or protein of HcCP were able to produce the antibodies against them, that means these proteins are able to produce the immunity and can be used as antigen against the infection.

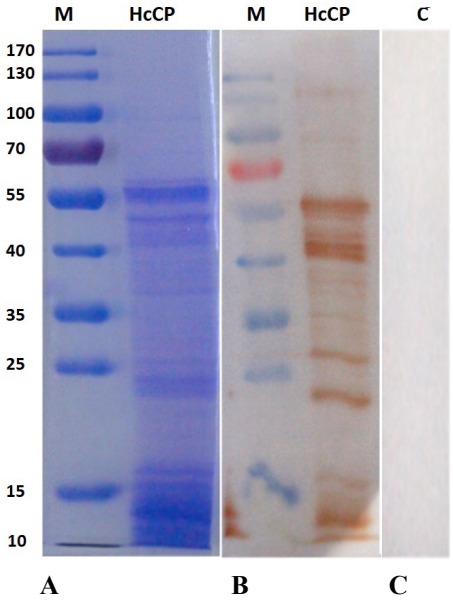

Fig. 1. Protein band pattern of HcCP analyzed by SDS-PAGE (A) and Western blot analysis of HcCP (B). M: Marker, HcCP: *H. contortus* crude proteins, C: Control using the normal goat sera.

Impact of HcCP on the shedding of eggs of H. contortus

In the present study, effects of the HcCP on the eggs output of *H. contortus* were evaluated. Eggs per gram (EPG) were determined by using the McMaster technique. For this purpose faecal samples were collected 1st, 2nd, 3rd, and 4th week after the injection of HcCP.

The finding of our research revealed that at 1st and 2nd week post immunization no eggs output were observed. Eggs were detected at 3rd week of immunization and 533.33± 33.33 EPG was recorded in the positive control and 133± 33.33 EPG was found in the vaccinated group. However, in the negative control group, no eggs were observed (Fig. 2A). Our findings indicated that egg production was significantly reduced (75.06%) in vaccinated group as compared to positive control.

At 4th week of immunization, in immunized group eggs output were recorded as 933.33±33.33 EPG and in case of positive control the EPG were recorded as 3950± 812.91, wich was significantly higher (Fig. 2B). Results revealed that egg shedding significantly decreased (76.37 %) in vaccinated group as compare to the positive control group.

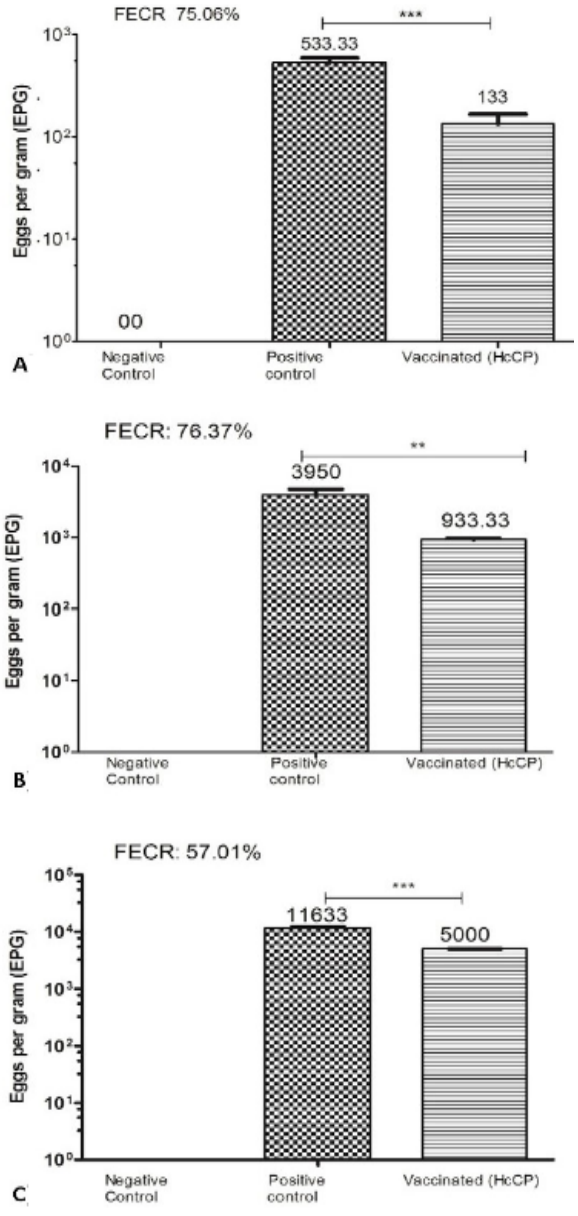

Fig. 2. Impact of HcCP on egg production at 3rd (A), 4th (B) and 5th (C) week post immunization. Eggs per gram (EPG) was shown as mean ± SD. FECR: Feacal egg count reduction. The data was calculated from three independent experiments (**$p<0.001$, ***$p<0.0001$).

At the 5th week of immunization, found that in vaccinated group eggs output were observed as 5000±

50.24 EPG and in case of positive control the eggs output were found very high (11633±633.33 EPG), which were significantly higher. However, non significant results were observed between the vaccinated and negative control group (Fig. 2C).

Effects of HcCP on blood parameters of goat

Impact of the HcCP on the blood parameter were also evaluated, After challenge infection blood samples were collected from all animals weekly. Blood samples were analyzed for hemoglobin (Hb) and PCV. As concerned Hb level, findings revealed that after one week of the infection no significant differences (P>0.05) were observed between the groups, Hb level were found as 9.80± 0.14 g/dl, 9.43± 0.23 g/dl and 9.79± 0.13 g/dl in negative control, positive control and vaccinated group. After second week of infection significant difference (P<0.05) were found between the negative control (10.61± 0.23 g/dl) and positive control (9.48± 0.12 g/dl), however no significant difference (P>0.05 g/dl) were found between vaccinated (10.13± 0.57 g/dl) and control groups. Statistically significant difference (P<0.05) were observed among positive control group (8.26±0.14 g/dl) and vaccinated group (9.34±0.23) at 3rd week of post infection, whenever Hb level were found significantly (P<0.0001) higher (11.32± 0.41) in negative control. At 4th and 5th week of infection, the Hb level were significantly (P<0.0001) decreased in positive control and vaccinated group as compare to the negative control group, however no significant (P>0.05) difference were recorded between the positive control and vaccinated group (Fig. 3).

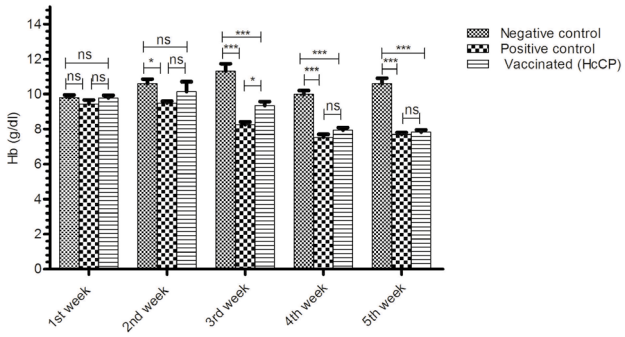

Fig. 3. Hemoglobin (Hb) level of the trial goats. The data are representative of three goats (ns P>0.05, *P<0.05, *** P<0.001).

PCV were also recorded during the trial period and research findings indicated that no significant differences (P>0.05) were observed among the all groups after the first week of challenge infection. After 2nd week of infection no significant difference (P>0.05) were found among the negative control (35.03±0.32%) and vaccinated group (34.56±0.18%), however significant differences (P<0.05) were found between the positive control (33.12±0.12%) and vaccinated group (Fig. 4).

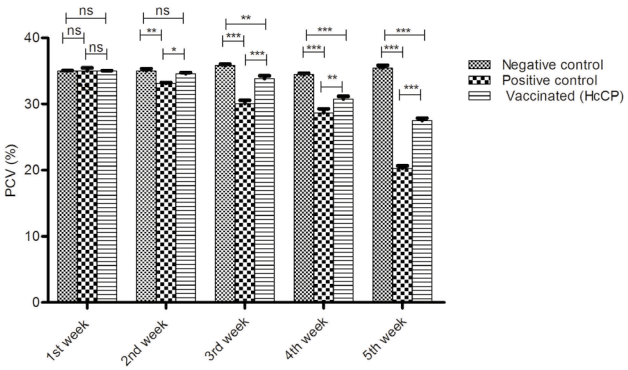

Fig. 4. Packed cell volume (PCV) of the trial goats. The data are representative of three goats (ns P>0.05, *P<0.05, *** P<0.001).

DISCUSSION

Haemonchosis is one of the most important and highly pathogenic diseases of small ruminants caused by *H. contortus*. Heavily infected animal can lose over 250 mL of blood per day, leading to decreased wool production, reduced carcass quality, anemia and death (Bambou *et al*., 2013). It was reported that small ruminants particularly sheep get resistance to the *H. contortus* related with breed, age and previous expose, however this immunological phenomenon are not very clear. Furthermore it was considered that genetic resistance might be closely associated to the host immune response (Williams, 2011). Here we have evaluated the immunogenicity of the HcCP.

In the present study HcCP were prepared from the homogenate of adult worms of *H. contortus*. Protein profile of HcCP were checked by SDS-PAGE different band pattern ranging from 10 to 170 kDa were observed and western blot analysis was used to identify the immunogenic proteins of HcCP and protein bands at 10-20 kDa, 24, 26 and 40-60 kDa were recognized by the antibodies against HcCP.

Previously, *H. contortus* excretory and secretory proteins (HcESPs) were analyzed and protein band patterns ranging from 13 to 180 kDa were detected by using the antibodies against the HcESPS (Gadahi *et al*., 2016c). *H. contortus* somatic antigen (Hc-23) was previously recognized as immunogenic component of HcCP and produced partial immunity in the trial goats (Fawzi *et al*., 2014a). Roberts *et al*. (2013) reported that *H. contortus* 11 kDa protein (aminopeptidase H11) as hidden antigen

and expressed in free living nematode (*Caenorhabditis elegans*). They suggested that recombinant protein of this hidden antigen could be used as vaccine candidate.

Previously, various scientists identified that these HcESPs as well as HcCPs are able to interact with the host cells during the infection and has properties to modulate the host immune response (Bassetto *et al*., 2011; Ehsan *et al*., 2017; Gadahi *et al*., 2016a, b; Godoy *et al*., 2015; Han *et al*., 2011; Souza *et al*., 2015; Yan *et al*., 2014).

In the current study antisera against the HcCP recognized the various somatic proteins indicated that somatic proteins *H. contortus* interacted with the host cells and produced the antibodies that means HcCP part play an active role in humoral immunity. Identification of these protein and phenomenon involved in the immunity need to be further investigation.

In the present study effects of the HcCP on the eggs output was evaluated. The finding of our study indicated that eggs shedding were started after the 3rd week post challenge. After 3rd week of infection, 75.06 % of faecal egg count reduction (FECR) was recorded in the vaccinated group as compared to positive control. However after 4th and 5th week of infection 76.36 % and 57.01 % FECR were found in the vaccinated group.

Previously, recombinant somatic antigen of *H. contortus* (rHcp26/23) was used as a vaccine in lambs and found no significant protective immunity and found higher eggs shedding in the immunized group than the control group (García-Coiradas *et al*., 2010). Contrary to the above findings, somatic protein (Hc23) isolated from the adult *H. contortus* was also used as vaccine and reported 70.67%–85.64% reduction in egg output at 45 days post infection (Fawzi *et al*., 2015; Fawzi *et al*., 2014b).

Previously, recombinant protein *H. contortus* 26 / 23 kDa (rHc26/23) and larval antigen was evaluated for immunization against infection and results of this study had shown 92.2% and 38.2% faecal egg count reduction (Kandil *et al*., 2017). Arab *et al*. (2013) evaluated the protective characteristics of *H. contortus* purified antigen in sheep and found that purified 26 kDa protein induced the partial immunity against the *H. contortus* infection resulted in 41.4% of faecal egg count reduction.

In the present study egg shedding reduction was recorded as 75.06 %, 76.36 % and 57.01 % at 3rd, 4th and 5th week post infection in the immunized goats. The findings indicated HcCP induces the partial immunity against the infection. On the basis of our findings we suggested that HcCP is a complex of numerous proteins, which proteins are able to induce the immune response need to be explored. Binding partner of these proteins in the host and the function of pathway by which they interacted may also be unveiled.

H. contortus is a hematophagous nematode parasite of the abomasum of small ruminant and causes severe anemia resulting in increased morbidity and mortality rates in a flock . A single worm is able to feed 0.05 ml blood daily, furthermore if the animal infected with 5000 worms resulted in loss of 250 ml of blood per day, ultimately infected animal become severely anemic in a short period (Clark *et al*., 1962).

In the haemonchosis blood loss and worm egg shedding are closely related each other, however blood analysis for the anemia may be useful indicator to assess the infection level (Rodríguez *et al*., 2015). Worm burden is not detected until the egg shedding, not although egg shedding needs 3 to 4 weeks, while as L_3 moult into L_4 has start to feed on blood meal and host become anemic during prepatent period (Hoste *et al*., 2001).

In the current study impact of the HcCP on the blood parameter was also evaluated. Blood samples were analyzed for the Hb and PCV. Our findings revealed that no significant differences was observed between the groups at the 1st week of challenge. However, after second week of challenge significant difference was found between the negative control and positive control, however no significant difference were found between vaccinated and control groups. Statistically significant difference was observed among positive control group and vaccinated group at 3rd week of post infection. At 4th and 5th week of infection, the Hb level was significantly decreased in positive control and vaccinated group as compare to the negative control group, however no significant difference was recorded between the positive control and vaccinated group.

Similarly to that of Hb results PCV % also decreased in all challenged groups, however at the 2nd week of post infection PCV level was significantly decreased in positive control as compare to vaccinated group. Previously, *H. contortus* somatic antigen rHcp26/23 was used as vaccine and animals were challenged with infective larvae (L_3) and reported transient fall in PCV and Hb level without differences between the gropus during the prepatent period (García-Coiradas *et al*., 2010). Results of this study are also in contrast with the results of Domínguez Toraño *et al*. (2000).

The hematological results of our study indicated that HcCP antigen might produce the partial immunity during the prepatent infection (early immunity), actual function of individual proteins of HcCP could be investigated to find out that these proteins increase or suppress the immune response during the infection.

CONCLUSION

Finally it has been concluded that results of present study indicated that HcCP induces the partial immunity against the infection. On the basis of present findings it has been suggested that HcCP is a complex of numerous proteins, which proteins are able to induce the immune response need to be explored. Binding partner of these proteins in the host and the function of pathway by which they interacted may also be unveiled.

Statment of conflict of interest

The authors declares no conflict of interest.

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