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IMMUNOLOGICAL RESPONSE OF SELECTED INDIGENOUS GOAT BREEDS OF PAKISTAN TOWARDS ARTIFICIAL INFECTION WITH HAEMONCHUS CONTORTUS

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The present study was planned to determine the immunological response of Dera Din Panah (DDP) and Nachi breeds of goats towards artificial infection with *Haemonchus (H.) contortus* which is considered among the most widely prevalent gastrointestinal nematodes (GINs) associated with the lowered production, elevated morbidity and mortality in goats under various farming systems. To this end, a total of 48 goats of DDP and Nachi breeds (24 of each breed) were administered with third stage infective larvae of *H. contortus* through early and late infection protocols. The serum and plasma samples were tested for immunoglobulins isotypes and histamine concentration through ELISA test, respectively. Adult worm antigens were used to detect the different antibodies in infected and control goats while histamine concentration was measured through commercially available histamine-ELISA kit. Results revealed that both breeds reflected a significant (P<0.05) difference in expression of IgG, IgE antibodies and concentration of histamine. Nachi breed showed a compromised immune response towards artificial infection with *H. contortus* as compared to DDP. Overall, lower production of antibodies (IgG, IgE) and plasma histamine depicted that Nachi goats are comparatively more susceptible to *H. contorts* infection as compared to DDP goats. In conclusion, difference in immune response towards *H. contortus* infection may formulate the basis of selective breeding of resilient goat breed (DDP) in the area.

Key words: Immunoglobins, Histamine, *Haemonchus contortus*, Nachi, Dera Din Panah, breed susceptibility.

INTRODUCTION

Goat (*Capra hircus*) is a multiuse animal which share significant role in the livelihood of resource poor farmers in developing countries like Pakistan. Nature has blessed Pakistan with 37 indigenous breeds of goats with an estimated population of 76.1 million heads (Anonymous, 2019) and ranked 3rdamong major goat producing countries, worldwide (FAO, 2011). Parasites have been recognized among major constraints to profitable livestock production systems round the globe (Nasir *et al*., 2018; Zafar *et al*., 2019; Ahmad *et al*., 2019; Batool *et al*., 2019; Li *et al*., 2019a, Li *et al*., 2019b). Gastrointestinal nematodes (GINs) impose severe constraints on goat production systems round the globe. Among various GINs, *Haemonchus (H.) contortus* is arguably the most prevalent and economically important parasite infecting the goat population worldwide (Shamim *et al*., 2018; Imran *et al*., 2018). As a voracious blood feeder, *H. contortus* is the principal cause of anaemia, hyper-gastrinaemia and alteration in GI secretions which leads to poor growth, compromised production/reproduction, elevated morbidity and mortality in infected host (Guo *et al*., 2016).

Haemonchosis leads to considerable economical losses in terms of poor weight gain (Nehra *et al*., 2019). Worldwide, 15% to 90% of the economy depends upon the livestock farming in different countries and 30-50% of the economic losses have been attributed to haemonchosis (Gebresilassie and Tadele, 2015; Ashram *et al*., 2017). The ultimate cost of 8900.09 million PKR losses has been reported from different districts of Pakistan (Qamar *et al*., 2011). Principally, GINs are controlled through anthelmintics (Ijaz *et al*., 2018) and pasture management, but emerging issue of anthelmintic resistance is of major concern (Verma *et al*., 2018). Furthermore, in developing countries like Pakistan, most of the goat farmers are resource poor and lack access to anthelmintic drugs or land management practices for mitigating the influence of GINs (Crook *et al*., 2016).Therefore, there is dire need to explore alternative parasite control strategies such as breeding for parasite resistant goats. Genetic resistance to GINs is well studied in both experimental (Riggio *et al*., 2013) and commercial flocks (Dlamini *et al*., 2019). Various studies have demonstrated that goat breeds are either resilient, resistant or susceptible to GINs (Chiejina *et al*., 2010; Chauhan *et al*., 2011; Periasamy *et al*., 2014). One of the promising tools to evaluate the resistance or susceptible status of goats is measurement of host's immune response in response to GINs infections particularly with *H. contortus* (Chevrotière *et al*.,

2012). This immune response is mediated through recruitment of globule leukocytes, mast cells and parasite-specific eosinophils in digestive mucosa (Meeusen *et al*., 2005) and production of parasite-specific immunoglobulins (Igs) particularly IgA and IgE in the infected hosts (Shaw *et al*., 1998). Increased expressions of IgA and IgE are linked with inherent resistance towards GINs infection in goats (Pernthaner *et al*., 2006). Furthermore, presence of histamine is also associated with GINs expulsion through increased abomasal sections and hypermotility (Miller, 1996).

In Pakistan, few goat breeds have been screened for their resilience/resistance towards *H. contortus* based on parasitological parameters (Baber *et al*., 2015; Shamim *et al*., 2016; Imran *et al*., 2018) while no reports are available on expression/ measurement of immune response in resistant or susceptible goat breeds of Pakistan towards natural or artificial infection with *H. contortus*. Thus, the present study was carried out to measure the expression of immunoglobulins and histamine in DDP and Nachi breeds of goats towards challenged infection of *H. contortus*.

MATERIALS AND METHODS

***Experimental design*:** A total of 48 female DDP and Nachi goats (24 of each breed) were housed and acclimatized for four weeks at the Small Animal Housing Facility, Department of Parasitology, University of Agriculture, Faisalabad. Goats were offered concentrate/hay and water *ad libitum* throughout the experiments. All the experimental goats were screened for GI parasites through qualitative, quantitative faecal examination and copro-culture prior to initiation of experiment. Experimental goats were grouped in separate pens based on their weight and other phenotypic characteristics. All the experimental protocols were opted as recommended by the Institutional Animal Care and Use Committee (IACUC), UAF (Anonymous, 2016).

Coproculture for harvesting of L_3 of H. contortus: Third stage infective larvae (L_3) of *H. contortus* were harvested through copro culture technique (Zajac and Conboy, 2011). Isolation of L_3 was carried out through Baermann's technique and stored at 4°C till further use.

***Infection protocol*:** The goats (n=48) were divided into 12 groups (six groups of each breed) with four goats per group. Experimental goats were infected through early and late infection protocols that were further divided into trickle and bolus forms of infection as described elsewhere (Shamim *et al*., 2016). Briefly, on day zero of experiment, an early bolus infection of 18,000 L_3 was administered to the first experimental group of both breeds (D1, N1). In early trickle infection, a total of 12000 L_3 were administered to second experimental group of both breeds (D2, N2) with an initial dose of 6000 L_3 followed by three successive doses of 2000 L_3 on every other day. Furthermore, a late bolus infection of 12000 L_3 was administered to D4, N4 groups followed by 6000 L_3 on day seven of infection to achieve a total of 18000 L_3. While, late trickle infection was given to D5, N5 groups with similar protocol as opted for early infection (mentioned above) except with administration of 6000 more L_3 (2000 L_3 on every other day) during second week of the experiment. Day wise outline of the experimental protocol is depicted in Table 1.

***Collection and processing of blood samples*:** Blood samples (10 mL for each subject) were collected aseptically directly from the jugular vein of goats. Isolation of sera was done in 2 mL Eppendorf tubes and kept at -20°C for further processing. Blood plasma was also collected for histamine determination in experimental goats.

***Immunoglobulin Isotypes Profiling*:** Mature *H. contortus* worms collected from abomasa of slaughtered goats were further used to prepare Adult Worm Antigens (AWA) as described by Kabagambe *et al*. (2000). Briefly, worms were hand-picked by using sterilized forceps and subjected to washing with Phosphate Buffer Saline (PBS). Washed worms were then kept in PBS (25 mL; pH-7.2) and homogenized through a tissue homogenizer. The homogenate was further centrifuged to collect the AWA by providing the suitable conditions and stored at -20°C. Immunoglobulin isotypes profiling was done through ELISA technique as described by Bambou *et al*. (2013) with some modifications. Briefly, 100 µL of crude AWA was used to coat the wells of ELISA microtiter plates followed by washing with 0.05% PBS in Tween 20 three to five times. The wells of ELISA plates were blocked with blocking buffer (bovine serum albumin and PBS) to prevent any non-specific binding and then washed with washing solution. Serum samples were then diluted and dispensed into wells of ELISA plate followed by incubation for two hours. Conjugate antibodies (Fitzgerald Industries International, USA) at dilution of 1:10,000 were then added into the wells of ELISA plate followed by incubation for one hour at the room temperature. After washing, substrate (Chromogen, Ultra TMB) was then added to each well and plates were incubated for 15 minutes. Reaction was stopped through stop solution. Separate ELISA plates were run for isotyping of IgG, IgA, IgM and IgE. Serum of colostrum deprived kid was taken as negative control. Absorbance of plates (optical density) was read through Microplate Reader (Serial No. 14309, Biorad, USA) at 450 nm. Mean optical density (OD) values of the negative control wells of ELISA plate were used to determine the cut-off point for the OD of

Table 1. Chronology of the events to determine the immune response of DDP and Nachi goats towards artificial infection with *Haemonchus contortus* (activity is indicated by "×" while "-" showed no activity)

Days	Activity	Groups					
		Early infection		Early Control	Late infection		Late Control
		Early Bolus *(N1, D1)	Early Trickle (N2, D2)	(N3, D3)	Late Bolus (N4, D4)	Late Trickle (N5, D5)	(N6, D6)
1	Faecal/blood collection, Deworming	×	×	×	×	×	×
2	Deworming	×	×	×	×	×	×
9 (7 days PD*)	FEC	×	×	×	×	×	×
16 (14 days PD)	FEC	×	×	×	×	×	×
23 (21 days PD)	FEC+ Blood	×	×	×	×	×	×
24	Artificial Infection *(H. contortus*)	18000 L_3*	6000 L_3	-	12000 L_3	6000 L_3	-
25	-	-	2000 L_3	-	-	2000 L_3	-
26	-	-	2000 L_3	-	-	2000 L_3	-
27	-	-	2000 L_3	-	-	2000 L_3	-
31 (7 days PI*)	-	×	×	×	6000 L_3	×	×
32	Artificial Infection *(H. contortus*)	-	-	-	-	2000 L_3	-
33	-	-	-	-	-	2000 L_3	-
34	-	-	-	-	-	2000 L_3	-
38 (14 days PI)	Blood	×	×	×	×	×	×
52 (28 days PI)	Blood	×	×	×	×	×	×
66 (42 days PI)	Blood	×	×	×	×	×	×
80 (56 days PI)	Blood	×	×	×	×	×	×

*N_{1-6} = Nachi; D_{1-6} = Dera Din Panah; PD = Post Deworming; PI = Post Infection; FEC = Faecal Egg Count; L_3 = Third stage infective larvae

ELISA method. A test sample was considered positive at the cut off value taken by adding the mean OD value.

***Plasma histamine*:** Histamine concentration in infected and negative control groups was measured through a commercially available histamine-ELISA kit (Labor Diagnostika, Germany) as per manufacturer's guidelines. Briefly, 25µL of acylated standard, control and plasma samples were poured into the wells of reaction plate. 100 µL of histamine antisera was then added into each well of plates and covered with an aluminum foil. Incubation of plate was carried out at room temperature for three hours. Washing of the plate was done four times through 300 µL with the washing buffer and dried by tapping of the inverted plate on an absorbent material. Enzyme conjugate (100 µL) was then added into each well of the plate. Again, washing of the plate was done four times through 300 µL of the washing buffer. Reaction plate was dried through multiple inverted tapping on an absorbent paper. 100 µL of the substrate was then added into each well of the plate in the dark place followed by incubation at room temperature for 25 minutes on a shaker. 100 µL stop solution was then added to stop the reaction. After 10 min, absorbance (optical density) of the plate was read through Microplate Reader (Serial No. 14309, Biorad, USA) at 450 nm. Results were presented as optical density (OD) for each sample by drawing of standard curve between OD values and histamine concentration.

***Statistical Analyses*:** ELISA results were analyzed and described in terms of mean OD values for immunoglobulins isotypes and histamine concentration and data were subjected to analysis of variance. All statistical procedures were carried out through SAS statistical software package (SAS, 2010).

RESULTS

The AWA was quantified as 4.022 mg/mL. The level of IgG was significantly (P<0.05) higher at 4th week Post Infection (PI) in early bolus group of DDP goats as compared to Nachi goats infected with same infection protocol. While, in case of early trickle groups of both breeds, the difference in values of IgG was significant (P<0.05) at 6th week PI. In late bolus and late trickle groups of both breeds, significant (P<0.05) difference in level of IgG was observed at 4th week PI (Fig. 1). The values of IgG in control goats was almost persistent at baseline which were different significantly (P<0.05) than those of infected groups of both breeds. Moreover, the difference in IgG levels started increasing from 2nd to 4th week

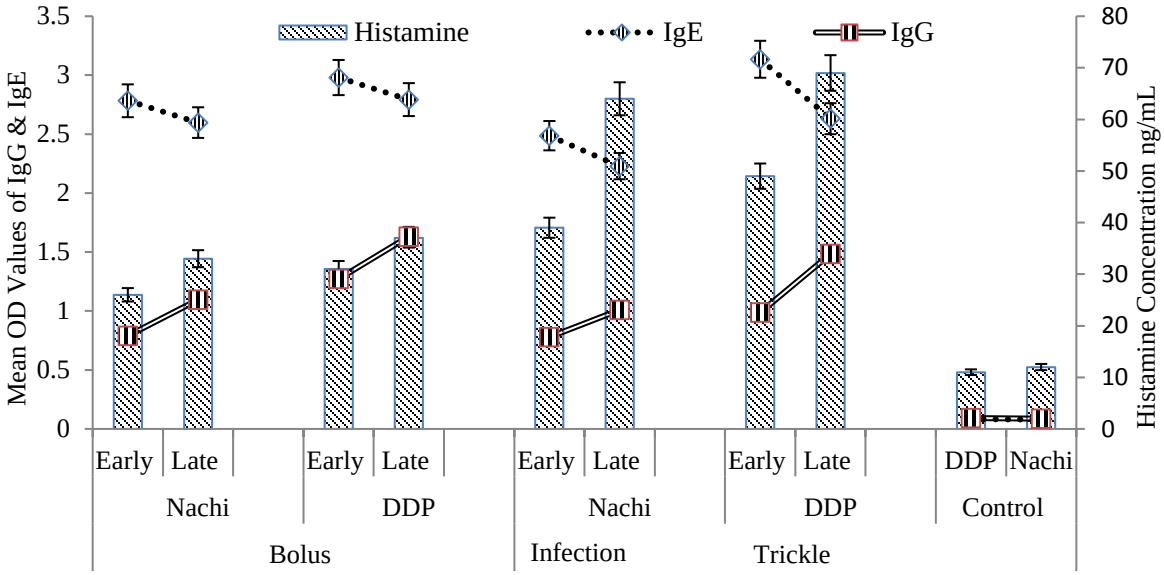

Figure 1. Comparison of immunoglobulins and concentration of histamine (means ± S.E) in Nachi and DDP goats infected with challenged infection of *Haemonchus contortus* through bolus and trickle infection protocols.

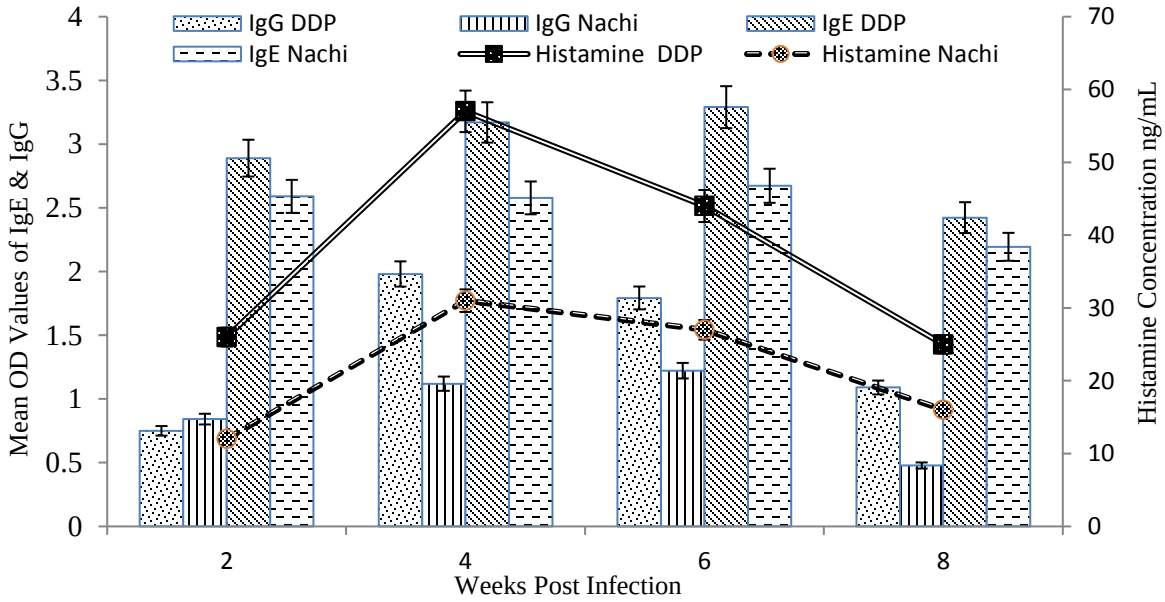

Figure 2. Comparison of immunoglobulins and concentration of histamine (means ± S.E) in Nachi and DDP goats infected with challenged infection of *Haemonchus contortus* at different time intervals.

PI but started declining at 6th to 8th week PI (Fig. 2). This pattern of increase/decline was more pronounced in Nachi goats as compared to DDP goats. In case of levels of IgE, a non-significant (P>0.05) difference was observed within early infected groups of both breeds of goats as well as different time intervals (weeks) PI. However, the difference in IgE levels was significant (P<0.05) in late bolus and trickle infected groups of DDP than same groups of Nachi goats (Fig.1). The differences were significant (P<0.05) in the concentrations of plasma histamine in both breeds at different time intervals (Fig. 2). However, no significant (P>0.05) differences were noticed within infected groups of goats. It is evident from the results of the current experiment that a persistent level of histamine was there in plasma of infected groups of both breeds of goats. However, higher concentration of plasma histamine was recorded at mid of the experiment (4-6th weeks).

DISCUSSION

The ability of a host to prevent the establishment of infective larvae in its GI tract is known as resistance. Resistant animals not only prevent the establishment of infection but also eliminate already established parasites (Maizels *et al*., 2012). Most frequently used markers for determination of host resistance are parasitological and immunological (Shamim *et al*., 2016). Immune system plays a significant role in the control of parasitic diseases particularly with those of GINs. Alternatively, expression of this system may be used for determination of inherited resistance of host (Kemper *et al*. 2010). A Th type 2 (Th2) antibody dependent response is exhibited during infection with GINs (Andronicos *et al*. 2010). It is documented that in resistant and immunized animals, level of antibodies (Abs) remained low during first week of post infection (Lacroux *et al*., 2006). The production of Abs at different time intervals PI was observed during 4th and 6th week. Pernthaner *et al*. (2006) reported that after 28 days PI, the production of Abs is increased.

In the present study, high level of IgG indicated its systemic origin (Smith, 1977). Resistant animals produced all types of Igs (Gomez-Munoz *et al*.,1999). During this investigation, IgG level remains elevated throughout the course of infection. It has been reported that in *H. contortus* infected sheep, the elevated level of IgG1 has also been reported by Schafer *et al*. (2015).In GINs infection, IgE is the most investigated Ig, and the level of IgE is expected to be elevated during infection. Level of IgE during haemonchosis has also been previously reported (Rodrigues *et al*.,2017; Nehra *et al*., 2019). Furthermore, IgE levels may increase up to 100-folds in case of helminths infection which is higher among other Igs (Shrivastava *et al*., 2018). However, the serum level of IgE is seems very low as compared to IgG levels. In small ruminants, the increased levels of IgE have been associated with resistance to GINs (de la Chevrotiere *et al*., 2012).

During current investigation, the level of IgE was observed to be significantly (P<0.05) increased in DDP goats compared with Nachi at different time intervals PI. The findings of the study are similar to that of Nehra *et al*. (2019) and Shrivastava *et al*. (2018). However, Albuquerque *et al*. (2019) have reported contradiction with the findings of the current study and reported that IgA is involved in local immune response against parasitic infection. The time mandatory to activate immune response and extent of that immune response is different in different breeds (Bowdridge *et al*., 2008). Furthermore, diverse mechanism of imitation of immune response is reported by different researchers.

It is recognized fact that infection with mature parasites leads to initiation of type 2 immune reaction (Lacroux *et al*., 2006) with greater levels of mastocytosis eosinophilia, IgG, IgA and IgE at the area of infection (Pernthaner *et al*., 2006; Bambou *et al*., 2013). Gill *et al*. (2000) speculated that various enzymes necessary for survival of the parasite neutralized by parasite-specific IgA and IgG. Similarly, Bottjer *et al*. (1985) observed the existence of IgG1 leads to marked change in parasite feeding.

Eosinophilic lethal effect against nematodes is aggravated by mast cell derivatives including histamine and macrophages and T-lymphocytes derived complement factors (Huang and Appleton, 2016). Histamine plays a role in immunity by immediately expelling the parasites and high concentration is observed in abomasal mucosa of resistant sheep. Histamine affect the immunological functioning of certain cells i.e. B-cells, dendritic cells, epithelial cells, T-cells, and granulocytes, reduction in type 2 cytokines secretion, IgE level, increase in blood eosinophil and decrease in brocho-alveolar eosinophil (Miyamoto *et al*., 2006;Bryce *et al*., 2006). Hypermobility and hyper secretions due to higher histamine concentrations is detrimental for fecundity and motility of worms and also helping in movement of Abs through abomasal lumen (Miller and Horohoy, 2006). Histamine released by mast cells results in peristaltic movements and leads to mechanical expulsion of parasites. Current study revealed that histamine was secreted during whole infection period. Minimum level in blood may be observed at day zero of infection (Laroche *et al*., 1991) while maximum concentration may be seen during 2nd to 6th weeks PI in goats. According to Harrison *et al*. (1999) higher level of histamine and Abs are observed in sheep infected *Trichostrongylus colubriformis*. The results of the present study showed that a persistent concentration of histamine in plasma of infected goats PI. Histamine is involved in resistance development again parasites, minimizing immunopathological complications and modification of immune responses (Vijayasarathi *et al*., 2015).

During present study, levels of IgA is not observed. IgA is a secretory antibody mostly appear in mucosal secretion, but higher levels maybe there in colostrum, milk intestinal fluid, saliva and urine. The primary role of IgA is to protect the lumen from all invading organisms like bacteria, virus and parasites (Day and Schultz, 2014). During secondary infection, the elevated production of IgA is credited with resistance of a parasite (Pfeffer *et al*., 2005). IgA is active antibody by nature, which is dynamically secreted from the epithelium of the abomasa and the relationship among infection and IgA Abs is very strong in many infectious diseases (Macpherson *et al*., 2008). IgM and IgA are established in abomasal mucus in nematode infections because they are main Ig isotypes in mucosal protection (Amarante *et al*., 2005). In animal, IgA Abs produce locally (Smith, 1977). However, it is reported that IgA also exists in sera of diseased animals and is secretory in nature. The specific Abs are produced against the specific larval infection in the animal which has been formerly attacked by that parasite and is able of identifying larval antigen (Bambou *et al*., 2013). Keeping in view the secretory nature of IgA,

further studies may be conducted in other body secretions of infected goats and its association with parasitic burden.

***Conclusions*:** The current study revealed that the resistance status in DDP breed might be attributable to elevated levels of immunoglobins (IgG, IgE) and plasma histamine as compared to Nachi. The levels of IgG, IgE and histamine were significantly different in both breeds of goats as well as in infected and control groups of DDP and Nachi goats. Moreover, the variation in response of selected goat breeds in current study will formulate the basis of selective breeding of resistant goat breeds. Selected breeding of resistant breed (DDP) in the area will enhance the economy of the herd owners in terms of negligible parasitic infections, cutting off treatment cost, low morbidity/mortality and high production. In the light of outcomes of present research selectively bred animals will be more productive and disease resistant. This practice will not allow the establishment of disease and consequently may reduce the use of anthelmintics. Ultimately, this effort will be a way forward to achieve the goal of secure food for human consumption.

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