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BIOCHEMICAL STUDIES AND SERODIAGNOSIS OF HAEMONCHOSIS IN SHEEP AND GOATS

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ABSTRACT

Haemonchosis is a serious health problem which causes lower production due to high morbidity, mortality, and cost of treatment and control measures. Therapeutical trials to control and treat haemonchosis were conducted by using various allopathic, homeopathic, herbal and biological products. A total of 120 sheep and goats were randomly divided into groups A, B, C, D, E & F and animals in group A, B, C and D were treated with Ivermectin, *Azedarachta indica* (neem Leaves) Powder, *Trematox* (a homeopathic drug), *EM-Biovet* (Effective micro-organisms) respectively. Whereas E and F were kept as infected untreated and uninfected control respectively. A total of 264 sheep and goat out of 300 blood samples (88%) were found positive for the *Haemonchuscontortus*. A range of haematological measures were significantly different between infected groups and uninfected controls at one or more time points. The concentrations of serum albumin, packed cells volume (PCV), Erythrocyte sedimentation rate (ESR), blood eosinophil number were significantly increased ($P<0.05$), while the concentration haemoglobin, total RBCs,total serum proteins, Albumin Globulin Ratio (A/G Ratio) were significantly decreased ($P<0.05$) in haemonchus infected animals. There were no significant differences among infected and control groups in total WBC counts, but it was relatively high in infected animals compared to healthy animals. The percentage of neutrophils was low while lymphocytes count, number of basophils and monocytes number in percentage were high. It was concluded that decreased hemoglobin concentration, total serum proteins, total RBCs, and A/G ratio were important indicators of haemonchosis in sheep and goats.

Key words: Haemonchosis, Haemonchus contortus, ELISA, sheep, goats, Pakistan.

INTRODUCTION

Haemonchosis caused by *Haemonchus contortus* and *Haemonchus placei* is the most important disease of small ruminants in Pakistan Maqsood*et al.,*(1996). Of these two species *Haemonchus contortus* is a predominant, highly pathogenic and economically important gastrointestinal parasite of sheep and goats Mortensen *et al.*, (2003). These parasites are common blood feeders that cause anaemia and reduced productivity and can lead to death in heavily infected animals Vatta*et al.,*(2001). It has been estimated that each worm sucks about 0.05 ml of blood per day by ingestion or seepage from lesions Urquhart *et al.,*(2000). In acute form, there is an anaemia and progressive dramatic fall in packed cell volume and hematocrit, which causes increase in appetence, the bone marrow eventually exhausted due to continuous loss of iron and proteins in the gastrointestinal tract and thus resulting into death.

The diagnosis of *haemonchosis* is usually based upon clinical signs and faecal examinations. Eggs are found in feces when the damage has already been done. So ELISA enables detection of sub clinical infection Almazan*et al.*, (2001). Furthermore, serodiagnosticstudies in large groups of animals benefit from ELISA which is more reliable in contrast to fecal examinations and less time-consuming. Keeping in view the importance of this disease the study was designed to record the different biochemical parameters from experimentally infected and control groups. The data thus obtained was helpful in diagnosing the disease at early stage as well as it was helpful for developing strategy for the control of *haemonchosis* in sheep and goats in Pakistan.

MATERIALS AND METHODS

EXPERIMENTAL PROTOCOL

A. Experimental animals: A total of 60 sheep kids and 60 caprine kids under two months of age were used. The study was conducted at a private sheep and goat farm.

B. Grouping of animals: Ten kids each of sheep and goats were kept as uninfected and untreated control groups A1 & A2 respectively and rest of the kids were infected by oral inoculation with 20000 *Haemonchus contortus* larvae each. The infected kids were divided into groups B1 & B2 (infected control) the groups C1 &C2, D1 & D2, E1 & E2, and F1 & F2 were maintained

as test groups (for sheep & goats) respectively. Groups C1 & C2 were administered with ivermectin, Animals in D1 & D2 were given *Azedarachta indica* (neem Leaves), Animals in E1 & E2 were injected with *Trematox*, while animals in group F1 & F2 were given *EM Biovet*.

C. Collection of blood samples: A total of 300 blood samples were collected out of the same animals from which faecal samples were positive for the Haemonchus contortus all the relevant informations were recorded on Performa regularly. Under aseptic measures 5ml of blood was drawn by vein puncture with the help of disposable syringe and transferred to a screw capped sterile test tube slowly to avoid haemolysis (Benjamin, 1981). All the blood samples were labeled with Identification number and date of collection. The samples were left for about an hour for blood clotting to occur. The clotted blood was separated with a fine loop and the samples were centrifuged at 3500 rpm for at least 5 minutes. The supernatant clear sterile fluid (serum) was aspirated with a pasture pipette and put in a screw capped vial and was stored at -20^0 C for analysis.

D. Biochemical analytical procedures: The clean non homolysed sera was prepared after blood coagulation and kept in clean vials at -20^{0}C until haemolysis. The serum was used for quantitative determination of total serum proteins, serum albumin and serum globulins Benjamin, (1981); Coles (1986).

E. Haematological examination: Blood samples from all animals were collected in vials containing ethylene diamine tetra acetic acid (EDTA),as anticoagulant, on zero, 18th and 28th days to determine the Red blood cells (RBC), white blood cells (WBC) , Haemoglobin estimation (Hb), Packed cells Volume (PCV) and Erythrocyte sedimentation rate (ESR) by the methods as described by Coles (1986); Coffin (1995).

F. ENZYME linked immunosorbant assay (elisa): Enzyme Linked Immunosorbant Assay (ELISA) was performed to diagnose *haemonchosis* at zero, 18th and 28th days. The results were compared with conventional diagnostic tests to compare their efficacy. All the sera from experimental animals were examined by ELISA as described by Almazan *et al.* (2001).

Statistical analysis: All the parameters including blood eosinophil count, packed cell volume and total serum protein level of the six experimental groups were compared using analysis of variance technique using DMR procedures.The data were analyzed statistically by using SPSS-15 for windows at 5 % level of probability Steel *et al*., (1997).

RESULTS AND DISCUSSION

Serodiagnosis of haemonchosis: Out of 300 blood samples collected 264 were found positive by serodiagnosis. Diagnosis of *haemonchosis* was based on clinical findings as well as laboratory tests. The most reliable method is the finding of eggs in the feces of infected animals. This method can not be used until the parasite attains sexual maturity. Therefore, it is necessary that these parasites should be controlled before they can cause damage to the stomach. Hence serological diagnosis should be preferred because *anti-Haemonchus* antibodies can be detected as early as one week post infection and thus can facilitate early chemotherapeutic intervention. Despite of the recent studies involving the diagnosis of *haemonchosis* using *Haemonchus contortus* antigens in ELISA test 264 out of 300 blood samples (88%) were found positive out of the same animals from which faecal samples were positive for the *Haemonchuscontortus.* These findings are in accordance with the results of Xuejuan*et al;* (1997), (92.79%),Nayebzadeh*et al.*, (2005), (53.6%),Mir*et al.,* (2008), (87.5%). It has been demonstrated that antigens of *Haemonchus contortus* elicit strong immune response and ELISA has potential source of diagnostics Brandt *et al.,* (1992); Mahannop*et al.,* (1992). ELISA is a reliable serological assay, which enables detection of subclinical infection and early infection, is needed Almazan*et al.,*(2001). Furthermore, sero-epidemiological studies in which large groups of animals must be examined might also benefit from a reliable ELISA. Such test is usually, in contrast to fecal examinations, less time-consuming. Xiaojun*et al.*, (2007) evaluated the diagnostic potential of ELISA. The diagnostic efficiency of the ELISA was 100%. Recombinant protein-based serological tests may achieve high sensitivity and specificity because of the high concentration of the immuno-reactive antigen and the lack of host protein components from the crude antigen preparations suitable antigen for the diagnosis of *Haemonchus contortus* infection Boonchit*et al.,*(2004). ELISA analysis clearly discriminated the positive sera from negative ones Rathore*et al.,*(2006). A positive correlation of antibody levels and worm burden was observed. Animals with higher worm burden had higher antibody titres.

Haematological values: A range of haematological measures were significantly different between infected groups and uninfected controls at one or more time points. The values of Total Serum Proteins (g/dl), Albumin (gm/dl), A/G ratio (gm/dl), RBC (10^6/ ul), PCV (%), Hb (gm/dl), ESR /hr, TLC (10^3/ ul), Neutrophils (%) and Lymphocytes (%) are given in Table 1, 2 and 3. The total serum protein concentration was relatively low in the four infected groups compared with control group. Infected animals of group B had significantly lower

values (P<0.001) compared to the treated four groups (C, D, E & F) and uninfected untreated control group animals. (Table 1, 2, 3) The present study revealed a marked reduction in haematocrit, haemoglobin and RBC count which confirmed the observations of early workers, Misra *et al*., (1996) who observed decreased values of haematocrit, haemoglobin, and RBC counts in lambs in relation to nematode and amphistome infection. The reduced RBC counts, Hb and PCV values in infected groups may be attributed to the bleeding of abomasa due to the injuries caused by the Haemonchus similar to that described by Abdel (1992).

Table 1: Blood picture of different groups in therapeutic trials of *haemonchosis* in sheep and goats on zero day

Parameters	A Group	B Group	C Group	D Group	E Group	F Group	Sig.
Total Serum Proteins (g/dl)	7.2±0.32a	6.3±0.14a	6.46±0.20a	6.12±0.22a	6.54±0.21a	6.91±0.21a	0.061^{NS}
Albumin (gm/dl)	5.35±0.24a	2.16±0.11b	2.61± 0.14b	2.5± 0.13b	2.71±0.17b	3.1± 1.12b	0.129^{NS}
A/G ratio (gm/dl)	0.95±0.02b	0.69±0.02b	0.67± 0.01b	0.61± 0.12b	0.78±0.03b	1.71± 0.04a	0.478^{NS}
RBC (X106/ul)	3.18±0.21a	2.34±0.15a	2.55± 0.24a	2.14± 0.13b	2.22±0.12b	2.69± 0.25a	0.395^{NS}
PCV (%)	30± 5.85a	25.5± 8.9b	20.6± 2.59b	22.7± 5.36b	23.9± 6.5b	24.75±5.64b	0.001*
Hb (gm/dl)	13.5±1.99a	12.9±2.11a	11.75±3.12a	12.42±1.81a	11.75±2.88a	10.7± 5.26b	0.061^{NS}
ESR /hr	3.6± 0.24b	4.2± 0.89a	4.4± 1.2a	3.9± 0.57a	4.17± 1.48a	4.27± 0.97a	0.009*
TLC(10^3/ ul)	7.5± 1.03b	11.8± 2.4a	10.92± 3.2a	9.98± 1.34a	11.5± 2.45a	10.8± 2.19a	0.069^{NS}
Neutrophils (%)	34± 3.22b	44± 4.46a	40.57±3.98a	31.59±2.87c	38.75±2.18b	36.12±3.43b	0.066^{NS}
Lymphocytes (%)	60± 4.1a	38.8±3.01b	43.21±3.21b	51.22±4.26a	45.1± 4.27b	48.25±4.19b	0.716^{NS}
Eosinophils (%)	2.5± 0.31b	15.7±2.75a	14.35±3.65a	15.57±2.68a	14.07± 2.2a	13.85±3.19a	0.077^{NS}
Basophils (%)	1.75±0.19a	0.1± 0.25b	0.52± 0.13b	0.35± 0.18b	0.27± 0.21c	0.59± 024b	0.413^{NS}
Monocytes (%)	1.75±0.11a	1.4± 0.09b	1.35± 0.18b	1.27± 0.07b	1.81± 0.16a	1.19± 0.17b	0.014*

Means with different letters are significantly different each other by Duncan test conducted at 5% significance level.
NS= Non significant, * Significant Group A: Healthy control Group B: Infected untreated
Group C: Treated with Ivermectin Inj. Group D: Treated with *Azedarchta indica (*Neem)
Group E: Treated with Trematox Inj. Group F: Treated with EM-biovet.

Table 2: Blood picture of different groups in therapeutic trials of *haemonchosis* in sheep and goats on 18th day.

Parameters	A Group	B Group	C Group	D Group	E Group	F Group	Sig.
Total Serum Proteins (g/dl)	6.14± 0.08a	6.34± 0.19a	6.81± 0.12a	6.59± 0.24a	6.69± 0.19a	7.34± 0.29a	0.041^{NS}
Albumin (gm/dl)	2.41± 0.12b	2.65± 0.17b	3.11± 0.20a	2.72± 0.19b	3.01± 0.19a	3.91± 1.14a	0.292^{NS}
A/G ratio (gm/dl)	0.6± 0.11b	0.71±0.01b	0.9± 0.10b	0.93± 0.15b	1.24± 0.05a	2.04± 0.12a	0.417^{NS}
RBC (X106/ul)	3.35± 1.01a	1.89± 0.89b	2.98± 1.28a	2.85± 1.64a	3.17± 1.23a	3.25± 1.07a	0.329^{NS}
PCV (%)	31.5± 5.52a	15.34±5.2b	28.2± 4.28a	26.4± 3.89a	27.3± 4.28a	27.57±4.5a	0.005*
Hb (gm/dl)	14.1± 2.64a	9.71± 1.98b	13.2± 2.19b	17.17±3.4a	13.21±1.5b	11.22±2.1b	0.085^{NS}
ESR mm/hr	3.5± 1.23b	4.5± 1.56a	3.85± 1.21a	3.71± 1.18a	3.91± 1.80a	4.18± 1.35a	0.012*
TLC(10^3/ ul)	8.2± 2.19b	10.2± 1.52a	9.75± 1.85a	9.39± 1.65a	10.23±1.9a	10.75±2.4a	0.069^{NS}
Neutrophils (%)	32.29±4.5b	45.98±8.7b	42.59±4.2b	51.27±5.8a	54.98±5.6a	50.91±5.3a	0.059^{NS}
Lymphocytes (%)	62.5± 6.41a	32.25±4.5b	48.2± 5.42b	35.9± 3.89b	33.8± 3.98b	37.5± 3.68b	0.725^{NS}
Eosinophils (%)	1.5± 0.15c	20.17±2.9a	6.42± 1.10b	10.28±2.1b	8.52± 1.56b	9.19± 2.59b	0.068^{NS}
Basophils (%)	1.91± 0.15a	0.35± 0.04c	0.89± 0.12b	0.75± 0.36b	0.55± 0.21b	0.65± 0.24b	0.446^{NS}
Monocytes (%)	1.8± 0.08b	1.25± 0.07c	1.9± 0.04b	1.8± 0.06b	2.15± 1.16a	1.75± 1.23b	0.029*

Means with different letters are significantly different each other by Duncan test conducted at 5% significance level.
NS= Non significant, * Significant Group A: Healthy control Group B: Infected untreated
Group C: Treated with Ivermectin Inj. Group D: Treated with *Azedarchta indica (*Neem)
Group E: Treated with Trematox Inj. Group F: Treated with EM-biovet

All infected groups tended to have a higher PCV at the final time point 28th day. Erythrocyte numbers and haemoglobin content of blood was suppressed most in animals with higher infection, RBC and Hb measures were also reduced compared to controls at days 18 and 28. Decline in total serum proteins in infected animals compared to control animals was similar to Mir *et al.,* (2007) who described decreased concentrations of total protein in sheep during *haemonchosis*. Decrease in total serum proteins in the present study may be attributed to haemodilution, a compensatory mechanism for the abomasalhaemorrhages caused by the invading larvae and

later on due to loss of large quantities of serum proteins into the gut and consequent increased fractional catabolic rate of albumin. The experimentally infected animals showed reduced albumin concentrations which was significantly lower ($P<0.05$) then non-infected animals. Also albumin/ globulin ratio was subnormal in infected animals. Globulin has been shown to contain immunoglobulin which is necessary for defense against parasitic infection. Abrahams-Sandi *et al.,* (2005) and Balic *et al.,* (2002) demonstrated an increase in both cellular and humoral response following parasitic challenge, which go on to suggest the increase in the serum globulin in this current study. Therefore, it can be deduced that these responses may be an immunologocal response against the *haemonchus contortus* challenge.

Table 2: Blood picture of different groups in therapeutic trials of *haemonchosis* in sheep and goats on 28th day.

Parameters	A Group	B Group	C Group	D Group	E Group	F Group	Sig.
Total Serum Proteins (g/dl)	7.2± 0.32a	6.21±0.20a	7.01±0.12a	6.91±0.28a	7.01±0.26a	7.51±1.05a	0.082^{NS}
Albumin (gm/dl)	3.35± 0.24b	2.16±0.12b	3.12±0.18b	2.93±1.21b	3.92±0.21a	4.12±1.21a	0.291^{NS}
A/G ratio (gm/dl)	0.95± 0.02b	0.65±0.02c	1.02±0.01b	1.04±1.10b	1.98±0.06a	2.11±0.04a	0.347^{NS}
RBC (X106/ul)	3.3± 1.02a	1.74±0.94b	3.2±0.68a	2.99±1.14a	3.25±1.10a	3.35±1.54a	0.003^{*}
PCV (%)	30.75±5.26a	10.32±3.56b	30.2±5.26a	29.6±4.58a	29.37±5.26a	30.15±4.27a	0.005^{*}
Hb (gm/dl)	15.8± 2.26a	8.6± 1.48b	14.44±2.45a	14.52±3.17a	14.89±2.24a	12.85±2.29a	0.006^{*}
ESR mm/hr	3.5± 1.27a	4.8± 2.21b	3.61± 1.94a	3.62±1.68a	3.45± 1.58a	3.95± 1.51a	0.009^{*}
TLC(10^3/ ul)	7.9± 1.23a	8.67± 2.24a	9.15± 2.56b	9.41± 2.49b	9.57± 2.58b	10.51±2.51b	0.071^{NS}
Neutrophils (%)	31.46±5.32b	42.39±4.43b	35.48±3.24b	56.69±2.65a	54.04±4.56a	51.24±4.36a	0.069^{NS}
Lymphocytes (%)	63.25±5.62a	31.5± 3.49b	55.7±4.38a	32.75±4.78b	35.75±4.36b	39.75±4.29b	0.727^{NS}
Eosinophils (%)	1.2± 0.09c	22.92±2.69a	5.11± 0.29b	6.58± 2.69b	7.21± 1.26b	6.15± 0.89b	0.077^{NS}
Basophils (%)	1.89± 0.26a	0.49±0.08b	1.21±0.05a	1.88±0.06a	0.65.07b	0.81± 0.08b	0.413^{NS}
Monocytes (%)	2.2± 1.01b	2.7± 1.21a	2.5± 1.23a	2.1± 1.24b	2.35± 1.27a	2.05± 1.28b	0.022*

Means with different letters are significantly different each other by Duncan test conducted at 5% significance level.
NS= Non significant, * Significant Group A: Healthy control Group B: Infected untreated
Group C: Treated with Ivermectin Inj. Group D: Treated with *Azedarchta indica (*Neem)
Group E: Treated with Trematox Inj. Group F: Treated with EM-biovet.

In the work described here, eosinophil, neutrophil and platelets measures were significantly affected by parasite. Relative to uninfected controls, platelet numbers were suppressed in the infected groups and eosinophils were elevated, with no evidence of a differential effect of parasite upon host responses. For neutrophils, however, at day 28, the group infected had enhanced numbers of circulating cells relative to uninfected controls, whereas, the other infected groups had lower numbers than uninfected animals. The effect of *haemonchosis* upon haemoglobin concentration was studied in the USA some time ago (Poeschel and Todd, 1972a, b), where significant effects were observed. A difference in infectivity between two *Haemonchus contortus* was also observed in different breeds of sheep Aumont*et al.,*(2003). The study described here confirms pathogenicity of *Haemonchus contortus*. Louvandini*et al.,* (2002) reported that the protein supplementation increased some characteristics of the resilience such as packed cell volume and body weight on calves in *haemonchosis*. In the present study, both sheep and goats had comparatively higher PCV and Hb levels with low infection levels. These two seemed to have become well adapted to the *haemonchosis* in experimental animals. These findings are in line with those of Baker *et al*. (1999). They observed that genetic differences among breeds and within-breed, resulting in variable resistance to infection by GIT nematodes. The PCV values were significantly lower ($P<0.01$) in infected animals. However, after treatment theses values become normal as was also reported by Amaranteet *al.,* (2004) and Yacob*et al.,* (2008). After treatment with different drugs the quality of blood loss by the parasites was minimized as was also reported by Yacob*et al.,* (2008). Woolaston*et al.*, (1996) studied the value of circulating eosinophil count and reported that circulating eosinophils (EOS) were higher in infected animals. It was also observed that PCV was negatively correlated with FEC which is in line with the findings of Gauly and Erhardt (2002). The infected animals showed reduced serum protein concentrations which significantly lower ($P<0.05$) in non-infected animals. These changes might be attributed to more blood loss, impairment of appetite. This was in agreement with Arzoun*et al.,* (1984). Anthony *et al.,* (2008) found that there is an inverse correlation between blood lymphocyte counts and worm fecundity and statistically significant correlation between worm size and fecundity. Lymphocytes counts in response to *Haemonchus contortus* infection induced a combination of humoral immunity, eosinophilia, and mast cell hyperplasia,

leading to reduction in worm size and fecundity. The difference in eosinophil counts between infected (C, D, E and F groups) and the control groups (B) was highly significant (P<0.001) and peak values were attained on day zero and then their values were obtained on different days after treatment with different anthelmintics. It was noted that the values become normal on day 28. Variable PCV and Hb levels were recorded in animals of various age groups in these studies might have occurred as a result of physiological effect due to undernourishment and non-availability of supplementary feed to these animals. In our study, the difference in eosinophil count between infected groups and the control groups was highly significant. Eosinophils are considered to be important elements in the response against *Haemonchus* infections Balic*et al.*, (2000). In the study, there was an increased number of circulating blood eosinophils in the animals infected with *Haemonchus* larvae. This was in agreement with Terefe*et al.* (2005). The animals infected with *Haemonchus contortus* showed considerable degree of blood eosinophlia as compared to the non-infected animals. The eosinophils mobilized against specific parasites were frequently found to cause immobility and death of larvae of homologous or heterologous parasites often in association with antibodies and/or other factors. Rainbird*et al.,* (1998) and Terefe*et al.,* (2005).The development of systemic and local tissue eosinophilia is characteristic of the host immune response towards helminth infection. However, opinion is divided on the role of eosinophils during infection, in terms of both their protective effect Klion and Nutman, (2004) and their ability to mediate inflammation Lee and Lee, (2005). Evidence for a protective role derives mainly from in vitro studies on parasite killing in the presence of eosinophils or their granular components Rainbird*et al.,* (1998). There is also some evidence suggesting that eosinophils may contribute to pathogenesis during parasitic infection Nickdel*et al.,* (2001). Moreover, it has previously been shown that a number of ovine parasitic gastrointestinal nematodes produce a factor(s) that promote eosinophil migration in vitro Wildblood*et al.,*(2005). This raises the possibility that helminths may actively promote eosinophil recruitment and activation, and utilize resulting tissue damage to aid their survival within the host. They assume that the mucosal cellular effector components in *Haemonchus contortus* were seriously affected by activated and concentrated eosinophils in the mucosal environment. Yacob*et al.*, (2008) reported the presence of an early and high eosinophilia and migration of the same cellular components into the abomasal and intestinal mucosae in the absence of nematodes in the gut. This was in agreement with Arzoun*et al.,* (1984). The haematocrit PCV) is an essential parameter, which may be used besides faecal egg count to describe resistance against nematode parasites in sheep Amaranteet *al.,* (2004); Gauly and Erhardt (2002). Eosinophillia and increased lymphocyte count observed in the present investigation is in agreement with the findings of Bhat and Sharma (1990) who concluded that eosinophillia is associated with antigenic stimulation or parasite burden. Incresed lymphocyte count may be due to proliferation lymphocytes due to excretory secretary product of *Haemonchus contortus*. The results of our study are similar to Mir *et al.,* (2007) who reported that ESR were significantly (p<0.05) elevated whereas decreased values of Hb, RBC, PCV, total serum proteins were observed in infected animals compared to control. The inhibition of monocytes seems an important defense strategy devised by the parasite; Clark *et al.,* (1962); Cox *et al.,* (1990); Karanu*et al.,* (1993); Jungi*et al.,* (1996).

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