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Research Article

HAEMOGLOBIN PHENOVARIANTS VS RESILIENCE STATUS, HAEMATOLOGICAL, BIOCHEMICAL AND MINERAL PROFILE IN GAROLE SHEEP NATURALLY INFECTED WITH *HAEMONCHUS CONTORTUS.*

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ABSTRACT: A total of 232 nos. of Garole sheep were evaluated for studying the variability of resilience to natural infection of *Haemonchus contortus* in relation to haemoglobin phenovariants as well as to know the association of haemoglobin phenovariants with several haematological parameters, biochemical parameters and serum macro and micro elements. This study revealed a significant variation (p<0.01) of haematological parameters, biochemical parameters and mineral status against resistance status and haemoglobin phenovariants. Significantly (P<0.01) less EPG count was recorded in Haemoglobin–AB type animals than the Haemoglobin-BB type animals. Haemoglobin-AB type animals also possessed significantly higher level of haemoglobin, packed cell volume, total leukocyte count, lymphocyte count, serum total protein, albumin, globulin, calcium, phosphorus, iron, copper and zinc than Haemoglobin-BB type animals. On the other hand Haemoglobin-BB type animals possessed significantly higher level of neutrophil and serum alkaline phosphatase enzyme than Haemoglobin-AB type animals. During selection of *H. contortus* resistant Garole sheep inclusion of haemoglobin type as an indicator trait may be helpful to achieve the goal. Measurement of haematological parameters, biochemical parameters and serum macro and micro elements in these cases may also be essential for diagnostic and therapeutic purposes.

Key words: Garole sheep, *Haemonchus contortus*, Haemoglobin phenovariants, Egg per gram, Haematological, Biochemical, Macro and micro elements.

INTRODUCTION

In small ruminants gastrointestinal parasites play a prime role for production losses. Most of the small ruminants have developed resistance against all commonly used anthelmintics (Waller and Prichard 1986,

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Prichard 1990, Windon 1991, Waller 1994). A further demand for organic products is increasing day by day and use of anthelmintics becomes an unacceptable mean of parasite control. Rapid urbanization also move towards more intense use of pastures will necessitate more frequent use of anthelmintics, further increasing the chances for selection of anthelmintic resistance in the parasite populations.

The aim of effective nematode control programmes is to protect animals from production losses and one such approach is to use genetically superior stock as an adjunct to current methods of control. There are some direct and indirect means to help make those selections. (Gasbarre and Miller 2000).

Resistance to *Hacmonchus contortus*, as measured by faecal egg count (FEC), has moderate heritability (0.22 to 0.43) indicating that selection and breeding of sheep for increased resistance is possible (Albers *et al.*, 1987, Bisset *et al.*, 1992, Woolaston and Piper 1996; Morris *et al.*, 1997).

The first genetic marker suggested for use in selection for resistance was haemoglobin type. Sheep have two alleles (A and B) for haemoglobin and several studies indicated that animals with haemoglobin type AA (Hb-AA) were more resistant than Hb-AB, which in turn were more resistant than Hb-BB to infection with *H. contortus* and its effects (Allonby and Urquhart 1976; Altaif and Dargie 1978a & 1978b; Preston and Allonby 1979; Roy *et al.*, 2013a). However, other workers were unable to confirm that association (Radhakrishnan *et al.*1972; Albers and Gray, 1986 and Kassai *et al.*, 1990).

Indicator traits usually reflect host response to infection, which includes some haematological and biochemical parameters (Khan *et al.*, 1988, Ahmad and Ansari 1989, Mottelib *et al.*, 1992, Yadav *et al.*, 1993 & 1997, Garcia *et al.*, 1994, Ghulam Rasool *et al.*, 1995, Stear *et al.*, 1995, Hayat *et al.*, 1996, Singh and Yadav 1998, Amarante *et al.*, 1999, Hooda *et al.*, 1999, Dhanalakshmi *et al.*, 2002, Vanimisetti 2003, Roy *et al.*, 2013b) that can be used as potential tool for selecting sheep with increased resistance. Several study quantitatively demonstrated that mineral metabolism was significantly affected by *H. contortus* infection in sheep (Singh and Yadav 1998; Sangwan and Sangwan 2000).

The main habitat of Garole sheep is the saline belt of Sunderban Delta of South 24 Parganas, some parts of North 24 Parganas and some saline zone of Purba Medinipur district of West Bengal, India. The climate is hot and humid. These sheep are native and is popular for its biannual lambing with multiple births and their ability to graze on aquatic weeds and grasses standing even in knee-deep water for hours together (Kar and Prasad 1992).

The research in connection to variability of haemoglobin phenovariants and its association with resistance level against natural infection of *H. contortus* as well as haematological & biochemical parameters and serum macro and trace elements levels in Garole sheep is very limited although infections exist and are widespread.

Realizing the importance of the subject, a research programme has been taken up in Garole sheep to study the relationship of response to natural infection of *H. contortus* with haemoglobin genotypes/phenovariants and haematological parameters, biochemical parameters and serum macro and trace elements levels. Further attempts were made to identify the marker(s) for the selection of *H. contortus* resistant sheep.

MATERIALS AND METHODS

Place and period of work

The research work was carried out during the period of December 2001 to December 2004 with 232 nos. of adult Garole sheep (*Ovis aries)*, maintained in a farm under National Agricultural Technology project on 'Animal Genetic Resource Biodiversity' of West Bengal University of Animal and Fishery Sciences, Mohanpur Campus, Nadia, West Bengal, India. The farm is situated at $88^0 32'$E longitude and $22^0 56'$N latitude with an altitude of 9.75 m above mean sea level and having sub- tropical humid climate. On the basis of temperature, relative humidity and rainfall, the year has been divided into three season *viz.* winter (from November to February), summer (from March to June) and monsoon (from July to October). During the experiment period temperature varied within the range of 7.27ºC to 39.98ºC and relative humidity varied from 47.18% to 97.69%.

General management practice:

All the experimental animals were allowed to graze on natural pasture from 8 A.M. to 3 P.M. in winter months. During summer and monsoon animals grazed in two shifts *viz.* from 7 A.M. to 10.30 A.M. and 2.30 P.M. to 5.30 P.M. The pasture comprised mainly of doob grass (*Cynodon dactylon*) with other grasses and leaves *viz.* goose grass (*Eleusine indica*), horse purseane (*Trianthema monogyna*) and anjan grass (*Cenchrus ciliaris*). In addition to grazing all the animals were offered about 50g concentrates after returning from grazing in the evening. Adlibitum water was provided to all the animals in all seasons. All the ewes and rams were dewormed for 3 times in a year with Fenbendazole or Albendazole or Rafoxanide (@10 mg/kg body weight orally) in three different seasons (summer, monsoon and winter). Animals were given dips from time to time to protect them from ectoparasites.

Collection of faecal sample and estimation of parasitic load:

Since sheep were maintained in a common natural pasture throughout the year, it was assumed that they got equal dose of *Haemonchus* infection from the grazing field. The faecal samples from each animal were collected rectally after one week of deworming and examined for *H. contortus* eggs. During each season two faecal samples were collected from each animal at five days interval and accordingly eggs per gram of faces (EPG) were determined following Stoll's Dilution Method as described by Soulsby (1982).

Collection of blood samples and determination of hemoglobin phenovariants:

About 5 ml of fresh blood was aseptically collected from each animal by puncturing jugular vein with 0.5 ml of anticoagulant (Sodium citrate) and accordingly hemoglobin polymorphism was conducted following the Starch gel electrophoresis method described by Smithies (1955).

Collection of blood for estimation of haematological parameters, biochemical parameters and mineral profiles:

From each animal about 10 ml of blood was collected by jugular vein puncture and out of this 5 ml was mixed with anticoagulant *i.e.* 4.0 mg EDTA for haematological examinations and rest 5 ml kept as such for collection of serum and collected serum was kept in deep freeze at (-) 20^0C till estimation of biochemical parameters and minerals.

During blood collection (from jugular vein)

Table 1: Haemoglobin type and Least squares means of EPG in adult Garole sheep.

Effects	**Observed Number**	**EPG** (Mean±SE)
Overall	232	775.822 ±20.189
Haemoglobin type:		**
Hb-BB	196	875.255±15.906[a]
Hb-AB	36	676.389±37.113[b]

Means under a particular effect in a column having different superscripts differed significantly.
**Significant at 0.01 level.

three thin blood smears were also made from each animal and Differential Leukocyte Count (DLC) was performed as per standard method of Schalm *et al.*, (1975). Hemoglobin level (g/dl) was determined by Cynmethaemoglobin Method (Cannan, 1958), Packed cell volume (PCV %) were determined as per standard method of Schalm *et al.*, (1975), Total Leukocyte Count (TLC) was enumerated by haemocytometer as per standard method of Schalm *et al.*, (1975) in terms of thousands per cubic millimeter ($10^3/mm^3$).

Serum total protein and albumin in each sample were determined by Modified Biuret and Dumas Methods of Reinhold (1953) in a photoelectric colorimeter using a yellow green filter supplied along with Diagnostic Reagent kit for the *in vitro* determination of total proteins and Albumin in serum (Manufactured by Span diagnostics Ltd.). The globulin fraction in the serum samples was calculated by substraction of serum albumin from total serum protein. The total serum protein, serum albumin and serum globulin values were expressed as g/dl of serum. Serum alkaline phosphatase activity was measured using 4-amino antipyrine by the method described by Kind & Kings (1954) using calorimeter and were expressed as KA Units.

Estimation of serum Calcium (Ca), Iron (Fe), Copper (Cu) and Zinc (Zn) was done by using atomic absorption spectrophotometer (Perkin Elmer, A analyst 100) following the specification ("Analysis of serum and plasma: Calcium and Magnesium" and "Analysis of serum and plasma: Copper and Zinc") of the instrument manual respectively. The value of Ca was expressed in mg/dl and that of Fe, Cu and Zn was in ppm (mg/ml). Serum phosphorus (P) level was measured calorimetrically following the method of Gomorri (1942). The concentration of serum phosphorus was then expressed in mg/dl.

Statistical Analysis:

All the data were subjected to Least-Squares analysis (Snedecor and Cochran 1967) for studying the variability of haemoglobin type and its effect on resistance status (EPG), haematological parameters, biochemical parameters and serum macro & trace elements levels in adult sheep. The model used for analysis was-

$Y_{ij} = \grave{\imath} + A_i + e_{ij}$

Where Y_{ij} is the observation on the i^{th} individual in j^{th} haemoglobin type.

Table 2: Haemoglobin type and Least squares means of EPG & Haematological parameters in adult Garole sheep.

Effects	Observed Number	EPG	Haemoglobin(g/dl)	PCV(%)	TLC (10^3/mm^3)	Lymphocyte (%)	Neutrophil (%)
		Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE
Overall	232	775.822 ±20.189	9.554±0.065	26.678±0.158	6.467±0.038	51.646±0.309	41.264±0.216
Haemoglobin type:		**	**	**	**	**	**
Hb-BB	196	875.255±15.906[a]	9.277±0.051[b]	26.161±0.124[b]	6.264±0.030[b]	50.510±0.244[b]	42.079±0.170[a]
Hb-AB	36	676.389±37.113[b]	9.832±0.120[a]	27.194±0.290[a]	6.669±0.070[a]	52.783±0.568[a]	40.449±0.397[b]

Means under a particular effect in a column having different superscripts differed significantly.
**Significant at 0.01 level.

Table 3: Haemoglobin type and Least squares means of EPG & Biochemical parameters in adult Garole sheep.

Effects	Observed Number	EPG	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Alkaline phosphatase (KA Unit)
		Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE
Overall	232	775.822 ±20.189	7.830±0.075	3.306±0.035	4.523±0.065	12.944±0.185
Haemoglobin type:		**	**	**	**	**
Hb-BB	196	875.255±15.906[a]	7.595±0.059[b]	3.133±0.027[b]	4.460±0.052	13.435±0.146[a]
Hb-AB	36	676.389±37.113[b]	8.066±0.138[a]	3.480±0.064[a]	4.587±0.120	12.454±0.340[b]

Means under a particular effect in a column having different superscripts differed significantly.
**Significant at 0.01 level.

Table 4: Haemoglobin type and Least squares means of serum minerals in adult Garole sheep.

Effects	Number	EPG	Calcium (mg/dl)	Phosphorus (mg/dl)	Iron (ppm)	Copper (ppm)	Zinc (ppm)
		Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE
Overall	232	775.822 ±20.189	6.614 ±0.124	4.841 ±0.053	2.593 ±0.024	0.816 ±0.011	1.389 ±0.012
Haemoglobin type:		**		**	**	**	**
Hb-BB	196	875.255 ±15.906[a]	6.388 ±0.098	4.674 ±0.042[b]	2.491 ±0.019[b]	0.768 ±0.009[b]	1.354 ±0.010[b]
Hb-AB	36	676.389 ±37.113[b]	6.840 ±0.228	5.008 ±0.098[a]	2.695 ±0.043[a]	0.864 ±0.021[a]	1.424 ±0.023[a]

Means under a particular effect in a column having different superscripts differed significantly.
**Significant at 0.01 level.

ì = General effect (Overall mean common to all observations);

A_i = Effect of the i^{th} haemoglobin type (l=1,2) and

e_{ij} = random error assumed to be normally distributed with zero mean and variance,$ó^2_e$.

Duncan's Multiple Range Test (Kramar, 1957) was performed to examine the significant differences between means whenever the analysis of variance showed significant differences for different factors.

RESULTS AND DISCUSSION

Haemoglobin phenovariants: Hb-A has faster electrophoretic mobility towards anode than Hb-B. Hb-AB had one component of Hb-B with slower mobility and other component of Hb-A with faster electrophoretic mobility. In our study most of the Garole sheep (196 nos.) were found to have Haemoglobin BB type (Hb-BB) and only 36 nos. of sheep were found to have Haemoglobin AB type (Hb-AB) presented in Table 1 & Fig.1. Report of Roy *et. al* (2013a) was in agreement with the results of our study. They also reported more number of Hb-BB type Garole sheep in comparison to Hb-AB type.

Haemoglobin type and EPG: Higher EPG count (875.255±15.906) was recorded in Hb-BB type animals in comparison to Hb-AB type animals (676.389±37.113) as presented in Table1 and Fig. 2. The reports of Allonby and Urquhast (1976), Alfait and Dargie (1978a and 1978b), Preston and Allonby (1979) were in agreement with the results of our study. They also reported that animals with haemoglobin type-AA (Hb-AA) were more resistant than Hb-AB, which were more resistant than Hb-BB to infection with *H. contortus* and its effects. Roy *et al.* (2013a) also reported the same trend in Garole sheep i.e. Hb-BB type sheep were facing more FEC than Hb-AB type against natural infection to *H. contortus*. Our study also revealed that haemoglobin-A locus was related with resistance. However, some studies were unable to confirm that association (Radhakrishnan *et al.*, 1972; Albers and Gray 1986; Kassai *et al.*, 1990).

Haemoglobin type and Haematological parameters: Haemoglobin type was found to have highly significantly effect ($P < 0.01$) on haemoglobin level. Hb-AB type animals had higher haemoglobin (9.832±0.120 g/dl) than Hb-BB type animals (9.277±0.051 g/dl) presented in Table 2 & Fig.-3. This difference was found to be associated with resistance level to *H. contortus.* Hb-AB type animals had lower EPG in comparison to Hb-BB type which imposes higher haemoglobin value in Hb-AB type animals compared to Hb-BB type. PCV level was varied significantly ($P < 0.01$) due to haemoglobin type of individuals. Hb-AB type animals showed higher level of PCV (27.194±0.290 %) as compared to Hb-BB type animals (26.161±0.124 %). This might be due to higher EPG count in Hb-BB animals. The effect of haemoglobin type on TLC was found to be highly significant ($P < 0.01$); the Hb-AB type animals have a TLC ($10^3/mm^3$) of 6.669±0.070 vs 6.264±0.030 in Hb-BB type animals. Significantly higher lymphocyte count ($P < 0.01$) was observed in Hb-AB type animals (52.783±0.568 %) as compared to Hb-BB type animals (50.510±0.244 %). Hb-AB type animals showed significantly ($P < 0.01$) lower level of neutrophil (40.449±0.397 %) in comparison to Hb-BB type animals (42.079±0.170 %). This may be due to superiority of Hb-AB type animals in response to natural *H. contortus* infection which had lower FEC than the Hb-BB type animals.

The findings of Preston and Allonby (1979), Altait and Dargie (1978b), Albers *et al.* (1987),

Khan *et al.* (1988), Ahamad and Ansari (1989), Yadav *et al.* (1993), Ghulam Rasool (1995), Hayat *et al*. (1996), Hooda *et al*. (1999), Dhanalakshmi *et al.* (2002), Vanimisetti (2003) and Roy *et al*. (2013b) were also in agreement with the results of this study with respect to significantly decreased level of haematological parameters except neutrophil count in heavily infected animals.

Haemoglobin type and Biochemical parameters: Hb-AB type animals possessed significantly ($P < 0.01$) higher serum protein (8.066±0.138 g/dl) than Hb-BB type animals (7.595±0.059 g/dl) presented in Table 3 & Fig.-4. However, Hb-AB type animals revealed significantly ($P < 0.01$) higher albumin values (3.480±0.064 g/dl) than those of Hb-BB type animals (3.133±0.027 g/dl). This significant variation as observed in the present study can be ascribed to lower FEC in Hb-AB type animals. Haemoglobin type had no significant effect on serum globulin level. But serum globulin level was higher in Hb-AB type animals (4.587±0.120 g/dl) than that of Hb-BB type animals (4.460±0.052 g/dl). The significantly ($P < 0.01$) higher alkaline phosphatase values (13.435±0.146 KAUnit) were associated with Hb-BB type animals than those of Hb-AB type animals (12.454±0.340 KA Unit).

Significant variations as observed in the present study can be ascribed to differences in FEC among the two groups. This may be compared well with the findings of Altaif and Dargie (1978), Yadav *et al.* (1993 & 1997), Singh and Yadav (1998), Hooda *et al.* (1999) and Roy *et al*. (2013a & 2013b) who also reported significant higher level of serum total protein, albumin and globulin, but lower level of serum alkaline phosphatase in sheep with lower FEC of *H. contortus*.

Haemoglobin type and Mineral profiles: Although haemoglobin type did not exert any significant effect on serum calcium level, the value was higher in Hb-AB type animals (6.840±0.228 mg/dl) in comparison to Hb-BB type animals (6.388±0.098 mg/dl) presented in Table 4 and Fig. 5. In our study it revealed that serum phosphorus, iron, copper and zinc level was significantly ($P < 0.01$) affected by the haemoglobin type. Hb-AB type animals had higher phosphorus (5.008±0.098 mg/dl) than Hb-BB type animals (4.674±0.042 mg/dl). Hb-AB type animals had higher serum iron (2.695±0.043 ppm) in comparison to Hb-BB type animals (2.491±0.019 ppm). Higher values of serum copper (0.864±0.021 ppm) were associated with Hb-AB type animals in comparison to Hb-BB type animals (0.768±0.009 ppm). Higher serum zinc level was recorded in Hb-AB type animals (1.424±0.023) than those of Hb-BB type animals (1.354±0.010).

Singh and Jadav (1998) also reported the same phenomena that resistance animals had higher phosphorus with lower FEC in comparison to more susceptible sheep. The decreased serum phosphorus level is an indication of heavy parasitism. This observation can be compared with the findings of Sangwan and Sangwan (2000) who also found significantly lower level of zinc in heavily *H. contortus* infected sheep.

CONCLUSION

This study revealed variation for haemoglobin phenovariants in Garole sheep along with a significant variation ($p< 0.01$) of resistance status to natural infection of *H. contortus*, haematological parameters, biochemical parameters and mineral status in respect to haemoglobin phenovariants.

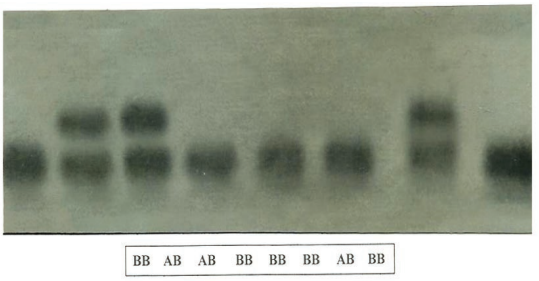

Fig. 1: Starchgel electrophoresis pattern of different haehoglobin phenovariants in adult Garole sheep

Haemoglobin–AB type animals showed significantly ($P<0.01$) better resistance than the Haemoglobin-BB type animals. Haemoglobin-AB type animals also possessed significantly higher level of haemoglobin, packed cell volume, total leukocyte count, lymphocyte count; serum total protein, serum albumin and globulin; serum calcium, phosphorus, iron, copper and zinc than Haemoglobin-BB type animals. On the other hand Haemoglobin-BB type animals possessed significantly higher level of neutrophil and serum alkaline phosphatase enzyme than Haemoglobin-AB type animals.

On the basis of the results it is concluded that variability of haemoglobin phenovariants and its association with haematological and biochemical parameters and mineral status in respect to natural infection with *H. contortus* is existed in Garole sheep and there is ample scope for increasing the resistance through selective breeding/assortive mating. During selection inclusion of haemoglobin type as an indicator trait may be helpful to achieve the goal early. Our study also quantitatively demonstrated that protein metabolism as well as the calcium, phosphorus, copper and iron metabolism was significantly affected by *H. contortus* infection in sheep. During infection impaired absorption and/or increased excretion; redistribution of elements among tissue pools may be the cause of disturbances in metabolism of minerals. Measurement of serum macro and trace elements in these cases may be essential for diagnostic and therapeutic purposes.

Further studies with large number of animals and artificial infection as well as identification of specific marker gene for resistance to *H. contortus* may be carried out as these are far away from the present study and this might give us a clear picture about the genetic basis of resistance to *H. contortus* infection in Garole sheep and helpful to develop a resistant population.

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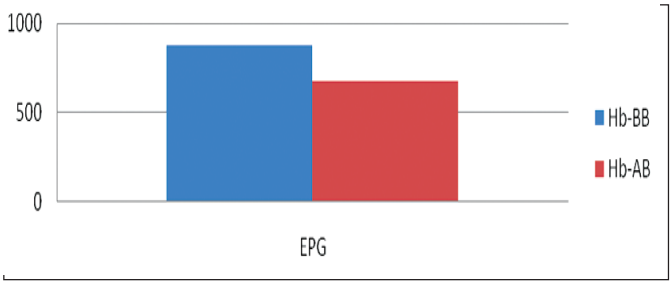

Fig. 2 : Haemoglobin type and EPG in adult Garole sheep.

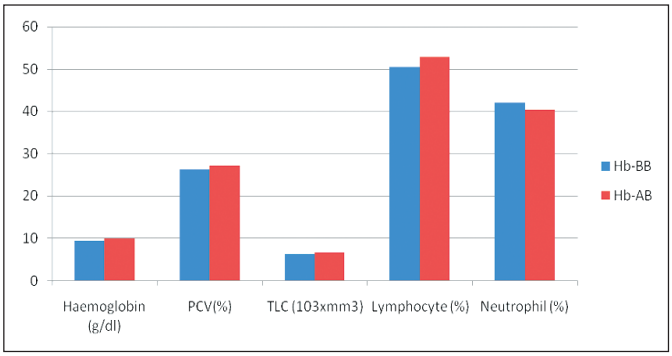

Fig. 3: Haemoglobin type and Haematological parameters in adult Garole sheep.

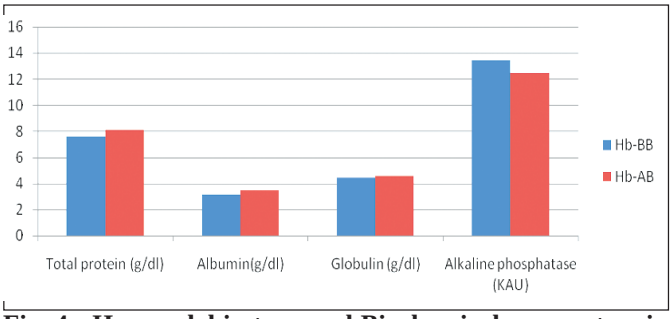

Fig. 4: Haemoglobin type and Biochemical parameters in adult Garole sheep.

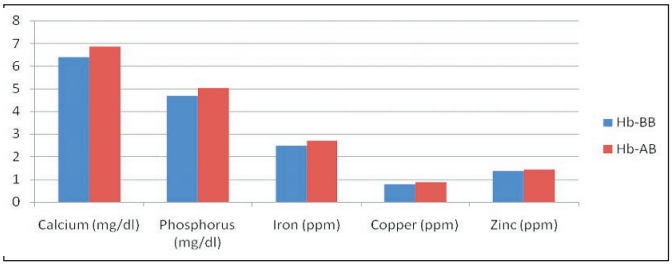

Fig. 5: Haemoglobin type and serum minerals in adult Garole sheep.

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