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ASSOCIATION OF PHYTOMINERAL WITH GASTROINTESTINAL PARASITES OF GRAZING SHEEP IN SIALKOT DISTRICT, PUNJAB, PAKISTAN

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The present study is based on idea of mineral supplementation in grazing animals through selected fodder which can indirectly reduce the threats of infections particularly of parasitic origin. To this end, correlation analysis of the trace elements including: copper (Cu), cobalt (Co), Manganese (Mn) and zinc (Zn) was done among feedstuffs, soils and grazing sheep. Moreover, the mineral analysis was compared to the gastrointestinal (GI) parasites during spring 2015 in grazing sheep of Sialkot district, Punjab, Pakistan. The overall prevalence of GI parasite species identified was 32.81%. A significant ($P < 0.05$) variation in the levels of Zn, Cu, Mn and Co was recorded in a total of eight collected forages. However, their levels in soils vary insignificantly. The results of present study indicated that, the burden of GI parasites was lower in animals having high level of Zn and Cu in sera. The trace element-rich forages (*Cichorium intybus* and *Cynodon dactylon*) preferably Zn and Cu found effective against parasitic infection, are advisable supplemental remedies to improve the trace element profile in grazing sheep. This mitigation strategy may ultimately improve the resilience against GI parasitic infections especially in the resource-poor countries like Pakistan. However, control studies are required to determine the exact mechanism of trace elements against GI parasites.
Keywords: Gastro-intestinal parasites, trace elements, sheep, prevalence.

INTRODUCTION

Sheep production in Pakistan has an imperative economic and social impact on rural small-holder farming systems due to their dependence on sheep for nutrition and income (Iqbal *et al*., 2005). Gastro-intestinal (GI) parasitic infections in young sheep, goat and cattle are a global problem having production losses up to 50% (Lashari and Tasawar, 2011). A variable prevalence (25.1 to 92%) of different GI parasitic species in sheep has been reported by Khan *et al*. (2010), Mehmood *et al*. (2013) and Raza *et al*. (2014) in different parts of the country. Anthelmintics are less effective due to increase in prevalence of parasitic resistant to drugs (Saddiqi *et al*., 2006), increasing public concern to pesticide residues in food chain and environment (Cooper *et al*., 2012) along with growing cost of treatment and labour. During the past decade, uses of plants with anthelmintic properties (ethno-veterinary medicine) are under consideration all around the world (Hassan *et al*., 2014).

Nature has endowed Pakistan with a variety of ecological zones. Agro-grazing animals and natural vegetation as their forage are the most important components of animal production (Shah *et al*., 2012). Climate in general and local conditions in specific determine the nature and kind of plants, their distribution, composition, nutritive value in different cropping belts, grazing sites as well as number and kinds of grazing animals (Maitima *et al*., 2009). Therefore, different cropping belts and grazing sites of Punjab have profound effects on the botanical and chemical composition of grazing/browsing forages in meadows (Rahim *et al*., 2011). Individual investigations have been conducted in different regions of Punjab province which indicated variable preferences of selection of plant species by small ruminants for grazing. Various plant species can minimize the degree of parasitic infections in animals as has been reported extensively (Hamad *et al*., 2013; Sindhu *et al*., 2014; Calixto Júnior *et al*., 2016). The fundamental principle inducing the antiparasitic effects is not clear (Anjos *et al*., 2012). However, direct or indirect effects of improved minerals and trace elements of the animals may reduce parasitic burden (Heckendorn, 2005).

The soil-plant-animal interaction is a complex system which has not been studied tolerably. Facts are required on relationship of minerals among soil, plant or forages, and animal sera to understand their association (Khan *et al*., 2007). Fragmentary data is available concerning the minerals status of soils, forages and small ruminants in Punjab (Khan *et al*., 2005; Khan *et al*., 2017). The present study was aimed to investigate soil-plant-sheep mineral profile correlation and the influence of serum mineral levels with the magnitude to egg per gram (EPG) in grazing sheep of Sialkot district, Punjab, Pakistan.

MATERIALS AND METHODS

***Sampling of faecal, blood, soils and forages*:** The study was conducted in Sialkot district (32° 30'0" N / 74° 31'0" E) having three administrative sub-divisions (tehsils) *viz*: (a) Daska, (b) Pasroor and (c) Sialkot. The study animals were indigenous breeds of sheep in Sialkot district, Punjab, Pakistan. Collection of faeces and sera (n=384) was done during Spring 2015 using standard protocols (Soulsby, 1982). Briefly, 5 g of faecal samples were collected per rectum in screw capped plastic bottles containing formalin and ten mL of blood was collected into commercially available vacutainers without anticoagulant and allowed to clot for 30-45 minutes. Two grazing sites (meadows) were selected from each tehsil of the study district for collection of grazed plants. The representative soil samples of the selected grazing sites from each tehsil of the study district were collected at depths of 0-15, 15-30, 30-60, 60-90 and 90-120 cm. The collected samples were appropriately labeled and shipped to the University of Agriculture, Faisalabad (UAF) for further processing and analyses.

***Coprological examination*:** The collected faecal samples were processed to identify endo-parasites through standard direct parasitological techniques and quantitative faecal analysis through "Modified McMaster test" as suggested by Soulsby (1982). Parasitic eggs were identified using standard identification keys.

***Pre-Treatment of forages, soils and serum samples for elemental analyses*:** The collected forage specimens were subjected to pre-treatment required before their processing for mineral profile determination through spectrophotometry. Briefly, the leaves of the collected plants were washed with 1% HCl followed by washing with distilled water. Air dried samples were further dried in drying oven (65 ± 5°C) and ground to powder by an electric grinding machine. One gram of each plant were separately taken into volumetric flasks and kept overnight in a solution of concentrated (concd.) HNO_3 and $HClO_4$ (Five mL each). Next day, five mL of concd. HNO_3 was added followed by digestion on a hot plate till materials became clear. After digestion, the materials were cooled, and made the volume up to 50 mL with de-ionized water (Miller, 1998), filtered through Whatman filter No. 42 and stored in air tight bottles for determination of mineral profile.

The collected sera were subjected to wet digestion following the method of Richards (1968). One mL of serum was taken into a digestion flask and 10 mL of concd. HNO_3 was added. Mixture was heated at 60-70°C for 15 minutes, and after cooling $HClO_4$ (five mL) was added in the flask. The contents of the flask were heated vigorously till the volume reduced to 1-2 mL. The contents were reconstituted up to 25 mL by adding de-ionized water.

The soils samples were ground in an acid washed porcelain pestle and mortar, filtered through 0.2 mm sieve, and dried (90°C; 24 h). Accurately weighed one gram of air-dried soil sample was taken into 50 mL conical flask, mixed with concd. HNO_3 (10 mL) and kept overnight. Next day, contents of the flask were heated, cooled and added with HNO_3 (one mL), $HClO_4$ (four mL) followed by heating (200°C) again till fumes of $HClO_4$ appeared. Samples were cooled and heated again to 70°C for one hour after adding 5 mL of 1:10 HCl. Finally, samples were allowed to cool and reconstituted volume up to 50 mL with 1% HCl. After filtration through Whatman No. 42 filter paper, filtrate was stored in plastic bottles and labeled appropriately for further analyses.

***Atomic absorption spectrophotometry for elemental analyses*:** Concentration (concn.) of Cobalt (Co), Copper (Cu), Manganese (Mn) and Zinc (Zn) from the digested forage, soil and sera samples were analyzed in triplicates with Atomic Absorption Spectrophotometer (AAS) using the standard protocols as described by Anan *et al*. (2001). The trace elements of forages (mg/Kg), soils (mg/Kg) and sera (mg/L) were compared with slandered values given by Rhue and Kidder (1983) for soil and plants and by Radostits *et al*. (2007) for sera/plasma.

***Statistical analyses*:** Relative prevalence of parasites in different tehsils and Sialkot district was calculated through: Prevalence (%) = [Number of positive samples / Total number of samples examined] x 100. Analysis of variance (ANOVA) of the data from forages, soils, and sera were computed using a COSTAT computer package (CoHort Software, 2003, Monterey, California, USA). The comparison of means was done by the same computer package, using Duncan's New Multiple Range Test. P values of less than 0.05 were considered significant statistically (Schork and Remington, 2010).

RESULTS

***Prevalence of GI parasites in grazing sheep of Sialkot district*:** The overall prevalence of GI parasites was 32.81%. Among tehsils, prevalence of GI parasites was insignificant and highest was found in Daska (33.78%) followed in order by Pasroor (32.33%), and Sialkot (32.03%). The frequency distribution of GI parasites in grazing sheep of various tehsils of Sialkot district is comprehensively presented in Figure 1. Variable results were found regarding association of intrinsic determinants like: sex, age and breed of sheep in the study tehsils. *Fasciola hepatica* (2.58%), *F. gigantica* (5.74%), *Haemonchus contortus* (27.43%), *Eimeria crandallis* (23.17%), *Oesophagostomum* sp., *Trichuris ovis* (9.39%), *Strongyloides* sp. (18.61%), *Cryptosporidium* sp. (16.91%)

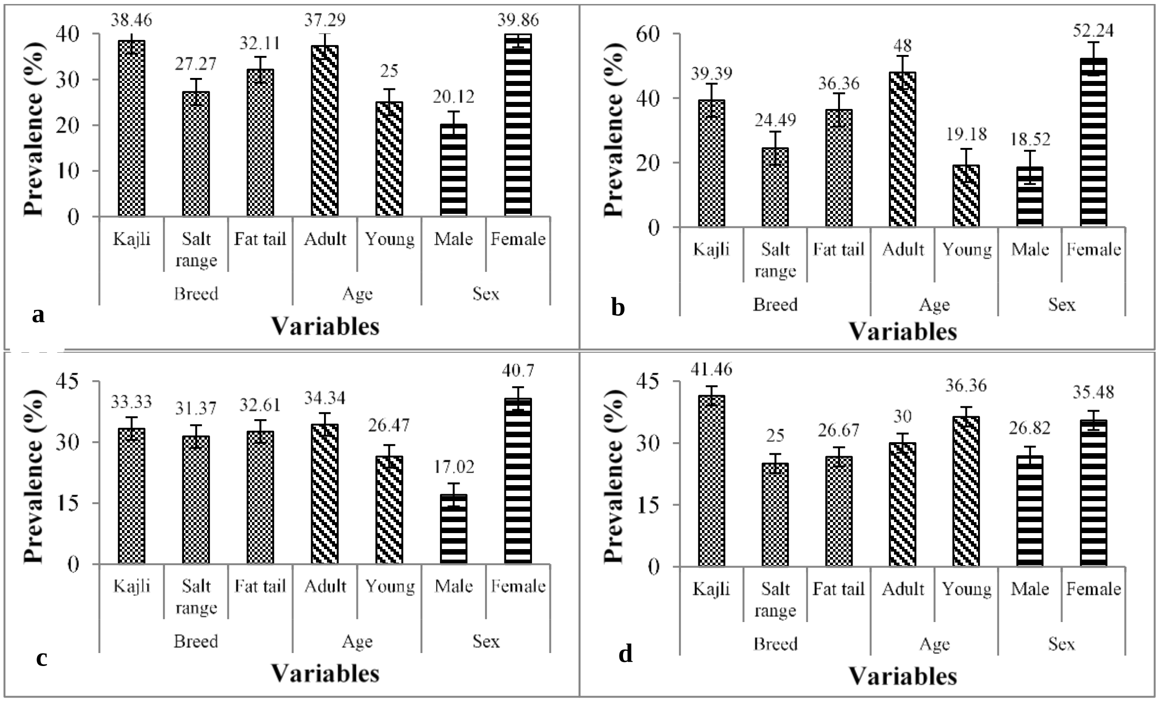

Figure 1. Prevalence of GI parasites in district and tehsils of Sialkot, Punjab, Pakistan during spring 2015.
a = frequency distribution of GI parasites in district Sialkot. b = frequency distribution of GI parasites in tehsil Daska. c = frequency distribution of GI parasites in tehsil Pasroor. d = frequency distribution of GI parasites in tehsil Sialkot.

and *Trichostrongylus* sp. (13.54%) were identified from the microscopically scanned faecal samples.

***Trace element profile of sera, soils and forages*:** Statistically, serum Zn concn showed variation (P < 0.05) among different tehsils. Concentration of Zn was maximum (1.38 ± 0.21) in sera of sheep belonging to tehsil Sialkot and minimum (0.76 ± 0.08) in tehsil Daska. Concentration of Cu varied significantly (P < 0.05) with a maximum (1.15 ± 0.09) and minimum (0.80 ± 0.30) levels in sera of sheep from tehsils Sialkot and Pasroor, respectively; however, those of Mn and Co varied insignificantly (P > 0.05) in sheep from Sialkot. Mean values of the study elements *viz*; Zn, Co, Cu and Mn in grazing field soils showed insignificant (P > 0.05) variation among soils of different tehsils. Eight forage species were recorded that were preferably consumed by sheep reared under open grazing system in different tehsils of Sialkot district. The identified species included: *Cichorium intybus, Coronopus didymus, Mazus reptans, Cynodon dactylon, Malva neglecta, Trifolium alexandrinum, Parthenium hysterophorus* and *Medicago polymorpha*. Levels of Zn, Cu, Mn and Co significantly (P < 0.05) varied in analyzed forages.

Table 1. Mean concentrations of selected trace elements (Zn. Cu. Mn. & Co.) in identified forages of district Sialkot, Punjab, Pakistan during Spring 2015.

Forages species	**Zn** (mg/kg) Mean ± SE	**Cu** (mg/kg) Mean ± SE	**Mn** (mg/kg) Mean ± SE	**Co** (mg/kg) Mean ± SE
Cichorium intybus	41.61 ± 0.16^{a}	37.29 ± 0.11^{b}	18.34 ± 0.22^{de}	1.20 ± 0.05^{a}
Coronopus didymus	21.53 ± 0.16^{c}	26.28 ± 0.19^{d}	32.18 ± 0.14^{b}	1.51 ± 0.03^{a}
Mazus reptans	38.31 ± 0.11^{a}	27.61 ± 0.13^{d}	21.61 ± 0.04^{de}	1.32 ± 0.05^{a}
Cynodon dactylon	29.28 ± 0.13^{b}	44.61 ± 0.07^{a}	17.91 ± 0.06^{e}	0.98 ± 0.03^{a}
Medicago polymorpha	37.80 ± 0.07^{a}	28.25 ± 0.09^{cd}	26.63 ± 0.12^{c}	1.18 ± 0.05^{a}
Malva neglecta	31.71 ± 0.16^{b}	29.29 ± 0.06^{cd}	34.59 ± 0.04^{b}	1.38 ± 0.06^{a}
Trifolium alexandrinum	38.31 ± 0.07^{a}	31.61 ± 0.07^{c}	22.61 ± 0.04^{cd}	1.16 ± 0.02^{a}
Parthenium hysterophorus	24.53 ± 0.06^{c}	43.72 ± 0.05^{a}	39.61 ± 0.05^{a}	1.04 ± 0.03^{a}
Mean	32.89 ± 7.29	33.58 ± 7.34	26.69 ± 8.00	1.22 ± 0.17^{a}

Mean sharing similar letters in a column are statistically insignificant (P > 0.05).

Maximum concn. (41.61 ± 0.16 mg/kg) of Zn was found in *Cichorium intybus*, and minimum (21.53 ± 0.16 mg/kg) in *Coronopus didymus*. Maximum concn. (44.61 ± 0.07 mg/kg) of Cu was found in *Cynodon dactylon* and minimum (26.28 ± 0.19 mg/kg) in *Coronopus didymus*. *Parthenium hysterophorus* and *Cynodon dactylon* contained maximum (39.61 ± 0.05 mg/kg) and minimum (17.91 ± 0.06 mg/kg) concn. of Mn, respectively. Concn. of Co was highest in *Coronopus didymus* (1.51 ± 0.03 mg/kg), and minimum (0.98 ± 0.03 mg/kg) in *Cynodon dactylon*. Variation in mean concns. of Zn, Co and Mn in forages from different tehsils were statistically insignificant ($P > 0.05$); however, those of Cu in forages from different tehsils varied significantly ($P < 0.05$) being maximum in tehsil Daska (39.81 ± 5.94 mg/kg) and minimum (29.01 ± 4.74 mg/kg) from tehsil Sialkot (Table 1).

Interaction Analyses:

***a. Analyses of serum trace elements with quantitative parasitic burden of sheep*:** Comparasion of trace elements of sera with mean EPG of each tehsil of Sialkot district is given in Figure 2. The mean concns. of Zn and Cu in sera were found inversely proportional to the mean EPG of sheep in all the three tehsils of Sialkot district. Whereas, mean concns. of Co and Mn showed variable results.

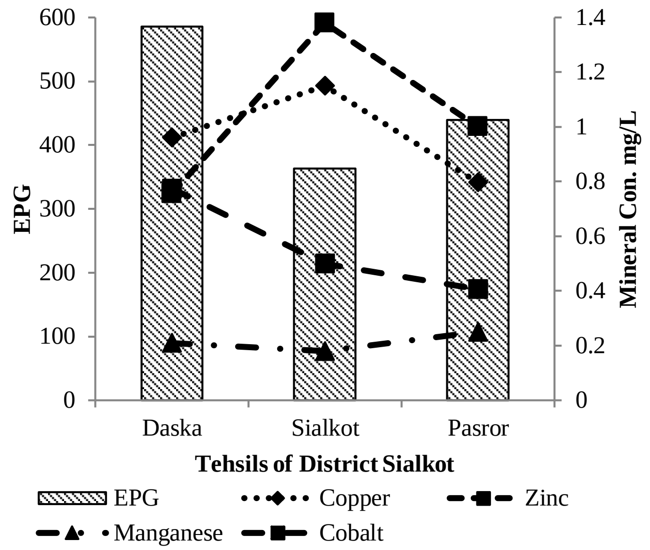

Figure 2. Comparasion of serum trace elements with mean egg per gram of each tehsil of Sialkot district, Punjab, Pakistan during spring 2015.

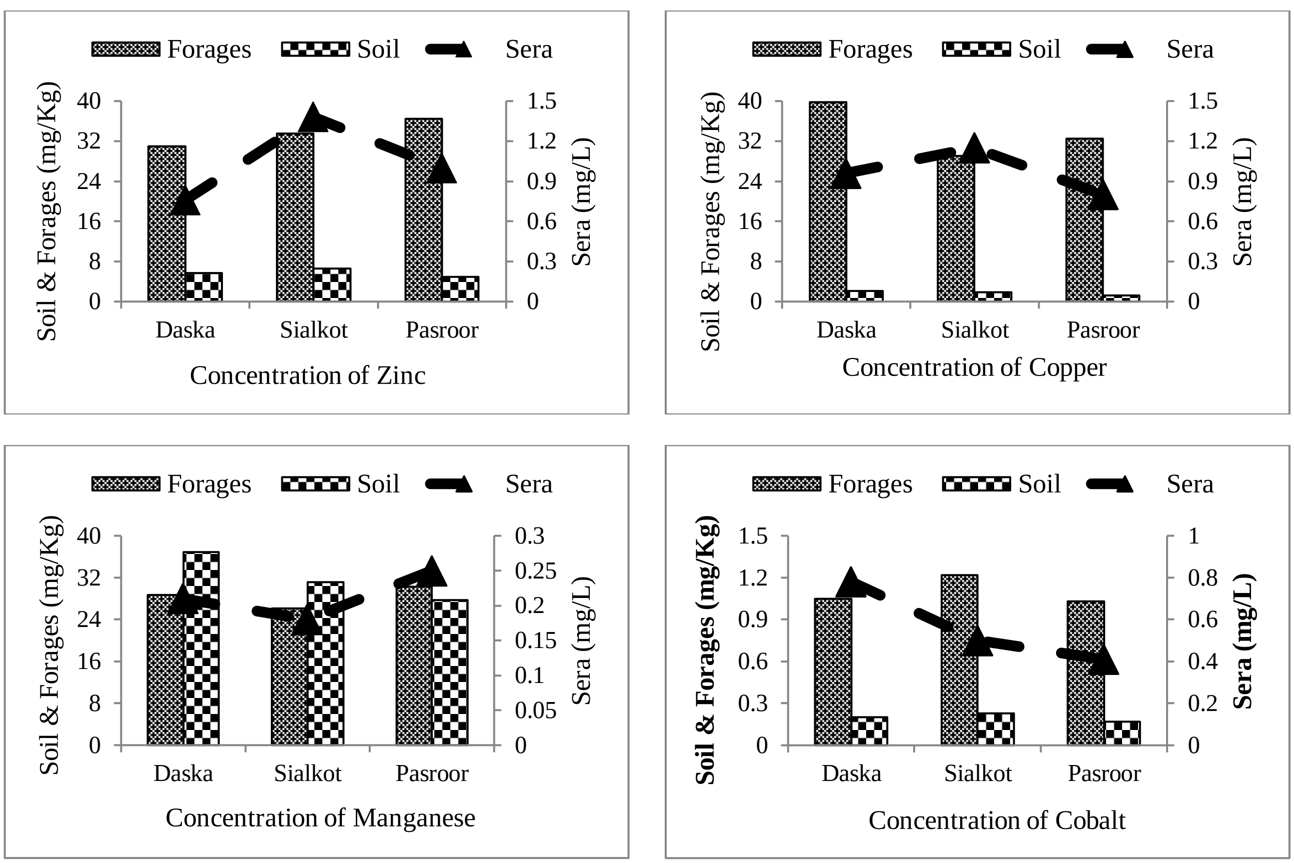

Figure 3. Association of trace elements of soil-plant-serum in each tehsil of Sialkot district during spring 2015.

***b. Soil-plant-serum mineral correlation analyses*:** Relationship of trace elements of soil-plant-serum each tehsil of Sialkot district is given in Figure 3. The mean concn. of Mn in serum was directly related to the mean concn. of Mn in forages in all tehsils of Sialkot district. The mean concn. of Co in forages and soil was also directly corelated in all tehsils of Sialkot district. Whereas, mean concns. of Zn and Cu showed variable results.

DISCUSSION

Minerals availability in an appropriate quantity is a pre-requisite for the health and productivity of livestock, while insufficient mineral intake or unavailability decreases the productivity (Khan *et al*., 2007). Under natural grazing conditions, forage plants are the major source for animals to obtain minerals (McDowell and Arthington, 2005). Mineral constituents of plants may vary widely among plant species, locations and may be pronounced seasonal changes. Minerals level in plants depends on interactions amongst various factors including; grazing management, stage of maturity, plant species, dry matter yield, soil type and climate (Khan *et al*., 2005). In grazing livestock mineral deficiencies or excesses can be predicated by regional reconnaissance and systematic mapping survey techniques in a specified region (McDowell and Arthington, 2005). Many plant species are available in pastures which can fulfil the mineral nutrients to grazing animals. Animals having inadequate supplies of mineral are more prone to GI parasitic infection as well as reduce their productivity. For determine the enough minerals availability for animals, appropriate analyses of mineral level in soil, forages and animal is essential. The soil, plant and animal is a complex system which has not been studied particularly in the developing countries. Facts are required on relationship of minerals among soil, plant and animals to boost the immunity of animals associated with the minerals.

The prevalence of GI parasites recorded in this study was lower than that recorded in different districts of Pakistan e.g. 32.6% in Sialkot (Rizwan *et al*., 2017), 35.4% in Peshawar (Shah *et al*., 2015), 40.25% in Loralai (Razzaq *et al*., 2013), 44.17% in Toba Tek Singh (Khan *et al*., 2010), 49.47% in Quetta (Kakar *et al*., 2013), 62.00% in Muzaffar Garh (Raza *et al*., 2007) and 72.08% in Lahore (Mehmood *et al*., 2013). Similarly, the prevalence of GI parasites determined in various parts of the world is also higher than that of this study e.g. 43.10% in Nigeria (Jegede *et al*., 2015), 62.02% in India (Bhat *et al*., 2014), 62.80% in German (Idris *et al*., 2012), 73.10% in Cameroon (Ntonifor *et al*., 2013), 81.10% in Bangladesh (Sangma *et al*., 2012), 86.71% in Iraq (Minnat, 2014) and 94.40% in Sri Lanka (Kandasamy *et al*., 2013). However, Ibrahim *et al*. (2008) in Saudi Arabia and Kantzoura *et al*. (2012) in Greece recorded lower prevalence of GI parasites i.e. 18.63% and 07.90%, respectively.

The higher prevalence of GI parasites may be related to the management of sheep under extensive pastoralism, inadequate animal care infrastructure and grazing nature of sheep which enhances the acquirement chances of infective larvae from contaminated herbage. Relatively lower prevalence of sheep GI parasites might be due to certain factors like host age and breed variation, parasitic species, epidemiological patterns (Tembely *et al*., 1997), environmental and management practices (Rizwan *et al*., 2016). Almost similar prevalence of GI parasites may be linked to the acquisition of worms by host species in these areas and may be joined with similar rainfall and humidity at these sites (Lashari and Tasawar, 2011).

The concn. of Zn in rangeland soils of the present study area is in line with Khan *et al*. (2006) and Aregheore *et al*. (2007). The lower concn. of Zn has been reported by Khan *et al*. (2008) and Ahmad *et al*. (2012); however, Vilallonga *et al*. (2012), Shisia *et al*. (2013), Silvanus *et al*. (2014) and Wang *et al*. (2014) recorded higher concn. of Zn in soils. Variation in soils Zn constituent may occur due to low pH and cultivation (Aubert and Pinta, 1977) because Zn solubility and susceptibility is more in soils with low pH and high rainfall localities. The concn. of Zn in selected forage species of present study was lower than values reported earlier (Hussain and Durrani, 2008; Habib *et al*., 2013; Lengarite *et al*., 2013; Nordlokken *et al*., 2015). However, Mountousis *et al*. (2009), Sher *et al*. (2011), Ahmad *et al*. (2012), Silvanus *et al*. (2014) have reported a higher concn. of Zn as compared to present study. The deficiency of Zn has been reported in ruminants grazing on forages low in Zn or high in compounds like: Cd, Fe, Mg, Mn, Mo and S, because these elements interact with Zn and decrease its utilization in ruminants (Ndebele *et al*., 2005). The observed serum Zn level in the examined sheep population is in coincides with Humann-Ziehank *et al*. (2008), Mohammed *et al*. (2013) and Dar *et al*. (2014). However, higher level of Zn in serum has been reported by Mohri *et al*. (2011), Kojouri *et al*. (2011), Donia *et al*. (2014), Wang *et al*. (2014) and Davoodi and Kojouri (2014), while, Suliman *et al*. (1988) reported lower serum Zn in sheep. The levels of Zn in serum were reported lower than the critical level before and after calving. In eastern Slovakia, the levels of Zn and Cu in serum samples were lower. Of the total number of samples, the amounts of Cu in serum were reduced in all production phases (Skalicka *et al*., 2016).

The grazing site soils of the selected areas have similar concn. of Cu as reported by Ure and Berrow (1982) and McBride (2001). Aregheore *et al*. (2007), Mapako (2011) and Wang *et al*. (2014) reported higher concn. of Cu in rangeland soils. However, Khan *et al*. (2004, 2006, 2008) reported low level of Cu in rangeland soil. Variation in the Cu availability can be affected by soil pH because its concn. declines as soil pH increases (Aubert and Pinta, 1977). Copper levels of identified forage species is well supported by Ramirez *et al*. (2001) and Khan *et al*. (2007); however, lower levels than recommended dietary requirements have been reported by Silvanus *et al*. (2014), Soni *et al*. (2014) and Nordlokken *et al*. (2015). It has been reported that low soil pH increases the Fe solubility which results into depressed Cu absorption by the plants (Beeson and Matrone, 1976). Lower Cu levels in forages may also be attributable to the presence of antagonist elements like: Mo, S and Ca (McDowell, 2003). The analyzed concn. of serum Cu has close similarity with those observed by Mohammed *et al*. (2013), Donia *et al*. (2014) and Dar *et al*. (2014). Reports are available which showed that sheep blood serum contains higher (Amer *et al*., 2014) and lower (Wang *et al*., 2014) concn. of Cu.

The similar level of Mn in the rangeland soils is reported by Espinoza *et al*. (1982) and Khan *et al*. (2006). Comparatively, higher level of Mn (Fardous *et al*., 2011; Shisia *et al*. 2013; Silvanus *et al*., 2014; Wang *et al*., 2014) and lower level (McDowell *et al*., 1982; Khan *et al*., 2004; Ahmad *et al*., 2012) have also been documented in the grazing site soils. Variation in the soil Mn level may occur due to its rapid oxidation and reduction under different soil environments. Similar levels of Mn have been reported by Mountousis *et al*. (2009) and Soni *et al*. (2014). However, relatively higher Mn levels in forage species have been reported by Habib *et al*. (2013), Silvanus *et al*. (2014), Udiba *et al*. (2014) and Nordlokken *et al*. (2015). Lower levels of Mn in certain forages consumed by livestock have also been reported (Aganga *et al*., 2000; Aganga and Mesho, 2008; Sher *et al*., 2011). Forage Mn levels depend on the levels of Mn in respective soils; however, it has been reported that the livestock get adequate amount of Mn even when grazed on plants from Mn deficient soils (Underwood, 1981). Similar picture of serum Mn concn. has been depicted by Khan *et al*. (2003) and Amer *et al*. (2014) in sheep. However, higher (Donia *et al*., 2014) and lower (Wang *et al*., 2014; Davoodi and Kojouri, 2014) levels have also been observed in sheep.

The observed Co level is similar with the range mentioned by McDowell *et al*. (1982). Comparatively higher (Mitchell and Gray, 2003; Yuan-Rong *et al*., 2011; Fardous *et al*., 2011) and lower (Khan *et al*., 2005; Khan *et al*., 2006) levels of Co have been documented in the grazing site soils. Trace elements of soils are directly linked to soil quality, pH, lime, electrical conductivity and organic matter. The presence of other elements can also suppress or enhance the mineral uptake (Mitchell and Gray, 2003). Cobalt levels in forage species is well comparable with those documented by Johannesson *et al*. (2007) and Nordlokken *et al*. (2015). However, lower level as compared to current estimation has been reported by Khan *et al*. (2008). Higher soil Mn concn. depresses Co uptake in forages. The absorption of Co by forages from the soil mainly depends upon the ratio of Co and Mn i.e. the higher the level of Mn in soil, the lower the level of Co in forages and *vice versa* (McKenzie, 1975). The estimated level of serum Co is comparable with Abdelrahman (2012); however, lower (Davoodi and Kojouri, 2014) and higher (Khan *et al*., 2003; Kozat *et al*., 2007) concns. have also been observed in sheep. Qudoos *et al*. (2017) reported an insignificant ($P > 0.05$) correlation of trace and ultra-trace minerals with EPG magnitude in naturally parasitized sheep. According to Khalil *et al*. (2016) Minerals of forages were not significantly affected by the seasons. They found some micro minerals like Mn, Se, Cu and Zn which were deficient in soil, forages and cattle.

***Conclusions*:** Overall prevalence of GI parasites was statistically insignificant and among factors only sex was found significantly associated with GI parasitism. Mean concns. of Zn, Cu, Mn and Co in grazing field soils showed insignificant ($P > 0.05$) variation. Concn. of selected trace elements was significantly ($P < 0.05$) varied in analyzed forages. Current investigation reflected an inverse correlation between certain trace elements i.e. Cu, and Zn with the magnitude of EPG in naturally parasitic infected sheep population. Overall, scale of EPG seems to be reduced in sheep flocks having optimum serum levels of these trace elements. The current investigation reveals an insignificant correlation of EPG with Mn and Co profile of sheep.

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