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Decolorization of Synozol Red 6HBN by Yeast, *Candida tropicalis* 4S, Isolated from Industrial Wastewater

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Abstract.- Azo dyes decolorizing yeast identified as *Candida tropicalis* 4S was isolated from wastewater treatment plant of textile and dyeing industry, Kot Lakhpat, Pakistan. The optimum temperature and pH for the growth of yeast were found to be 30ºC and 7, respectively. *C. tropicalis* 4S was capable of decolorizing Synozol Red 6HBN up to 79%, 90% and 93% after incubation of 7, 14 and 21 days, respectively. Phyto- and microbial toxicity studies revealed that dye degraded products did not show any toxicity. *C. tropicalis* 4S can be exploited for bioremediation of wastewater containing azo dyes.

Keywords: Synozol Red 6HBN, *Candida tropicalis*, azoreductase, decolorization, degradation.

Dyes are colored substances that when applied to fibers give them a permanent color which is able to resist fading upon exposure to sweat, light, water and many chemicals, including oxidizing agents and microbial attack (Rai *et al*., 2005). It is estimated that 280,000 tons of textile dyes are discharged in such industrial effluents every year worldwide (Jin *et al*., 2007). With the increasing use of a wide variety of dyes, pollution by dye wastewater is becoming increasingly alarming. Among these azo dyes are important colorants and are characterized by the presence of one or more azo groups (–N=N–) and constitute the largest class of dyes having extensive applications in textiles, papers, leathers, gasoline, additives, foodstuffs and cosmetics (Chen *et al*., 2009).

The release of textile and dye-house effluent may cause abnormal coloration of the surface water and this creates the greatest immediate environmental concern with regard to water quality, and directly affects the aquatic flora and fauna. It has been found that purified forms of many azo dyes are directly mutagenic and carcinogenic (Chen, 2002).

In the last decade, several microorganisms have been investigated for decolorization of reactive dyes and its effectiveness depends on the adaptability and the activity of selected microorganisms (Aksu *et al*., 2007; Jadhav *et al*., 2007; Dave and Dave, 2009; Aftab *et al*., 2011; Ilyas and Rehman, 2013). Microbiological decolorization and degradation is an environmental-friendly and cost competitive alternative to the chemical decomposition process (Saratale *et al*., 2009b, 2010; Aftab *et al*., 2011).

Very little work has been done to explore the decolorization ability of yeast, and it has mainly been studied with regard to biosorption. Some ascomycetes yeast species, such as *Candida tropicalis, Debaryomyces polymorphus, Candida zeylanoides* (Yang *et al*., 2003) and *Issatchenkia occidentalis* (Ramalho *et al*., 2004), have been used to carry out putative enzymatic biodegradation and consequent decolorization of different azo dyes.

This study aims to investigate the potential of *C. tropicalis* 4S to decolorize an azo textile dye, Synozol Red 6HBN. The rate dependent environmental parameters such as temperature, pH and metal ions were studied. The phyto- and microbial toxicity of the products formed after degradation were also studied.

Materials and methods

Isolation and screening of yeast strains

Samples were collected from Kot Lakhpat industrial areas, Lahore. Isolation was done by spreading 100 µL of wastewater sample on yeast extract peptone-dextrose (YEPD) agar plates. YEPD plates were prepared by dissolving 2g of peptone,

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2g of dextrose and 1g of yeast extract and 2g of agar in 100 mL distilled water. In the preliminary study, Synozol Red 6HBN [CI Reactive Red 195; MF: $C_{31}H_{19}ClN_7Na_5O_{19}S_6$ (Fig.1)] was selected for determining the decolorization ability of the 34 yeast isolates. YEPD broth medium containing 20 µg/mL of dye was used for screening of yeast incubated at 30°C.

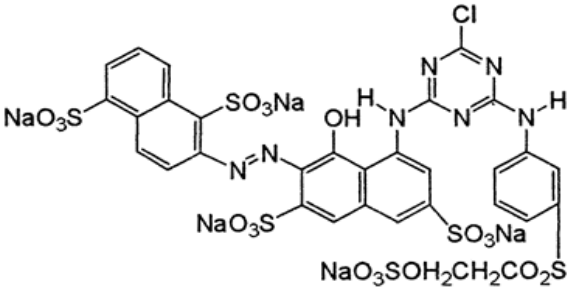

Fig. 1. Structure of Synozol Red 6HBN (CI Reactive Red 195).

Yeast identification

DNA was isolated (Masneuf-pomarède *et al*., 2007) and polymerase chain reaction (PCR) was performed by using two oligonucleotide yeast 18S rRNA primers, the forward primer (ITS5; 5-GGAAGTAAAAGTCGTAACAACG-3) and the reverse primer (ITS-4; 5-TCCTCCGCTTATTGATATGC-3) (Larena *et al.*, 1999). The PCR consisted of 35 cycles, each of denaturation at 94°C for 4 min, annealing at 55°C for 2 min, and elongation at 72°C for 10 min. PCR reaction mixture contained 3 µL of PCR buffer, 3 µL of dNTPs, 2.5 µL of each forward and reverse primer, 0.5 µL of taq polymerase, 2.5 µL of $MgCl_2$, 5 µL of nuclease free water and 6 µL of genomic DNA.

The PCR products was extracted by the thermo scientific gene JET gel extaction kit method. The sequencing was carried out by Genetic analysis system model CEQ-800 (Beckman) Coulter Inc. Fullerton, CA, USA. Nucleotide sequence similarities were determined using BLAST (NCBI database; http://www.ncbi.nlm.nih.gov/BLAST).

Microorganism and maintenance conditions

The yeast culture was maintained on YEPD agar plates at 4°C. The yeast culture was also maintained in salt medium containing 10 g/L glucose, 1 g/L $(NH_4)_2SO_4$, 0.15g/L KH_2PO_4, 0.1g/L K_2HPO_4, 0.1g/L $MgSO_4 \cdot 7H_2O$, 0.026g/L $FeSO_4$ and 0.086g/L $CaCl_2$. The pH of the medium was adjusted to 7.0-7.2 and was sterilized at 121°C for 20 min. The 20 µg/mL of Synozol Red 6HBN was added to the cell culture on 2nd day of incubation. After 7 days of incubation cells were harvested by centrifugation (6000 *g*, 10 min), washed twice with sodium phosphate buffer (50 mM, pH 7.0) and lysed by sonication for 2-3 times at 4°C for 15 s with a 60 s interval. The lysate was centrifuged at 4000rpm for 10 min.

For the determination of optimum temperature of the yeast YEPD broth medium was inoculated with fresh yeast culture and kept at 20, 30, 37, 50 and 70°C under shaking conditions. For determining optimum pH 100 mL YEPD broth medium with pH 5, 6, 7, 8, and 9 was inoculated with fresh yeast culture and incubated at 30°C for 24 h. Growth was monitored by taking the optical density at 600 nm.

Effect of dye on growth

For analyzing the effect of dye on the growth of yeast isolate YEPD broth medium (100 mL) was prepared and inoculated with yeast culture in 250 mL of Erlenmeyer flask and marked as control. Another flask containing 100 mL of salt medium was inoculated with culture and 20 µg/mL of Synozol Red was also added in it and marked as treated. The flasks were incubated at 30°C in shaker (120 rpm) and 3 mL sample was withdrawn after regular time interval of 4, 8, 12, 16, 20, 24, 28 and 32 h. The absorbance was taken at 600nm.

Enzyme activity assay

Yeast cells were harvested by centrifugation at 4000 rpm for 10 min and an appropriate amount of lysis buffer (SDS and mercaptoethanol: 1:1 Check spelling) was added to the cell pellet and then sonicated at 4°C for 15 s with a 60 s interval 2-3 times and centrifuged at 4000 rpm for 10 min. The lysate or sonicated suspension was used for determining and analyzing the activity of crude enzyme. Azo reductase activity was determined by initiating the reaction by addition of 200 µl of 100 µM NADH in enzyme reaction mixture comprising

200 µl of 25µM Synozol Red 6HBN, 600 µL of crude enzyme and 1 ml of 25mM tris-HCl buffer with pH 7.4. The reaction was incubated at 30°C for 30 min and relative activity of the enzyme was measured by taking absorbance at 436 nm using UV-VIS spectrophotometer (UV-4000, Germany). One unit of azoreductase can be defined as the amount of enzyme required to decolorize 1 µmol of Synozol Red 6HBN per min.

Effects of temperature, pH and metal ions

The activity of azoreductase enzyme was checked by incubating the enzyme reaction at temperature (30-90°C) by standard enzyme assay. The optimum pH of the crude enzyme was investigated over a pH range of 4.0-9.0. The enzyme was kept at 30°C for 30 min in various buffers (50 mM) and the residual azoreductase activity was determined under standard assay conditions. The buffer systems used comprised of 50 mM sodium acetate buffer pH (5.0-6.0), 50 mM sodium phosphate buffer pH (7.0-8.0) and 50 mM Tris-HCl buffer pH (9.0). For each pH a reaction mixture without enzyme (control) was prepared under the same condition and was used to measure the possible changes in absorbance. The effect of metal ions ($FeCl_3$, $CoCl_2$, NaCl, $CuSO_4$, $ZnCl_2$, $MgCl_2$ and $MnCl_2$; 1 mM each separately) on the azoreductase activity was also investigated.

Decolorization studies

For decolorization study, a loop full culture of yeast isolate from YEPD plate was used to inoculate 250-mL Erlenmeyer flask containing 100 mL salt medium under optimal growth conditions (pH 7.0; 30°C). The flask was kept at 30°C with shaking (130 rpm) for 2 days. The 48 h grown culture was incubated with Synozol Red 6HBN (20 µg/mL) for 7, 14 and 21 days under aerobic condition. The decolorization process was monitored by taking 5 mL sample at regular intervals and O.D was measured spectrophotometrically at 436 nm. Control had no inoculated yeast culture except dye. Experiment was run in triplicate. The percentage decolorization was calculated (Saratale *et al*., 2009a) as follows:

$$\% \text{ decolorization} = \frac{\text{Initial absorbance} - \text{Observed absorbance} \times 100}{\text{Initial absorbance}}$$

Microbial and phytotoxicity

Toxicity effects were studied using dye degraded metabolites of 7 days old filtrate of yeast isolate. LB plates were inoculated with *Bacillus cereus* and *Azotobacter* and incubated at 37°C for 24 h. The yeast culture supernatant was then poured into the wells of the plate. The plates were incubated at 37°C for 24 h. Control contained distilled water in place of yeast culture supernatant. The effect was measured in terms of a zone of inhibition (diameter; cm) after 24 h of incubation at 37°C. To know the phytotoxic effect of Synozol Red decolorized products *Vigna radiata* was used. The pot containing four seeds of *V. radiata* was watered by 10 mL of yeast decolorized water per day after centrifugation at 4000 rpm for 10 min. Control pot was watered with distilled water at the same time. Growth was monitored after 7 days of incubation under 1:1 light and dark period (Ilyas and Rehman, 2013).

Results and discussion

Growth characteristics of C. tropicalis

The nucleotide sequences coding for the 18S rRNA gene (GenBank database under accession number JN009853) after BLAST query revealed that this gene is 100% homologous to *Candida tropicalis*.

The isolate showed maximum growth in YEPD medium (Control). Yeast isolate also showed good growth in dye containing salt medium but it was less as compared to control (data not shown). The optimum temperature and pH of the yeast was found to be 30°C and 7, respectively.

Effects of temperature, pH and metal ions on enzyme activity

It was determined that *C. tropicalis* 4S showed maximum azo reductase activity at 30°C (108%) and pH 7 (Fig. 2A). The decline at higher temperatures can be attributed to the loss of cell viability or the denaturation of azo reductase enzyme (Chang *et al*., 2001a; Saratale *et al*., 2009c).

The effects of pH (Fig. 2B) may be correlated to the transport of dye molecules across the cell membrane, which is considered as the rate limiting step for the decolorization (Chang *et al*., 2001b).

The enzyme activity increased 37% in the

presence of K^+ (Fig. 2C). The presence of heavy metal ions in the industrial effluents is a major obstacle which could potentially affect the activity of the reductase enzymes.

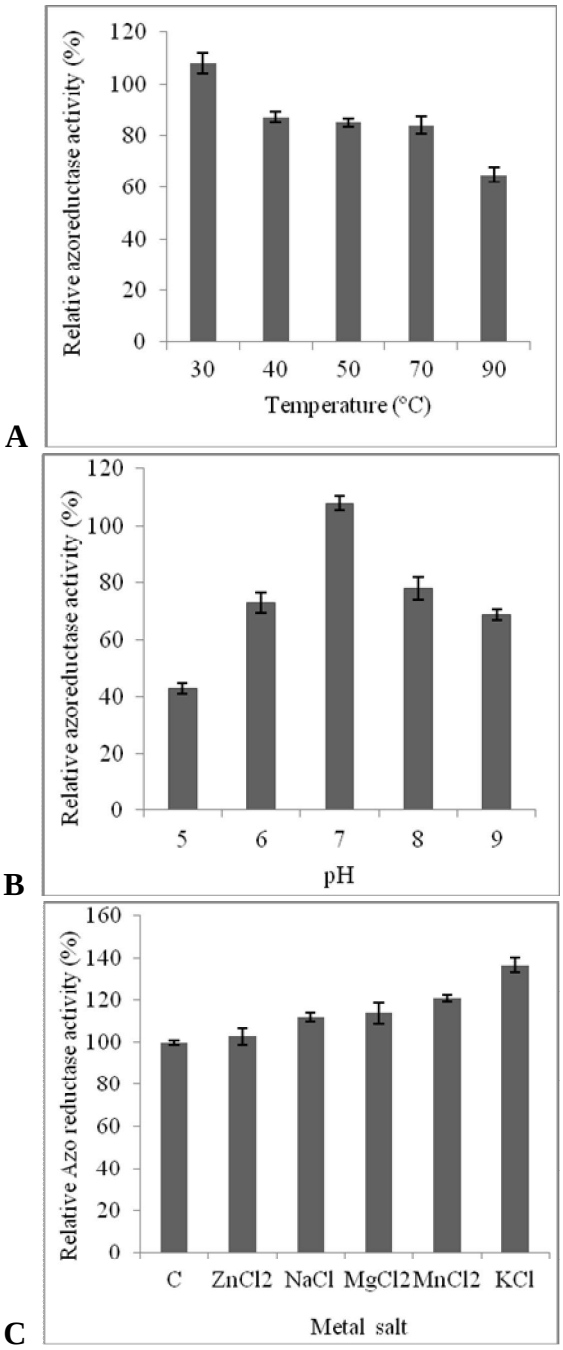

Fig. 2. Effect of (A) temperature (A), pH (B) and metal ions (C) on the enzymatic activity of azo reductase.

Dye removal

The color removal was 79% after 7 days, 90% after 14 days and 93% after 21 days. Some studies also show that yeast species act as a promising dye adsorbent and are able to uptake higher dye concentration (Sˇafarˇı'kova' *et al*., 2005), while *Galactomyces geotrichum* MTCC can decolorize triphenylmethane, azo and reactive high exhaust textile dyes (Jadhav *et al*., 2008a). Another detailed study was conducted on the decolorization of Navy Blue HER by using *Trichosporon beigelii* NCIM-3326, with the enzymatic mechanism and toxicity of the degradation products also reported (Saratale *et al*., 2009a).

In general, during azo dye degradation initial reductive cleavage of the azo bonds takes place, with the help of azoreductase enzymes which results into the production of colorless solutions containing potentially hazardous-aromatic amines (Van der Zee and Villaverde, 2005) which are further degraded aerobically or anaerobically (Joshi *et al*., 2008).

Microbial and phytotoxicity

Microbial toxicity and phytotoxicity results revealed no germination inhibition of plant by Synozol Red 6HBN metabolites. The growth of *V. radiata* was as good as its growth was observed in distilled water. This indicates that the yeast decolorized/degraded dye supernatant was safe for irrigation.

In the present study *C. tropicalis* 4S exhibited maximum ability to decolorize Synozol Red 6HBN upto 93%. It was observed that biodegradation efficiency is strongly affected by pH, temperature and type of metal ions used. Thermal stability of azo reductase was found to be 30ºC at pH 7. Biodegraded products of Synozol Red 6HBN by *C. tropicalis* 4S have shown that these metabolites are safe and non-toxic for microbial flora and agricultural crops; thus this treated wastewater can be at-least used for irrigation purposes. Therefore the nature of the metabolites and their biodegradability require further investigation for complete mineralization of these compounds by the yeast strain.

References

Aftab, U., Khan, M.R., Mahfooz, M., Ali, M., Aslam, S.H. and

Rehman, A., 2011. *Pakistan J. Zool*., **43:** 1-8.

Aksu, Z., Kilic, N.K., Ertugrul, S. and Donmez, G., 2007. *Enzyme Microb. Technol*., **40:** 1167–1174.

Chang, J.S., Chou, Y.P. and Chen, S. Y., 2001a. *Process Biochem.*, **36:** 757-763.

Chang, J. S., Chou, C., Lin, Y., Ho, J. and Hu, T. L., 2001b. *Water Res*., **35:** 2841-2850.

Chen, B.Y., 2002. *Process Biochem*., **38:** 437-440.

Chen, B.Y., Lin, K.W., Wang, Y.M. and Yen, C.Y., 2009. *J. Hazard. Mat*., **166:** 187–194.

Dave, S.R. and Dave, R. H., 2009. *Biresour. Technol*., **100:** 249–253.

Ilyas, S. and Rehman, A., 2013. *Iranian J. environ. Hlth. Sci. Engineer*., **10(12):** 1-9.

Jadhav, J.P., Parshetti, G.K., Kalme, S.D. and Govindwar, S.P., 2007. *Chemosphere*, **68:** 394-400.

Jadhav, S.U., Jadhav, M.U., Kagalkar, A.N. and Govindwar, S.P., 2008a. *J. Chin. Inst. Chem. Engrs*., **39:** 563-570.

Jin, X., Liu, G., Xu, Z. and Tao, W., 2007. *Appl. Microbiol. Biotechnol*., **74:** 239-243.

Joshi, T., Iyengar, L., Singh, K. and Garg, S., 2008. *Bioresour. Technol*., **99:** 7115-7131.

Larena, I., Salazar, O., Goncalez, V., Julian, M. C. and Rubio, V., 1999. *J. Bacteriol.,* **75:** 187-194.

Masneuf-pomarède, I., Le jeune, C., Durrens, P., Lollier, M., Aigle, M. and Dubourdieu, D., 2007. *Sys. appl. Microbiol.*, **30:** 75-82.

Rai, H., Bhattacharya, M., Singh, J., Bansal, T.K., Vats, P. and Banerjee, U. C., 2005. *Crit. Rev. environ. Sci. Technol*., **35:** 219-230.

Ramalho, P.A., Cardoso, M.H., Cavaco-paulo, A. and Ramalho, M.T., 2004. *Appl. environ. Microbiol*., **70:** 2279-2288.

Sˇafarˇi'kova', M., Pta' Cˇkova', L., Kibrikova', I. and Sˇafarˇi'k, I., 2005. *Chemosphere*, **59:** 831-835.

Saratale, R.G., Saratale, G.D., Chang, J.S. and Govindwar, S. P., 2009a. *J. Hazard. Mat*., **166:** 1421-1420.

Saratale, R.G., Saratale, G.D., Kalyani, D.C., Chang, J.S. and Govindwar, S. P., 2009b. *Bioresour. Technol*., **100:** 2493-2500.

Saratale, R.G., Saratale, G.D., Chang, J.S. and Govindwar, S. P., 2009c. *Bioresour. Technol*., **110:** 3897-3905.

Saratale, R.G., Saratale, G.D., Chang, J.S. and Govindwar, S.P., 2010. *Biodegradation*, **21:** 999–1015.

Van der zee, F.P. and Villaverde, S., 2005. *Water Res*., **39:** 1425-1440.

Yang, Q.M., Yang, K., Pritsch, A., Yediler, A., Hagn, M., Schloter, A. and Kettrup, S., 2003. *Biotechnol. Lett*., **25:** 709–713.

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The Association of Some Heavy Metals With Rheumatoid Arthritis

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Abstract.-The objective of the present study was to investigate the association of some heavy metals with rheumatoid arthritis. The results indicated that concentration of Cu was significantly lower in the serum of RA +ve patients compared to RA -ve individuals. Non-significant relationship was observed between concentration of Zn, Co, Pb, and Ni with RA +ve patients. Similarly the concentration of these metals had no significant relationship with sex or age of RA +ve patients.

Keywords: Rheumatoid arthritis, heavy metals.

Rheumatoid arthritis (RA) is an inflammatory disorder which affects many tissues and organs but mainly the synovial joints. Although there are many factors implicated in RA but human leukocyte antigen (HLA) gene like direct repeat 4 (DR4) drugs, chemicals, bacteria, some viruses like hepatitis C virus, Epstein Bar virus and metals have been considered as the causative agents (Lawrence *et al.,* 1998). Several researchers have also mentioned heavy metals as one of the contributing factors of RA (Naveh *et al*., 1997; Hannson *et al*., 1978; Ala *et al*., 2005; Yazar *et al*., 2005). Chronic exposure to heavy metals, especially lead (Pb), mercury (Hg) and cadmium (Cd) affects the immune system. Heavy metals also reduce the number of existing B and T cells (Lehmann *et al*., 2011). Resultantly, the immune system attacks on its self molecules, which can lead to RA and other joint diseases (Lehmann *et al*., 2011). Scudder *et al*. (1978) reported significantly higher levels of Cu in RA patients compared to healthy objects. Level of copper in RA patients was found to be correlated with the age *i.e.,* decrease with increasing age (Farid *et al.,* 2005). However, RA patients were found to be deficient in Zn level (Naveh *et al*., 1997; Ala *et al*., 2009). Alegre *et al.* (1984) observed though normal concentration of Zn in RA patients. In the present study we evaluated the contribution of some heavy metals (*i.e*., Cu, Zn, Pb, Co and Ni) in causing RA.

Materials and methods

Blood samples of patients, suspected of RA, were collected from different diagnostic laboratories in Sargodha. Age and gender of the patients was also noted. About 5 ml blood was taken from each patient with the help of sterile disposable syringe, centrifuged at 3,000 rpm for 2 min for serum extraction. The serum was further processed through wet acid digestion and analyzed by atomic absorption spectrophotometry (AAS).

Two sample t-test was used to compare the heavy metal concentration of RA+ve and control groups, RA+ve males and females and two age groups *i.e.,* below 35 years and above 35 years. Minitab (Version 15) was consulted for calculating means, standard errors and t-test. The difference was considered significant if P value was less than 0.05.

Results and discussion

Level of Cu in RA+ve patients was significantly lower than control group (df =37; t= 2.09; P = 0.04), however, there was no difference in the levels of Zn, Pb, Co and Ni between RA+ve and control groups (df =37; t= 1.24; P = 0.22 for Zn, df =55; t= 0.18; P = 0.85 for Pb, df =59; t= 0.49; P = 0.62 for Co and df =57; t= 1.82; P=0.07 for Ni, respectively). Figure 1A depicts the comparison of heavy metals between RA+ve and control groups. RA+ve males and females did not differ in heavy metal's concentrations (Table I). The RA+ve females had higher concentration of Cu, Pb, Co and Ni as compared to RA+ve males, while Zn concentration were higher in RA+ve males than females (Table I). Non-significant differences were also recorded when we compared the heavy metals concentrations of two age groups (Fig.1B).

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Results obtained from our study showed that Cu concentration was significantly lower in RA+ve patients than RA-ve individuals. These findings are comparable to Olak *et al*. (2001) and Afridi *et al*. (2012) but do not match the findings of Colak *et al*. (2001) who observed a high concentration of Cu in RA+ve patients. Strecker *et al*. (2013) recorded lower concentration of Cu in patients erythrocytes and in serum. However, we did not find any difference in heavy metal concentration of RA+ve patients of different age groups. Farid *et al*. (2005) reported that RA+ve females had elevated level of Cu in comparison to RA+ve males while during present study a non-significant difference was observed in the two sexes.

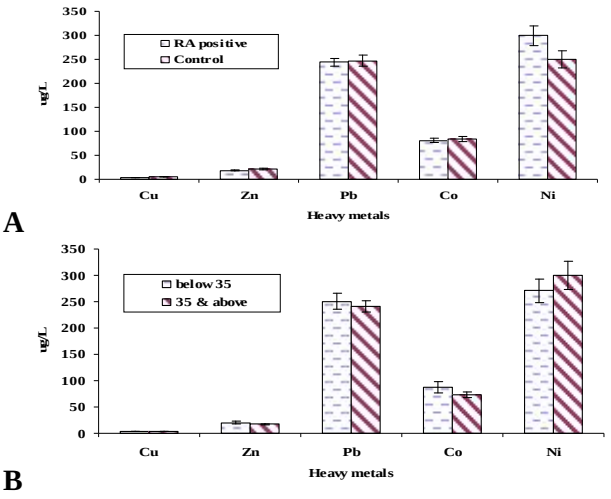

Fig. 1. The mean (±SE) concentrations (µg/L) of heavy metals among RA+ve and control groups (A) and two age groups of RA+ve individuals (B).

In the present study, the results showed non-significant correlation of Zn with RA+ve and control, RA+ve male and female and with any age group. The findings were in accordance with the findings of Tapeiro and Tew (2003) who also did not found any correlation between Zn concentration and RA. However, the findings are contrary to those of Afridi *et al*. (2012), Farid *et al.* (2005) and Olak *et al*. (2001) they reported deficiency of Zn in RA patients. Farid *et al.* (2005) observed high levels of Zn in RA+ve females than RA+ve males while our findings did not find any difference.

Table I.- The mean (±SE) concentrations (µg/L) of heavy metals among RA^{+ve} males and females.

Metal	RA^{+ve} Males	RA^{+ve} Females	df	t-value	P-value
Cu	3.66±0.30	4.60±0.41	18	-0.78	0.44
Zn	19.10±1.26	17.39±1.52	23	0.97	0.34
Pb	240.5±11.5	250.6±14.2	23	0.55	0.58
Co	71.99±4.98	80.08±9.71	12	0.78	0.45
Ni	277.8±24.6	330.5±36.2	19	1.20	0.24

In the present study the Pb concentration does not vary significantly in the serum of RA+ve patients when compared with RA-ve individuals. Hannson *et al.* (1978) also observed that Pb concentration of RA+ve patients was not different form normal. However, Afridi *et al.* (2012) observed high concentration of Pb in the hair samples of RA+ve patients. The Pb concentration in RA+ve males and female was not found significantly different in our study. Similarly no significant relation of Pb concentrations was found with age of RA patient during present studies.

The concentration of Co and Ni observed in our study was not significantly different between RA+ve and RA-ve individuals. It was also not varying in the two sexes in RA patients and no significant relation was found in two age groups of below 35 years and 35 years and above.

The variations in result of present study with already reported result may be due to variation in genetic race, climatic changes and any other environmental factor.

Conclusion

It is concluded from the study that a significant correlation does exist between low concentration of Cu and RA+ve patients. On the other hand non-significant association between RA and concentration of Zinc, lead, cobalt and nickel was found. Moreover there is no significant correlation between sex and age groups of RA patients with concentration of all the studied heavy metals.

References

Afridi, H.I., Kazi, T. G., Brabazon, D. and Naher, S., 2012. *Clin. Lab.* **148**: 139-147.

Ala, S., Shokrzadeh, M., Shoja, A. M. and Saravi, S. S., 2009. *Pak. J. biol. Sci*., **12**: 1041-1044.

Alegre, C., Baro, J. and Obach, J., 1984. *Arthritis Rheum.*, **27**: 1073-1074.

Colak, M., Kemal, N., Ayhan, O., Avcy, P. and Bulut, V., 2001. *Romatizma Cilt*., **16**: pp.2.

Farid, Y. Z., Alaa, K., Mohammed and Israa, S., 2005. *Iraqi J. med. Sci*., **4**: 49-56.

Hannson, L., Hunnan-Seppala, A. and Mattila, A., 1978. *Scand. J. Rheumatol.*, **7**: 237-240.

Lawrence, R. C., Helmick, C. G., Arnett, F. C., Deyo, R. A., Felson, D. T. and Giannini, D. H., 1998. *Arthritis Rheum*., **41**: 778-789.

Lehmann, I., Sack, U. and Lahmann, J., 2011. *Met. Ions Life Sci.*, **8**: 157-185.

Naveh, Y., Schapira, D., Ravel, Y., Geller, E. and Scharf, Y., 1997. *J. Rheumatol*., **24:** 643-646.

Scudder, P. R., Al-Timimi, D., Zoob, B. C. and Dormandy, D. L., 1978. *Ann. Rheum. Dis*., **37**: 67-70.

Strecker, D., Miersecki, A. and Radomska, K. 2013. *Ann. Agric. Environ. Med*., **20**: 312-316.

Tapiero, H. and Tew, K. D., 2003. *Biomed. Pharmacother*., **57**: 399–411.

Yazar, M., Sarban, S., Kocyigit, A. and Isikan, U. E., 2005. *Biol. Trace Elem. Res.*, **106**:123- 132.

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Chemical Composition and Mineral Contents Differentiation in Hairs of Some Wild Animal Species

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Abstract.- The present study investigates the hair chemical traits of some wild animal species. American black bear *(Ursus americanus)*, blue nile monkey *(Cercopithecus mitis*), barbary sheep *(Ammotracus lervia*), bactrian camel *(Camelus bactrianus*) and llama (*Lama glama*) were studied at Giza Zoo, Giza, Egypt. Fat, ash, nitrogen and total protein content as well as the concentration of some minerals (calcium, magnesium, potassium, sodium, phosphorus and copper) were determined on hair. Results showed that the American black bear reported the highest values among all species for fat, ash, nitrogen and total protein, while the other species could be arranged as follows: blue nile monkey, llama, bactrian camel and barbary sheep, respectively. From our findings, it was found that the darkest hair showed the higher concentration of minerals than lighter colored hair. Thus, it can be concluded hair coloration is highly correlated to chemical traits and mineral concentration in hair of different wild animal species.

Keywords: Hair, chemical composition, minerals, wild animals.

Hair is a complex tissue consisting of several components, and its chemical composition varies based on water content (Robbins, 2012). The main hair component is the protein, which is 65-95% of total weight, mainly as keratin and keratin-associated proteins as well as condensed polymer of amino acids. Other constituents are water, lipid, pigment and inorganic minerals as trace elements (Chojnaka *et al.*, 2006).

Hair may be described as crystalline, cross-linked and orientated polymorphic protein structure. Most of the extractable keratinous protein is contained within cortical cells, but significant and important tractions are present within cuticle (Powell and Rogers, 1986; Dawber, 1996). Recently published papers, on the detail of keratin production, reported differences between individuals, having an important forensic significance (Sen, 2010).

The main hair proteins are three structurally related keratins, the low sulphur, high sulphur and high tyrosine and glycine keratins, where the sulphur content derived from amino acids (Dawber, 1996; Dunnett and Lees, 2003). There are five primary sources of elements to growing hair which include matrix cells, the connective tissue papilla, eccrine secretions, apocrine secretions, and the epidermis. The elements obtained through ingestion or environmental exposure are applied to hair through endogenous deposition from the connective tissue papilla and matrix cells. Eccrine sweat exogenously deposits salts high in sodium and potassium on the hair. Other trace elements deposited through sweat include nitrogen, calcium, phosphorus, magnesium, copper, manganese and iron (Ambrose, 1993; Larsen, 1997; Yousafzai *et al*., 2013).

Therefore, the main objective of this study was to investigate the differentiation between some wild animal species depending on their biological differences among the chemical and mineral composition of hairs to acquire valuable information for the experts in field.

Materials and methods

Samples of hair were obtained from five animal species from Giza Zoo, Giza, Egypt; a carnivore, American black bear *(Ursus americanus)*, a primate, blue nile monkey *(Cercopithecus mitis*), and three artiodactyls, barbary sheep *(Ammotracus*

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lervia), bactrian camel *(Camelus bactrianus*) and llama (*Lama glama*) from four animal per species (two males and two females, respectively).

Hair samples collected from animals' back were analyzed in triplicate for ash (967.05), total protein and nitrogen (Kjeldahl N × 6.25, 990.03), ether extract (945.16) contents according to AOAC (2006). The ash was wet with sulfuric and perchloric acids and diluted with distilled water. Hair samples from the selected species were subjected to acid digestion according to the method proposed by Mahaffy *et al.* (1981), Digests were filtered (Whatman No. 1) and diluted to 25 ml deionized water (Julshman, 1983). The resultant solutions were analyzed by flame atomic absorption spectrophotometer (Agilent 6890 Series GC). All results were expressed on dry matter basis.

The results were expressed as mean ± standard error of the means (SEM). Data were statistically analyzed by a one-way ANOVA using the GLM procedure of IBM SPSS Statistics software (version 21.0). Significance implies $P<0.05$ (Steel and Torrie, 1993).

Results

Table I shows chemical analysis of hair of different species, which revealed that American black bear has the greatest values for fat, ash, nitrogen and total protein among all wild species, followed by blue nile monkey, llama, bactrian camel and barbary sheep. In the present study, the data for mineral concentration of hairs showed that American black bear had the highest level for Ca, Mg, K, Na, P and Cu followed by llama, blue nile monkey, bactrian camel and barbary sheep ($P < 0.001$; Table I).

Discussion

Difficulties with a proper interpretation of results of hair analysis were mainly due to the absence of well-defined reference concentration ranges; thus, problems associated with differentiating between endogenous and exogenous deposition and inconsistency of hair concentration anomalies with nutritional status occurred (Rodushkin and Axelssom, 2000). The difficulties in establishing reference ranges were also due to the natural variance in hair composition as a possible consequence of age, sex, diet, hair color, ethnical and geographic origin (Miiekeley *et al.*, 1998; Sharma *et al.*, 2004).

Unlike most tissues, hair is formed in relatively short period of time, after which it is expelled from the body's continuing metabolic process. The concentration of most trace elements are high in hair when compared with other body tissues or fluids. The trace elements in fact accumulated during growth are sealed into the hair (Katz and Katz, 1992).

Most attention on mineral incorporation into hair focused on the uptake within the hair follicle during hair synthesis. During the processes of mitosis, differentiation, maturation and melanin synthesis, macro- and microelements enter the newly formed hair cells. As the hair shaft is keratinized, a cement-like protein fills all intercellular spaces and binds the cortical cells of the hair shaft together; moreover, the hair is rich in sulfur containing amino acids that presumably provide ligands for stable hair mineral bonds (Combs, 1987).

Our results showed that the American black bear had the fat, highest concentration of ash, nitrogen, total protein and minerals. The dark hair showed higher concentration of minerals than the light colored hair. This finding is in agreement with the previous results reported by O'Mary *et al.* (1970) who compared hair from Holstein and Herford cows and found that Holstein black hair contained more Na, P, Mg, Ca and K than Herford red hair. Furthermore, these result also agreed with those of Hall *et al.* (1971) who observed that ash content of white hair was lower than pigmented hair and that also some minerals appear to influence the color. Meanwhile, Feughelman (2002) and Szpoganicz *et al.* (2002) stated that minerals, such as Cu, have an high affinity for melanins which are granules of pigment providing the natural colouring material in keratin fibers, and this may explain the mineral concentration in the studied species in relation to the hair color level. So, the increased protein content (consequently keratin) could be responsible for increasing the melanin content in hair, and thus the binding sites of minerals into hair (Dunnett and Lees, 2003). In conclusion, from our findings it can be concluded the hair coloration is

Table I.- Crude fat, ash, nitrogen, total protein contents (%) and concentration of minerals (ppm) in the hairs of studied species.

Items	American black bear	Blue nile monkey	Barbary sheep	Llama	Bactrian camel	SEM	*P*-value
Crude fat, %	2.00[a]	1.80[c]	1.60[f]	1.75[d]	1.69[e]	0.001	0.020
Ash, %	2.38[a]	1.89[c]	1.40[f]	1.65[d]	1.59[e]	0.002	<0.001
Nitrogen, %	15.00[a]	14.00[c]	11.00[f]	13.03[d]	12.23[e]	0.051	<0.001
Total protein, %	93.74[a]	87.80[c]	73.76[f]	81.24[d]	78.12[e]	0.022	<0.001
Calcium, ppm	130.00[a]	98.00[d]	65.00[f]	111.66[c]	80.33[e]	0.401	<0.001
Magnesium, ppm	99.00[a]	54.66[d]	21.00[f]	70.66[c]	40.33[e]	0.452	<0.001
Potassium, ppm	118.00[a]	91.00[d]	62.00[f]	99.00[c]	80.33[e]	0.512	<0.001
Sodium, ppm	145.00[a]	71.00[d]	45.66[f]	114.66[c]	68.30[e]	0.531	0.002
Phosphorus, ppm	161.00[a]	100.33[d]	71.33[f]	130.66[c]	80.66[e]	0.493	<0.001
Copper, ppm	61.00[a]	20.66[d]	10.33[f]	48.66[c]	15.66[e]	0.394	<0.001

Means within the same raw within each group carrying different superscripts are significantly different ($P < 0.05$); SEM, standard error of the means.

highly correlated to the chemical composition and mineral levels of hairs in different wild animal species.

References

Ambrose, S.H. and Norr, L., 1993. In: *Prehistoric human bone. Archaeology at the molecular level* (eds. J.B. Lambert and G. Grupe). Springer-Verlag, Berlin, pp. 1–37.

AOAC, 2006. *Official methods of analysis*, 18th Edition. Association of Official Analytical Chemists, Gaithersburg, MD, USA.

Chojncka, K., Gorecka, H. And Gorecki, H., 2006. *J. environ. Toxicol. Pharmacol.*, **22**:52- 57.

Combs, D.K., 1987. *J. Anim. Sci*., **65**:1753-1758.

Dawber, R.M.D., 1996. *Clin. Dermatol.*, **14**:105-112.

Dunnett, M. And Lees, P., 2003. *Res. Vet. Sci*., **75**: 89-101.

Feughelman, M., 2002. *J. Appl. Polym. Sci*., **83**: 489-507.

Hall, R.F., Sanders, W.L., Bell, M.C. and Reynolds, R.A., 1971. *J. Vet. Res*., **32**:1613.

Julshman, K., 1983. *Analysis of major and minor elements in molluscus from Norway*. Ph.D. thesis, Institute of Nutrition Directorate of Bergen Nygardsangen, Norway. Fisheries. P.O. Box 4285, N-5013.

Katz, S.A. and Katz, RB., 1992. *J. Appl. Toxicol*., **12**:79-84.

Larsen, C.S., 1997. *Bioarchaeology: interpreting behavior from the human skeleton*. Cambridge University Press. pp. 270-301.

Mahafy, K.R., Caper, S.G., Gladen, B.C. and Fowler, BA., 1981. *J. Lab. Clin. Med*., **98**:463-481.

Miekeley, N., Das Carneiro, M.T.W. and Porto Da Silveira, C.L., 1998. *Sci. Total Environ.*, **218**:9-17.

O'mary, G.C., Bell, M.C., Snead, N.N. and Butts, W.T., 1970. *J. Anim. Sci*. **54**:391-398.

Powell, B.C. and Rogers, G.E., 1986. In: *Biology of the integument* (eds. J. Bereiter-Hahn, A.G. Matoltsy and K.S. Richards), vol 2. Springer, Berlin, pp. 695–721.

Robbins, C.R., 2012In: *Chemical and physical behavior of human hair*. Springer, Berlin, Heidelberg, pp. 105-176.

Rodushkin, I. and Axelsson, M.D., 2000. *Sci. Total Environ.*, **262**:21-36.

Sen, J., 2010. *Anthropologist*, **12**:47-58.

Sharma, R., Singh, L.C., Tanveer, S., Verghes, P. and Kumar, A., 2004. *Bull. environ. Cont. Toxicol*., **72**:530-534.

Szpoganicz, B., Gidanian, S., Kong, P. and Farmer, P., 2002. *J. Inorg. Biochem*., **89**:45-53.

Yousafzai, A.M., Khan, W. and Hasan, Z., 2013. *Pakistan J. Zool*., **45**:1727-1734.

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Detection of *Plasmodium falciparum* infection in *Anopheles stephensi* in Punjab, Pakistan*

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Abstract.- In understanding the transmission dynamics in area-specific malaria epidemiological studies, the determination of *Plasmodium* sporozoite infection rate in the field caught *Anopheles* mosquitoes remains an integral component. In present study the *Plasmodium falciparum (P. falciparum)* sporozoite infection rate of female *Anopheles stephensi (An. stephensi*) mosquitoes caught from Districts Kasur and Shiekupura, Province Punjab was detected. A nested polymerase chain reaction (nested PCR) was used to amplify 205 bp DNA of a small subunit ribosomal RNA (ssu rRNA) gene specific to *P. falciparum* from field caught *An. stephensi*. The *P. falciparum* sporozoite infection rate was higher, *i.e.* 10% in *An. stephensi* from District Shiekhupura followed by 6% sporozoite infection rate in mosquitoes collected from District Kasur. These results may contribute to plan and assess malaria control strategies in Punjab, Pakistan.

Key Words: *Anopheles stephensi*, *Plasmodium falciparum*, nested PCR, sporozoite infection rate

Malaria is one of those ancient parasitic diseases which persisted through the centuries, quite ineffective of the developments made by mankind in the field of health care. The third world countries like Pakistan are still affected by this disease quite regularly in spite of all the malaria control strategies made by government and health care organizations (Dash *et al*., 2007). Female mosquitoes of more than 30 *Anopheles* species act as vector for malaria worldwide (WHO, 2012; Sinka *et al*., 2012). About 23 *Anopheles* species have been reported in Pakistan. Of which two species, *i.e.*, *Anopheles culicifacies* and *An. stephensi* acts as primary and secondary malaria vectors in the country, respectively (Rowland *et al*., 1997; Ilahi and Suleman, 2013; Mahmood *et al*., 1984; Jahan and Hussain, 2011).

The emergence of vector borne diseases and the evolution of parasite density can be assessed by understanding the vector parasite relationship (Inci *et al*., 2012). One of the integral components in the transmission dynamics of malarial epidemiological studies is detection of *Plasmodium* sporozoites in the field caught *Anopheles* mosquitoes (Mahapatra *et al*., 2006; Tangin *et al*., 2008). The vectorial capacity of a mosquito depends upon susceptibility to *Plasmodium* sporogony, its anthropophagic behavior and enough longevity to become infective to human beings (Dash *et al*., 2007). The efficacy of the malaria control program also depends upon the epidemiological studies of malaria prevalence by detecting *Plasmodium* in mosquitoes (Tangin *et al*., 2008; Bass *et al*., 2008; Hasan *et al*., 2009, Li *et al*., 2001).

In present study the *P. falciparum* infection rate of the field caught *An. stephensi* from two Districts of Punjab, Pakistan was determined. This was done by amplification of *P. falciparum* ssrRNA gene through nested PCR.

Materials and methods

The Kasur and Shiekhupura districts of Province, Punjab, Pakistan were selected for mosquito collection. These areas were selected on the basis of malaria endemicity and having suitable climatic conditions for mosquito breeding.

After approved consent of the villagers from these Districts, mosquitoes resting on different surfaces from animal sheds were collected by using mouth aspirators (WHO, 1975). Female *An. stephensi* were identified upto species level according to key provided by (Amerasinghe *et al*., 2002).

After species identification, the head and

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thorax of 100 field caught blood fed females of *An. stephensi* were separated and placed in 1.5 ml tubes at -20ºC till DNA extraction.

The DNA was extracted with slight modifications as described by Collins *et al*. (1987).

The genus *Plasmodium* was confirmed by PCR using primers
rPLU5 (CCTGTTGTTGCCAAACTTC) and
rPLU6 (TTAAAATTGTTGCAGTTAAAACG) (Snounou *et al*., 1993) with minor modifications. Briefly, 25 µL of reaction mixture containing 2.5 µL of extracted DNA, 12.5 µL of PCR master mix (2x) (Fermentas, Germany), 1 µL of forward (10 pmoles/ µL) and reverse (10 pmoles/ µL) primers each (Oligos, GeneLink, USA), and 8 µL of DEPC water was subjected to thermocycler (ThermoElectron, Finland) under following conditions; initial denaturation at 94°C for 4 min followed by 35 cycles, each of 94°C for 30 seconds, annealing at 55°C for 1 min and extension at 72°C for 1 min. The reaction was ended with final extension made at 72°C for 10 min followed by holding at -20°C till further processing and electrophoresis on 0.8% agarose yielding 1.1 kb product against the standard ladder of 100bp (Fermentas, Germany). Artificially infected mosquitoes served as positive control, whereas, normal saline was used as negative control.

Sporozoites of *P. falciparum* was confirmed using amplified PCR product and nested PCR was done using primers
rFAL1
(TTAAACTGGTTTGGGAAAACCAAATATATT) and
rFAL2
(ACACAATGAACTCAATCATGACTACCCGTC) described by (Snounou *et al*., 1993) with minor modifications. Briefly, 25 µL of reaction mixture containing 1 uL of extracted DNA, 12.5 µL of PCR Master Mix (Fermentas, Germany), 1 µL of forward (10 pmoles/ µL) and reverse (10 pmoles/µL) primers each (Oligos, GeneLink, USA), and 9.5 µL of DEPC water was subjected to thermocycler (ThermoElectron, Finland) under following conditions; initial denaturation at 94°C for 4 min followed by 35 cycles of each of 94°C for 30 seconds, annealing at 57°C for 1 min and extension at 72°C for 1 min. The reaction was ended with final extension made at 72°C for 10 min followed by holding at -20°C till further processing and electrophoresis on 1.8% agarose yielding 205 bp product against the standard ladder of 50bp (Fermentas, Germany). Artificially infected mosquitoes served as positive control, whereas, normal saline was used as negative control.

Results and discussion

Figure 1 shows amplified DNA band of 205 bp length for *P. falciparum* ssrRNA gene. A total of 8% *P. falciparum* sporozoite infection rate was observed in these mosquitoes. The *P. falciparum* sporozoite infection rate was higher, (10%) in *An. stephensi* from District Shiekhupura followed by 6% sporozoite infection rate in mosquitoes collected from District Kasur.

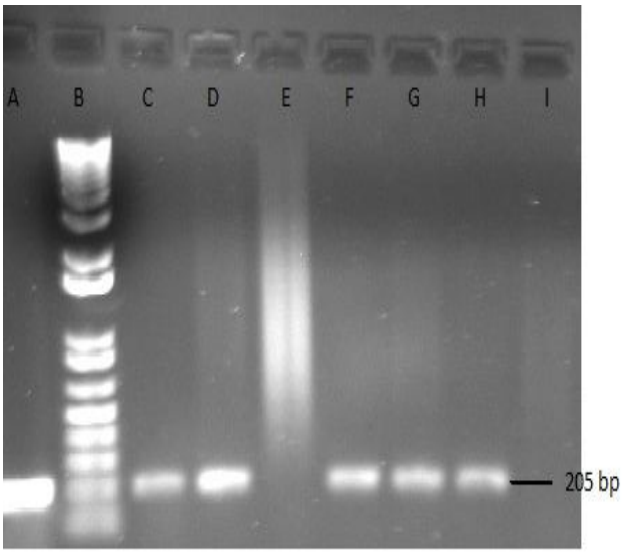

Fig. 1. Amplification of *P. falciparum* from head and thoraces of *An. stephensi* from both districts. A, positive control; B, ladder; C-D, DNA bands of mosquitoes of District Kasur; F-H, DNA bands of mosquitoes of District Shiekhupura.

The main prerequisite for malaria vector control is vector surveillance. Therefore knowledge about vector biology and geographical distribution plays key role in modulating successful vector control strategies (Tangin *et al*., 2008). Salivary gland dissection and its microscopic examination is impractical, time consuming and cannot

differentiate *P. falciparum* from other species of the genus Plasmodium (Bass *et al*., 2008; Hasan *et al*., 2009).

In Pakistan *An. culicifacies* is known as primary malaria vector while *An. stephensi* is known as a secondary malaria vector (Ali *et al*., 2007; Husain and Talibi, 1956; Mahmood *et al*., 1984; Reisen and Boreham, 1982). Over the last 3 decades a major shift in the malaria vector species composition has been observed the relative increase in the abundance of *An. stephensi* to *An. culicifacies*, which was highly prevalent in the 1980's (Reisen *et al*., 1982, Klinkenberg *et al*., 2004). Secondly some investigation also reported the peak transmission of falciparum malaria in the month of October when *An. culicifacies* was completely disappeared while *An. stephensi* was still prevailing (Rowland *et al*., 2002). Similar changes in the relative abundance of these two malaria vectors were also observed in different studies (Ali *et al*., 2013; Ilahi and Suleman, 2013, Herrel *et al*., 2004). The low density of *An. culicifacies* and high density of *An. stephensi* is the plausible explanation that *An. stephensi* may impart important role in the transmission of *falciparum* malaria in these areas. Therefore the present study was planned to estimate the *P. falciparum* sporozoite infection rate of *An. stephensi* from Districts Kasur and Shiekhupura.

The results of the present study clearly show that detection of *P. falciparum* from *An. stephensi* confirming its capacity as a potent malaria vector in Punjab, Pakistan. In accordance with these results several studies had also shown high significance of use of PCR for detection of sporozoite infection rate of *Anopheles* mosquitoes (Kumari *et al*., 2009; Mahapatra *et al*., 2006). Although this procedure would be more expensive, but is more sensitive and specific in detecting parasites in field-caught mosquitoes and evaluating the effectiveness of control measures (Li *et al*., 2001; Wilson *et al*., 1998).

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References

Ali, N., Hume, J.C., Dadzie, S. K. and Donnelly, M. J., 2007. *Med. Vet. Ent.*, **21:** 265-269.

Ali, N., Khan, M.K. and Aisha, K., 2013. *Pakistan J. Zool.*, **45:** 503-510.

Amerasinghe, F. P., Mukhtar, M. and Herrel, N., 2002. *J. med. Ent.*, **39:** 28-35.

Bass, C., Nikou, D., Blagborough, A. M., Vontas, J., Sinden, R. E., Williamson, M. S. and Field, L.M., 2008. *Malar. J.*, **7:** 177.

Collins, F.H., Mendez, M. A., Rasmussen, M.O., Mehaffey, P.C., Besansky, N. J. and Finnerty, V., 1987. *Am. J. Trop. Med. Hyg.*, **37:** 37-41.

Dash, A. P., Adak, T., Raghavendra, K. and Sing, O. P., 2007. *Curr. Sci.*, **92:** 1571-1578.

Hasan, A. U., Suguri, S., Sattabongkot, J., Fujimoto, C., Amakawa, M., Harada, M. and Ohmae, H., 2009. *Malar. J.*, **8:** 182.

Herrel, N., Amerasinghe, F.P., Ensink, J., Mukhtar, M., Van Der Hoek, W. and Konradsen, F., 2004. *Med. Vet. Ent.*, **18:** 141-152.

Husain, M. Z. and Talibi, S. A., 1956. *Pak. J. Hlth.*, **6:** 65-72.

Ilahi, I. and Suleman, M., 2013. *Int. J. Innov. appl. Stud.*, **2:** 454-463.

Inci, A., Yildirim, A., Njabo, K. Y., Duzlu, O., Biskin, Z. and Ciloglu, A., 2012. *Vet. Parasitol.*, **188:** 179-184.

Jahan, N. and Hussain, N., 2011. *Pakistan J. Zool.*, **43:** 915-919.

Klinkenberg, E., Konradsen, F., Herrel, N., Mukhtar, M., Van Der Hoek, W. and Amerasinghe, F. P., 2004. *Trans. R. Soc. trop. Med. Hyg.*, **98:** 442-449.

Kumari, S., Parida, S.K., Marai, N., Tripathy, A., Hazra, R.K., Kar, S.K. and Mahapatra, N., 2009. *Southeast Asian J. trop. Med. Publ. Hlth.*, **40:** 713-719.

Li, F., Niu, C. and Ye, B., 2001. *Chin. Med. J. (Engl.),* **114:** 654-657.

Mahapatra, N., Marai, N. S., Ranjit, M.R., Parida, S. K., Hansdah, D.P., Hazra, R. K. and Kar, S.K., 2006. *J. Vector Borne Dis*., **43:** 191-194.

Mahmood, F., Sakai, R. K. and Akhtar, K., 1984. *Trans. R. Soc. trop. Med. Hyg,*, **78:** 607-16.

Reisen, W. K. and Boreham, P.F., 1982. *J. med. Ent.*, **19:** 98-103.

Reisen, W.K., Mahmood, F. and Parveen, T., 1982. *J. med. Ent.*, **19:** 86-97.

Rowland, M., Hewitt, S., Durrani, N., Bano, N. and Wirtz, R., 1997. *Trans. R. Soc. Trop. Med. Hyg.*, **91:** 252-255.

Rowland, M., Mohammed, N., Rehman, H., Hewitt, S., Mendis, C., Ahmad, M., Kamal, M. and Wirtz, R., 2002. *Trans. R. Soc. trop. Med. Hyg.*, **96:** 620-626.

Sinka, M. E., Bangs, M.J., Manguin, S., Rubio-Palis, Y., Chareonviriyaphap, T., Coetzee, M., Mbogo, C.M.,

Hemingway, J., Patil, A. P., Temperley, W. H., Gething, P. W., Kabaria, C. W., Burkot, T. R., Harbach, R. E. and Hay, S. I., 2012. *Parasit. Vect.*, **5:** 69.

Snounou, G., Viriyakosol, S., Zhu, X.P., Jarra, W., Pinheiro, L., Do Rosario, V.E., Thaithong, S. and Brown, K.N., 1993. *Mol. Biochem. Parasitol.*, **61:** 315-320.

Tangin, A., Komichi, Y., Wagatsuma, Y., Rashidul, H., Wataya, Y. and Kim, H. S., 2008. *Biol. Pharm. Bull.*, **31:** 703-708.

WHO, 1975. *Manual on practical entomology in Malaria - Part II: Methods and techniques*. World Health Organization, Geneva, Switzerland

WHO 2012. *World malaria report 2012*. World Health Organization, Geneva, Switzerland, . http://www.who.int/malaria/publications/world_malaria_report_2012/wmr2012_full_report.pdf.

Wilson, M. D., Ofosu-Okyere, A., Okoli, A. U., Mccall, P. J. and Snounou, G., 1998. *Trans. R. Soc. trop. Med. Hyg.*, **92:** 482-483.

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Detection of Enzootic Bovine Leucosis in Suspected Herd

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Abstract.- Bovine Leukemia Virus (BLV) was detected in blood sera and leukocyte samples of 5 of 50 (10%) dams and 5 of 11 (45.4%) calves of Enzootic Bovine Leucosis (EBL) suspected herd by ELISA. All of the samples were also tested by nested-PCR for detection of proviral BLV DNA in which 5 of 50 (10%) dams and 3 of 11 (27.2%) calves were positive for BLV specific 341 bp product. This is the first report of (EBL) in calves in Turkey.

Key words: Bovine leukemia virus, calves, cattle, ELISA and nested-PCR.

Bovine Leukaemia Virus (BLV) (Retroviridae: Orthoretrovirinae: Deltaretro virus) the causative agent of Enzootic Bovine Leucosis (EBL) is responsible for neoplastic disease and persistent lymphocytosis of cattle (Willems and Kettmann, 2002). The main source of BLV is B lymphocyte (Panei *et al*., 2013). Embryos, semen and uterine fluids are the source of BLV provirus. Some farm practices such as tattooing, dehorning, rectal palpation, injections blood collection and iatrogenic transmission (Willems and Kettmann, 2002), tabanids and other large biting fly vectors are also the routes of BLV transmission (Oshima *et al*., 1981).

Clinically, diagnosis of EBL is difficult. The methods based on viral envelope protein gp51 such as agar gel immunodiffusion (AGID) and enzyme linked immunosorbent assay (ELISA) are commonly performed in laboratory diagnosis of BLV. EBL infection confirmation with PCR is checked in cattle with doubtful serological results (Beier *et al*., 1998).

EBL infection has been reported in different countries (Mahmood *et al*., 2012; Mohammadabadi *et al*., 2011). It has been reported in Turkey for the first time in 1966, though the first entry of disease is unknown. Up to this time, EBL was detected in cows by ELISA and PCR (Burgu *et al*., 2005; Bulut *et al*., 2009).

Aim of the this study was detect BLV in dams and their calves in EBL suspected herd. This is the first report of BLV in calves in Turkey.

Materials and methods

In this study, a dairy herd of private farm in Samsun, northern Turkey were investigated for EBL. This herd exhibited EBL like symptoms such as lymphadenopathy, descreased milk yield, loss of appettite and weight. Blood serum and leukocyte samples of 50 dams and 11 calves (4 of 1-6 months old and 7 were 0-30 days old) were collected. and stored at - 20°C untill analysis.

All the serum samples were tested for the detection of antibodies against BLV using commercial ELISA kit (Institut Pourquer, Fransa) according to the manufacturer's instructions.

All of the dams and calves were investigated by n-PCR. For this, proviral DNA was extracted directly from leukocyte fractions of all samples using the viral DNA extraction kit (QiaAmp, Qiagen, Germany) and then used for detection of BVL proviral DNA using n-PCR developed by Belak and Ballagi-Pordany (1993). Primers are detailed in Table I. n-PCR were performed under the following steps: For the first PCR, 5 cycles each of 94°C for 45 s and 60°C for 60 s and 72°C for 90 s, 1 cycle 72°C for 7min. For the second (nested) PCR, 5 cycles each of 42°C for 45 s, 60°C for 1 min and 72°C for 90 s and 30 cycles each of 94°C for 45 s, 55°C for 1 min and 72°C for 90 s and final extension at 72°C for 7 min. Second-round PCR products were analysed on agarose gel (1.5%) electrophoresis at 80 V for 30 min.

Results and discussion

Antibodies against BLV were detected in 10 of 61 (16.3%) serum samples; 5 of 50 (10%) dams

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Table I.- The primers used to n-PCR for detection of proviral BLV DNA.

Genomic region	Sequence	Nucleotid position
OBLV1	(5'-CTT-TGT-GTG-CCA-AGT-CTC-CCA-GAT-ACA-3')	5029
OBLV6	(5'-CCA-ACA-TAT-AGC-ACA-GTC-TGG-GAA-GGC-3')	5442
OBLV3	(5'-CTG-TAA-ATG-GCT-ATC-CTA-AGA-TCT-ACT-GGC-3')	5065
OBLV5	(5-GAC-AGA-GGG-AAC-CCA-GTC-ACT-GTT-CAA-CTG-3')	5376

and 5 of 11 (45.4%) calves were seropositive. Leucocyte samples of 61 animals were investigated for proviral BLV DNA using n-PCR; 8 of 61 (13.1%) leukocytes samples, 5 of 50 (10%) dams and 3 of 11 (27.2%) calves were found positive for proviral BLV DNA showing specific product of 341 bp. All of the seropositive dams and three of five seropositive calves were found proviral BLV DNA positive.

BLV presence in cattles were detected by PCR in various countries of the world (Mohammadabadi *et al*., 2011; Belak and Ballagi-Pordany, 1993; Panei *et al*., 2013). Mekata *et al*., 2014 was indicated approximately 15% of newborn calves born to infected dams are infected with BLV by the perinatal infection route. In addition, more than 40% of newborn calves born from cattle with high viral loads are infected with BLV after birth. Bulut *et al*. (2009), were detected 24.6% BLV proviral DNA rates by n-PCR in cattle. But no study is present about BLV nucleic acid in calves in Turkey. In this study, five cows (10%), three calves (27.2%) (one calf was 1-6 months old and 2 calves were 0-30 days old of them) were detected positive for BLV nucleic acid by n-PCR. EBL is a notifiable disease in Turkey, so all of the seropositive dams and 3 of BLV proviral DNA positive calves were culled out according to eradication rules.

BLV seroprevalences were found 0.5%-34.4% in private farms enterprises in different region in Turkey (Burgu *et al*., 2005; Bulut *et al*., 2009). In this study, 5 of 50 dams (10%) and 5 of 11 calves (45.4%) (two of four calves were 1-6 months old and three of seven calves were 0-30 days old) were found seropositive by ELISA against BLV.

In a study conducted by Ferrer and Piper (1981), all of calves which were colostrum fed until 3-6 months old, and most of the calves fed with colostrum for 7-9 months old have been found seropositive. Based on these data, the maternal immunity is effective until 3-6 months old in calves. In our study, all of the calves have been fed first (colostral period) by their mothers' milk in the enterprises. The seropositive dams' calves (2 calves were 1-6 months old and 3 calves were 0-30 days old) were found seropositive by ELISA. If the investigation is performed only used by ELISA test, these positivities are considered as maternal immunty. However, in this study all of the seropositive calves were investigated by n-PCR. Seropositive three of five calves were detected n-PCR positive. So, detection of BLV nucleic asid in three calves were interpreted as these calves were infected with BLV.

All of the new borns should be tested by PCR in seropositive herds. Thus, the decision of infected calves or maternal immunity may be more accurate. The early detection of BLV can be performed confidentally in this way and virus positive calves can be removed from the herd earlier by n-PCR assay. This is one of the important strategies for EBL eradication. We recommend that all the calves should be investigated by PCR in BLV positive herds for further studies.

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References

Beıer, D., blankensteın, P. and Fechner, H., 1998. *Dtsch. Tierarztl. Wochenschr*., **105**: 408-412.

Belak, S. and Ballagı-Pordany, A., 1993. *Mol. Cell. Probes*, **7**: 241–248.

Bulut, H., Gulaçtı, I. and Sozdutmaz, I., 2009. *Fırat. Univ. Hlth. Sci. Vet. J*., **23**: 39-42.

Burgu, I., Alkan, F., Karaoğlu, T., Bılge-Dagalp, S., Can-Sahna, K., Gungor, B. and DEMIR, B., 2005. *Dtsch. Tierarztl. Wochenschr*., **112**: 271-274.

Ferrer, J.F. and Pıper, C.E., 1981. *Cancer Res*., **41**: 4906-4909.

Mahmood, F., Khan, A., Khan, M.Z., Hussaın, R., Gul, S.T. and Sıddıque, A.B., 2012. *Pak. Vet. J*., **32**: 511-514.

Mekata, H., Sekıguchı, S., Konnaı, S., Kırıno, Y., Honkawa, K., Nonaka, N., Horıı, Y. and Norımıne J., 2014. *Vet. Rec*., 10.1136/vr.102464, 3-4.

Mohammadabadı, M.R., Soflaeı, M., Mostafavı, H. and Honarmand, M., 2011. *Gen. Mol. Res*., **10**:2658-2663.

Oshıma, K., Okada, K., Numakunaı, S., Yoneyama, Y., Sato, B. and Takahashı, I., 1981. *Jpn. J. Vet. Sci*., **43**: 79-81.

Paneı, C.J., Takeshıma, S., Omorı, T., Nunoya, T., Davıs, W.C., Ishızakı, H., Matoba, K. and Aıda, Y., 2013. *BMC Vet. Res*., **9**:95.

Wıllems, L. and Kettmann, R., 2002. In: *The springer index of viruses* (eds.C.A. Tidone and G. Darai), Springer, Berlin, pp. 1014-1019.

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Effects of Induced Molting on Semen Quality of Indigenous Chicken Aseel

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Abstract.- Aseel is the very famous indigenous chicken breed of Pakistan which is facing the reproductive issues. In the modern poultry industry, molting is economically used for the improvement of reproductive performance. So the current experiment was designed to investigate the effect of molting on semen quality of indigenous aseel chicken. Roosters (n=16) were divided into two groups, molted (n=8) vs non molted (control) (n=8) group. Molting was performed through the method of feed restrictions. After the molting phase semen was analyzed for five weeks. The semen quality was significantly improved in terms of volume, concentration, motility, live sperms and reduced morphological defects by the molting. It was concluded that molting could be used for improvement of semen quality of indigenous aseel chicken to cover the reproductive problems.

Key words: Indigenous chicken, aseel, molting, semen quality

Aseel is the very famous indigenous chicken breed of Pakistan which is mostly reared in rural areas or as a game bird for fighting purposes. The aseel breed is known for its stamina, pugnacity, majestic gait, and dogged fighting properties. The aseel is favored in rural areas because of their adaptability to harsh environment and immunity to the diseases (Jatoi *et al.*, 2014). The meat and eggs of aseel are supposed to have medicinal as well as aphrodisiac properties as they contain higher amount of protein, iron and amino acids (arginine, lysine, threonine and valine) as compared to the exotic birds (Mohan *et al.*, 2008).

However, aseel breed is facing the problems of poor growth rate, late maturity, less persistency and number of egg production, broodiness and low fertility and hatchability rates (Amjad *et al.*, 2012). The low fertility may be due to the poor semen quality and there is no baseline data available about semen quality of Aseel.

Molting is the natural physiological phenomenon of anorexia, broodiness, reproductive quiescence, shedding and replacement of feathers in female birds. Molting causes rejuvenation of reproductive organ which increases their reproductive potential. Molting is usually connected with hens, but it may also result in improvement in the semen quality of roosters. The reproductive capacity of chicken males starts to decline after 50 weeks of age so molting could be used to regain their reproductive capacity. There is no report available about the effect of molting on semen quality of indigenous aseel chicken. The current study was conducted to investigate the effects of molting on semen quality of indigenous aseel chicken.

Materials and methods

Aseel roosters (n=16) having 3.5-4 kg body weight and 1-1.5 years of age were selected on the basis of initial semen quality and caged individually. Males were divided into two groups equally, molted (n=8) vs non molted (control) (n=8). Molting was performed through the method of feed withdrawal by providing the 50 grams of maize feed daily. The 24 h water availability was guaranteed by the automatic watering system. No artificial light was provided. The duration of molting phase was 5 weeks. After the rest of 2 weeks semen analysis was performed. At the same time, non molted group was feed with 100 g of standard breeder diet once daily. The lightening schedule was 16h light and 8h darkness. The availability of water was 24 h.

Semen collection was performed once in a

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week by the method of abdominal massage (Riaz *et al.*, 2004) for five weeks. During the whole research, same time, same person and same place were used for semen collection to avoid the stress on males. Ejaculates containing any contamination of urine, feaces or blood were rejected. The following variables of each ejaculate were measured collected from individual male. Volume of ejaculate was measured by using insulin syringe. The motility was estimated by placing a drop of semen and normal saline, on clean and pre-warmed slide (37^{o}C). The cover slip was applied and motility was checked under 100x of light microscope. At least three fields were observed before final decision. The concentration of each ejaculate was measured by Hemocytometer technique using the dilution rate of 1:500 (Riaz *et al.*, 2006). The eosin nigrosin staining technique was used to estimate live spermatozoa by following the standard protocol.

Data were presented as Mean ± S.E.M and analyzed using unpaired student t-test for semen parameters of molted and non-molted roasters. A P-value of <0.05 was considered as significant. All data were analyzed using statistical software SAS (version 9.2).

Results and discussion

The data were analyzed and presented in Table I. The volume (0.15 vs 0.32 ml), motility (60.7 vs 70.5%), concentration (1.53 vs 3.22 x10^{9}/ml) and live sperms (64.2 vs 72%) were significantly ($p<0.05$) lower in non-molted males as compared to molted males, respectively. The morphological defects (8.2 vs 6.8%) were significantly lower ($p<0.05$) in molted males as compared to non molted males.

Table I.- Effect of molting on semen quality of indigenous aseel chicken.

	Non molted	Molted
Volume (ml)	0.15±0.4^{a}	0.32±0.7^{b}
Concentration (x10^{9})	1.53±0.2^{a}	3.22±1.2^{b}
Motility (%)	60.78±2.2^{a}	70.5±2.5^{b}
Livability (%)	64.26±2.5^{a}	72.02±2.3^{b}
Morphological defects (%)	8.22±0.6^{a}	6.88±0.5^{b}

$^{a-b}$ denote difference between semen quality of molted and non molted groups ($P < 0.05$)

The process of molting is characterized by complex physiological events, which affect endocrine and reproductive system. One of the most significant post-molt profit is the rejuvenation of the reproductive system of birds which result in enhanced reproductive performance (Khan, 2011). Molting is an economical process in layers but in male birds molting is not commonly practiced. The results of current experiment show that the semen quality in terms of volume, concentration, motility, live sperms and morphological defects was improved ($p<0.05$) by the molting. In this experiment molting was used first time for the improvement of aseel semen quality so no data available for comparison. Similar results were documented by (Khan *et al.*, 2012) where in broiler breeder, molting enhances the semen quality and fertility.

On the basis of findings of the current research, it was concluded that molting could be used for improvement of semen quality of indigenous aseel chicken.

References

Amjad, I., Akram, M., Sahota, A.W., Javed, K., Hussain, J., Sarfraz, Z. and Mehmood, S., 2012. *J. Anim. Pl. Sci.*, **22:** 848-852.

Jatoi, A.S., Iqbal, M., Sahota, A.W., Akram, M., Javed, K,. Jaspal, M.H., Mehmood, S., Hussain, J., Hameed, T,. Khan, M.S., Abbas, Y., Ahmad, S. and Ishaq, H.M., 2014. *Pakistan J. Zool.*, **46:** 1565-1571.

Khan, R.U., Rahman, Z.U., Javed, I. and Muhammad, F., 2012. *Anim. Reprod. Sci.*, **135:** 85-90.

Khan, R.U., 2011. *World Poult. Sci. J.*, **67:** 297–308.

Mohan, J., Sastry, K.V.H., Moudgal, R.P. and Tyagi, J.S., 2008. *Int. J. Poult. Sci.*, **43:** 379-381.

Riaz, A., Aleem, M. and Ijaz, A., 2006.. *Korean J. Poult. Sci.*, **33:** 165-169.

Riaz, A., Aleem, M., Ijaz, A., Saeed, M.A. and Latif, A., 2004. *Br. Poult. Sci.*, **45:** 823-827.

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Survey of Plant Parasitic Nematodes in Different Regions of Khyber-Pakhtun-Khwa

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Abstract.- In this study, five different localities *viz.*, Dergai, Warsak, Dera Ismail Division, Agriculture Research Institute Tarnab and Peshawar University of Khyber-Pakhtun-Khwa province, were surveyed for plant parasitic nematodes. Sixteen species of plant parasitic nematodes out of which 11 are being reported for the first time in this region, and 4 previously reported in Pakistan. The highest numbers of nematode species were found in Dera Ismail Khan, followed by Dergai, Warsak and Agriculture Research Rnstitute Tarnab.

Keywords: Parasitic nematodes.

Plant nematodes are tiny worms usually from 0.25 mm to 3 mm long in size with tapering ends. They are usually found in the soil around the roots of fresh-water plants (Zuckerman and Richard, 1981; Bird and Bird, 1991). Some species are found within the tissues of host plants and lead to great economic loss (Siddiqi, 1962; Timm and Ameen, 1960). Among plant parasitic nematodes, root-knot nematodes are the most destructive ones (PiedraI *et al.*, 2006). They attack more than 2000 species of plants including almost all cultivated plants (Troccoli *et al*., 2008). The plant parasitic nematodes also act as vectors for soil born viruses (Ahmad and Zainul-Abedin, 1975).

Majority of the known endo- and ecto-parasitic nematodes of plants belong to order Tylenchida. Some species of nematodes belong to order Rhabditida, which inhabit soil and decaying organic matter (Ahmad and Zainul-Abedin, 1975). Many species of nematodes, belonging to order Dorylaimida are also frequently found in the soil around the roots of plants (Pokharel and Larsen, 2008). Most of the injury that nematodes cause to plants is related in some way to the feeding process (Pokharel and Larsen, 2008; Ahmed *et al*., 1973).

Here the main objective of this study was to explore the occurrence of plant parasitic nematode in various areas of Khyber-Pakhtun-Khwa (KPK), Pakistan.

Materials and methods

In order to study plant parasitic and soil inhabiting nematodes of fresh-water, soil samples from the field of tomato, maize, okra and banana plants and root samples of the said plants were collected from Dergai, Warsak, Dera Ismail Division (D.I.Khan), Agriculture Research Institute, (A.R.I.) Tarnab and Peshawar University of KPK province.

About 400 g of soil from each sample was placed in a large basin and covered with water. Soil was allowed to soak, lumps were broken down with fingers and stones were removed. The muddy mixture was stirrered and poured through sieve into another enamel basin. After cleaning, about 350 g of soil sample was put in a funnel. The bottom opening of the funnel was closed with plastic and rubber band. Water was then put in the soil sample. After 48 h, it was examined. Nematodes present in the soil could not retain themselves in the mud and were gathered in water in the lower part of the funnel. The same procedure was adopted for all the other soil samples and a large number of nematodes were collected. Nematodes were preserved in 70% alcohol plus 5% glycerine**.** Temporary slides were prepared for identification of the nematodes (Tarjan *et al*., 1977; Ingham and Merrifield, 1996; Mekete *et al*., 2012; http://bpp.oregonstate.edu/files/bpp/webfm/pdf/bot499/2-NEMATODE%20MORPHOLOGY%20, 2013).

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Table I.- Comparative account of nematodes previously reported in Pakistan and that of our findings in different areas of KPK province.

Family / specimen		Reported previously in Pakistan	Our findings (in KPK regions)
Order Tylenchida			
Family Tylenchidae	*Ditylenchus angustus*	Sindh region	Dergai and D.I. Khan
Family Belonolaimidae	*Histotylenchus siddiqi*	Not reported in Pakistan	Dergai and D.I. Khan
Family Pratylenchidae	*Pratylencus pratensis* or *Pratylenchus irregularis*	Sindh region, Peshawar (KPK)	Dergai, Warsak and D.I. Khan
Family Hoplolaimidae	*Haplolaimus seinhorstei*	Sindh region	Warsak
	Helicotylenchus dihystera	Sindh region and Tarnab (KPK)	Warsak and D.I. Khan (Only females)
	Acontylus vipriensis	Not reported in Pakistan	Dergai and D.I. Khan
Family Heteroderoloidae	*Meloidogyne incognita* or *Meloidogyne javanica*	Sindh region	Dergai Warsak and D.I. Khan
Family Aphelenchidae	*Aphelenchus avnea* or *Aphelenchus Agricola*	Punjab regions	Dergai and D.I. Khan
	Seineura fuchs	Not reported in Pakistan	Dergai and D.I. Khan
	Cephalobus persegnis	Punjab	Dergai
	Eucephalobus steiner	Sindh region	Dergai and D.I. Khan
Family Tylenchorhynchidae	*Tylenchorhynchus annulatus* or *Tylenchorhynchus martini*	Sindh region, Tarnab (KPK)	Dergai, Warsak and D.I. Khan
Order Dorylaimida	*Dorylaimus dujardin*	Punjab	A.R.I. Tarnab
	Lebronema thorne	Not reported in Pakistan	A.R.I. Tarnab
	Trichodorus Pakistanensis	Punjab, Abbottabad and Haripur (KPK)	Warsak, Tarnab
	Xiphinema indium	Punjab	Dergai

Results

Fifteen genera and 16 different species were identified from soil gathered around the roots of host plants. Twelve species belonged to the order Tylenchida, whereas four belonged to order Dorylaimida. Out of 16 identified species, 11 species are reported first times in the KPK province (Table I), while four are reported first time in Pakistan (Table I). All the species of nematodes were parasitic except *Cephalobus persegnis.*

Data analysis revealed occurrence of 15 nematodes species in the soil of D.I.K. (*Ditylenchus angustus, Tylenchorhynchus annulatus, T. martini, Histotylenchus siddiqi, Pratylencus pratensis, P. irregularis, Helicotylenchus dihystera, Acontylus meagre, Meloidogyne incognita, M. javanica, Aphelenchus avnea, A. agricola, Seineura fuchas, Eucephalobus steiner, Dorylaimus dujardin*), followed by 12 species in Dergai (*Tylenchus angustus, Tylenchorhynchus martini, Histotylenchus siddiqi, Pratylenchus irregularis, Acontylus meager, Meloidogyne incognita, M. javanica, Aphelenchus agricola, Seinura fuchas, Cephalobus persegnis, Eucephalobus steiner, Xiphinema indium*), five species in Warsak (*Tylenchorhynchus martini, Pratylenchus irregularis, Haplolaimus sienhorsti, Tylenchus dihystera, Trichodorus* sp.) and 3 species in A.R.I. Tarnab (*Haplolaimus seinhorstei, Trichodorus pakistanensis, Lebronema thorne*). No nematode was found in Peshawar University.

Discussion

Plant parasitic nematodes feed on living plant tissues, using needle like oral stylet (Thiery *et al*., 1999; Anwar and Chaudhry, 1973). These parasitic nematodes inject enzymes into the host cell before feeding to partially digest the cell contents before being sucked into the gut (Thiery *et al*., 1999; Smiley *et al*., 2008).

We found the following nematodes in the soil

around or within the roots of maize, tomato, banana and okra crops of KPK province. Maize: *Ditylenchus angustus, Tylenchorhynchus annulatus, Pratylencus pratensis, Lebronema thorne, Trichodorus pakistanensis, Haplolaimus seinhorstei, Helicotylenchus dihystera, Meloidogyne incognita, M. javanica, Aphelenchus avnea, Cephalobus persegnis, Eucephalobus steiner, Dorylaimus dujardin.* Tomato: *Histotylenchus siddiqi, Acontylus vipriensis, Meloidogyne incognita, M. javanica, Seineura fuchs, Cephalobus persegnis, Eucephalobus steiner, Lebronema thorne.* Banana: *Dorylaimus dujardin, Ditylenchus angustus, Meloidogyne incognita.* Okra: *Histotylenchus siddiqi, Xiphinema indium.*

Acontylus vipriensis was previously reported in the soil and roots of *Eucalyptus* and *Acacia* species in Australia (Bird and Bird, 1991; Pokharel and Larsen, 2008). We found *Acontylus vipriensis* within the roots of tomato from Dergai and D.I. Khan regions. *Seineura fuchas* are predatory in nature (Thiery *et al*., 1999; Endo, 1979). During our study, we found the *Seineura* nematodes in the soil around the roots of tomato plants from Dergai and D.I. Khan regions. *Lebronema thorne* are associated with *Eucalyptus* seedlings (Pokharel *et al*., 2009; Maqbool, 1992). These nematodes were found around the roots of maize and tomato plants in Warsak of KPK. *Ditylenchus angustus* has been reported from Bangladesh in rice and potato (Timm and Ameen, 1960; Ahmad and Zainul Abedin, 1975) and in sugarcane crop from Sindh region of Pakistan (Ahmad and Zainul Abedin, 1975; Khan *et al*., 1973). During the survey, *Ditylenchus angustus* was found in the root soil of maize and banana plants from Dergai and D.I.Khan regions. *Haplolaimus seinhorstei* species is previously reported from Sindh region of Pakistan in sugarcane (Williamson, 1999; Souza and Baldwin, 1998), and from Bangladesh in tobacco and maize (Ahmad and Zainul Abedin, 1975; Khan *et al*., 1973). *Aphelenchus avnea* was previously reported from India and Punjab province of Pakistan within the roots of sugarcane and pea plants (Timm and Ameen, 1960; Maqbool, 1992). *Cephalobus persegnis* species was previously found as free living in the soil around the roots of bean plants in southern region of Punjab, Pakistan (Maqbool, 1992; Akhtar, 1962). *Eucephalobus steiner* species was found in the soil around the roots of maize and tomato from Dergai and D.I.Khan regions. *Dorylaimus steiner* has formerly been reported as free-living nematodes around the roots of egg-plants (Ahmed *et al*., 1973) or gram plants (Anwar and Chaudhry, 1973) in the southern region of Punjab, Pakistan, however in the current study it was found in the soil around the roots of maize crop and banana plants from D.I.Khan region.

Species of *Xiphinema* have been reported from different regions, *viz., Xiphinema americanum* in U.S.A. (Siddiqi, 1962), *Xiphinema indium* in sindh (Ahmed *et al*., 1973) Punjab (Akhter, 1962) and *Xiphinema pratense* also in Punjab (Akhter, 1962; Khan and Anwar, 1973). But current study identified *Xiphinema indium* within the roots of okra plants from Dergai region of KPK.

Conclusion

Fresh-water plant parasitic nematodes do exist in various regions of KPK province of Pakistan but can be controlled through regular alternation of crops, use of biological predators or use of sun-heating in summer after ploughing.

Authors' contributions

Jabbar Khan designed the project, did the sample collection, identification of nematodes and manuscript writing. Rehana Gul, Inam Ullah Khan, Muzammil Ahmed Khan and Shahid Niaz Khan helped in sample collection and in critical review of manuscript. All the authors read and approved the final manuscript.

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References

Ahmed, M., Saeed, M. and Khan, S., 1973. *Pak. J. Res.*, **16**: 121-125

Ahmad, M. and Zainul-Abedin, M., 1975. Studies on parasitic nematodes associated with sugarcane crop in Sindh region. *Res. Bull., Biol. Res.* Complex, University of Karachi, Karachi, Pakistan.

Akhter, S.A., 1962. *Pak. J. Agric.*, **13**: 140-144.

Anwar, S.A. and Chaudhry, N.A., 1973. *J. agric. Res. Punjab*, **11**: 89-92.

Bird, A.F. and Bird, J., 1991. *The structure of nematodes*. 2nd Edition Academic Press San Diego, pp. 316.

Endo, B.Y., 1979. *J. Ultrastruc. Res*., **67:** 1-14.

http://bpp.oregonstate.edu/files/bpp/webfm/pdf/bot499/2-NEMATODE%20MORPHOLOGY%20, 2013)

Ingham R. and Merrifield, K., 1996. *A guide to nematode biology and management in mint.* Integrated Plant Protection Center, Oregon State University, Corvallis. Public. No. 996, pp. 38.

Khan, A.R. and Anwar, S.A., 1973. *J. agric. Res. Punjab*, **11**: 123-128

Khan, A.R., Zeb, A., Shah, F.A., Khan, M. and Gul, A., 1973. *Pak. J. Phytopatol.,* **5**: 23-26.

Maqbool, M., 1992. *Pak. J. Sci.*, **21**: 72-76

Mekete, T., Dababat, A., Sekora, N., Akyazi, F. and Abebe, E., 2012. *A manual for nematology. Identification key for agriculturally important plant-parasitic nematodes*, International Nematode Diagnosis and Identification Course, Eskisehir, Turkey.

PiedraI, B.A., Garcia-Alvarez, A., Diez-Rojo, M.A., Ros, C., Fernandez, P., Lacasa, A. and Bello, A., 2006. *Biores. Technol.,* **98**: 2846-2851

Pokharel, R. and Larsen, H.J., 2008. In: *Western Colorado Research Center Annual Report – 2007* (ed. R. Godined), Agricultural Research Station, Colorado State University. Ft. Collins 76. pp. 22-28.

Pokharel, R.R., Larsen, H.J., Hammon, B., Gourd, T. and Bartolo, M., 2009. In: *Western Colorado Research Centre, Colorado State University. Annual Report* (ed. R. Godin), pp. 39-44.

Siddiqi, M.R., 1962. *Nematologica*, **8**: 11-14.

Smiley, R.W., Yan, G.P. and Handoo, Z.A., 2008. *Pl. Dis*., **92**:1136.

Souza, R.M. and Baldwin, J.G., 1998. *J. Nematol.,* **30**: 275-290

Tarjan, A.C., Esser, R.P. and Shih L. and Chang, S.L., 1977. *J. Water Pollut. Cont. Fed*., **49**: 2318-2337.

Thiery, M.G., Greene, A.E. and Bird, D.M., 1999. *J. Pl. Physiol*., **120**: 934.

Timm, R.W. and Ameen, M., 1960. *Pak. J. Agric.*, **3**: 24-27.

Troccoli, A., De Luca, F., Handoo, Z.A. and Di Vito, M., 2008. *J. Nematol*., **40**: 190-196.

Van Den Berg, E., Subbotin, S.A., Handoo, Z.A. and Tiedt, L.R., 2009. *J. Nematol.,* **11**: 523-540.

Williamson, V.M., 1999. *J. Curr. Opin. Plant Biol*., **2**: 327-331.

Zuckerman, B.M. and Richard, R.A., 1981. *Plant parasitic nematodes*. Academic Press, New York.

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