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Isolation of Malathion Degrading Chromogenic *Pseudomonas aeruginosa* Strains From Insecticide Impregnated Soils

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Abstract.- Soil samples from an insecticide contaminated site were processed for isolation of malathion degrading bacteria. Selective medium containing malathion as sole carbon source was used for this purpose. Four isolates were identified as through ribotyping *Pseudomonas*. The bacteria, yielded water soluble greenish pigment both in nutrient broth as well as in the select media in a growth responsive fashion. These strains can be used for bioremediation of malathion contaminated soils or waters and add to the microbial diversity being conserved by workers from yet polluted environments. Chromogenic nature of these malathion degrading bacteria can make their action visible in select applied sites.

Key words: Bioremediation , malathion, *Pseudomonas*

Pesticides geared increased agricultural productivities (Blain, 1990; Hashmi *et al.,* 2004) have been accompanied with contamination of soil and water environments (Cisar and Synder, 2000; Getenga *et al.,* 2000; Tse *et al.,* 2004) and created hazards for human health including cancer (Van Maele-Fabry and Willems, 2003; Engel *et al.,* 2005) and adverse effects on fertility (Golec *et al.,* 2003).

Malathion is one of the general purpose organophosphate (OP), household and agricultural pesticides recommended for control of insects including the stored grains pests (Saleem and Shakoori, 1987; Shakoori and Saleem 1989; Wester and Cashman, 1989; Ali *et al*., 2007). It has quite earlier been found toxic for a variety of organisms (Butler, 1963; Culley and Applegate, 1967; Tagatz *et al*., 1974).

Different bacteria have been reported for successful bioremediation of polluted habitats, including insecticide contaminated soils and waters (el-Deeb *et al.,* 2000; Bhadhade *et al.,* 2002; Mohamed *et al*, 2010). Several bacteria such as *Pseudomonas* (Gill and Ballesteros, 2000); *Actinomycetes* (De-Schrijver and De-Mot, 1999); *Escherichia coli* (Elashvili *et al.,* 1998); *Arthrobacter* sp. (Ohshiro *et al.,* 1996); *Flavobacterium* sp. (Mallick *et al.,* 1999) and *Rhodococcus* sp. (Parekh *et al.,* 1994) capable of completely mineralizing OPs have been described. In fact, malathion has been used as a general insecticide since over the decades. Resultantly, water and soil habitats got contaminated with the insecticide and a large number of bacteria capable of degrading malathion have been reported. For instance various species of *Bacillus* genus capable of varying levels of malathion degradation have been isolated from soils (Singh *et al*., 2011; Kumari *et al*., 2012, Thabit and Naggar, 2012), while *Pseudomonas* sp. upto 100% malathion degradation efficiency have been demonstrated by different workers (Rosenberg and Alexender, 1979; Abo-Amer, 2007; Goda *et al*., 2010; Thabit and Naggar 2012; Pankaj *et al*., 2013).

This paper reports malathion degrading bacteria from a locality having been influenced for more or less a decade by the run off of an insecticide formulating unit. The bacterial isolates appear good candidates for rehabilitation of soils contaminated with malathion following provision of other ingredients of a minimal medium. Moreover, the chromogenic ability of these *Pseudomonas aeruginosa* isolates may be helpful in assessing their activities in fluid environments.

Materials and methods

Samples were collected in sterilized glass bottles from insecticide influenced soils including the one that had been receiving drainage of a factory involved in formulation of pesticides. The samples were processed by mixing 10g of soil in 100 ml of

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autoclaved water and keeping the mixtures on orbital shaker (Digitek instruments) at 100 rpm for 2 hours at 30±1ºC. From each processed soil sample, 0.5 ml was spread over the surface of a selective medium with the help of glass spreader. The selective medium contained 0.5% malathion (commercial grade 50%), as sole source of carbon, 0.1% K_2HPO_4, 0.5% NH_4NO_3, 0.2% $MgSO_4$ and 1.5% agar in distilled water. The medium was routinely autoclaved and then 20µl of separately autoclaved mineral solution containing $FeSO_4$ 10%, $CaCl_2$ 10%, $CuNO_3$ 0.5%, zinc powder 0.5% and $MnCl_2$ 0.5% (w/v) was added. The inoculated plates were incubated at 37ºC for 48 h. The bacterial growth was then streaked for pure culturing on nutrient agar and again on the selective medium. The isolates were preserved in glycerol stocks (10 % glycerine) for further use.

Each of the isolates was allowed to grow in minimal broth of the selective medium (without malathion) at 37ºC for four days. O.D. of the inoculated broths was then noted at 600nm daily to declare their autotrophic/heterorophic nature.

Isolates expressing promising growths in the selective medium were identified by amplifying and sequencing their 16SrDNA as described by Ghauri *et al*. (2003). Accordingly genomic DNA was isolated from overnight cultivated culture in nutrient broth. Amplification of 16SrDNA was then carried out by using forward (fDI) AGAGTTTGATCCTGGCTCAG and reverse (rP1) ACGG (ACT) TACCTTGTTACGACTT primers. Two times ReadyMix PCR Master Mix (Abgene) was used. Each reaction vial contained 25 µl master mix and the addition of the template (1 µl) and primers (fD1 and rP1, each 1µl) resulted in a final volume of 50 µl with nanopure water. The master mix contained 1.25 U Taq DNA polymerase, 75 mM TRIS-HCl (pH 8.8 at 25ºC), 20 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.01% (v/v) Tween 20, 0.2 mM each of dATP, dCTP, dGTP, dTTP, precipitant, and red dye for electrophoresis. The reaction mixture was heated at 95ºC for 5 min. Amplification was carried out in thirty cycles. Each cycle comprised 30 s at 95ºC, 40 s at 55ºC and 2 min at 72ºC. The final extension was carried out for 10 min at 72ºC. Five µl amplicons were run on 1.5% agarose gel along with 1 kb 5M0313 Fermentas DNA ladder.

The PCR products were sequenced commercially (Macrogen Inc., Seoul, Korea). The gene sequences were compared with others in the Gene Bank databases using the NCBI BLAST (www.ncbi.nlm.nih.gov). Gene sequences of 16S rDNA of selected organisms were obtained from Gene Bank and aligned with gene sequence of our isolates using CLUSTALX.

Results and discussion

Inoculation of the processed soil samples and subsequent incubation yielded small colonies over surface of the selective medium indicating malathion consuming nature of the bacteria. Chemotrophic nature of the bacteria was confirmed by inoculating and incubating them in the minimal medium, where no growth was recorded upto 96 hrs.

Growth of these bacteria in nutrient broth indicated greenish pigment production by the isolates 2A, 3B and S1. These isolates also yielded the pigment when cultivated in the selective medium broth.

The partial sequence of the 16S rDNA of the isolates reported in this study had homology with *Pseudomonas* spp. (Table I).

Table I.- Relatedness on the basis of 16S rDNA homology.

Isolate code	Nearest Relative	Similarity (%)
3B	*Pseudomonas aeruginosa* (EF151192)	98%
2A	*Pseudomonas aeruginosa* strain MY 06 (DQ083947)	97%
3A	*Pseudomonas putida* strain NAA (DQ864462)	99%
S1	*Pseudomonas aeruginosa* strain SWD (DQ859983)	98%
S2	*Acinetobacter baumannii* sp. QN6 (DQ640274)	97%

The present study brings support to the generalization that continuous exposure to a drastic environment exerts selective pressure for microorganisms which results into appropriate ecological succession characterized with appearance of pollutant(s) resistant/utilizing microbes. Bacteria

Table II.- An overview of bacterial diversity reported for malathion degrading potential.

Name of bacteria	Isolation locality	Enzyme(s) reported for degradation	Degrading efficiency	Reference(s)
Pseudomonas	Soil	-[a]	100%	Rosenberg and Alexander (1979)
Serratia marcescens	Degraded Cattle Bone	-	3.33-7.49%	Kannan and Vanitha (2005)
Pseudomonas aeruginosa AA112	Soil	About 3 Proteins of high M.W.	Complete degradation	Abo-Amer A.E. (2007)
Brevibacillus sp. Strain KB$_2$	Soil	Carboxylesterase activity	36.22% in 7 days	Singh *et al*. (2011)
Bacillus cererus strain PU	Soil	Carboxylesterase activity	49.31% in 7 days	Singh *et al*. (2011)
Lysinibacillus sp. KB$_1$	Soil	Carboxylesterase activity	20% in 7 days	Singh *et al*. (2012)
Bacillus sp.	Soil	-	-	Kumari *et al*. (2012)
Pseudomonas aeruginosa	Soil	-	T-value at P$\leq$ 0.05 0.0149	Thabit and Naggar (2012)
Bacillus pseudomycoides	Soil	-	T-value at P$\leq$ 0.05 0.0077	Thabit and Naggar (2012)
Bacillus licheniformes	Soil	-	T-value at P$\leq$ 0.05 0.0079	Thabit and Naggar (2012)
Pseudomonas xanthomarina		-	70.5% in 2 days	Pankaj *et al*. (2013)
Pseudomonas sp.	Soil	-	High degrading activity	Goda *et al*. (2010
Pseudomonas putida	Soil	Carboxylesterase enzyme	High degrading activity	Goda *et al*. (2010)
Micrococcus lylae	Soil	-	Low	Goda *et al*. (2010
Pseudomonas aurofaciens	Soil	-	Low	Goda *et al*. (2010)
Acetolacter liquefaciens	Soil	- -	Low	Goda *et al*. (2010)

[a]Information was not available

are well known for being capable of adapting themselves to environments exerting a selective pressure by utilizing a myriad of man made toxic chemicals including pesticides.

Drained water from a pesticide formulating unit had altered the effected soils and the microbial community of the reported location. The bacteria reported in this communication used malathion successfully for their energy requirements, as sole carbon source. The isolates 3B, 2A, SI showed maximum homology with *Pseudomonas aeruginosa*. Production of green pigment in nutrient and the selective media broths, as observed in this study, is also a characteristic biochemical feature of *P. aeruginosa*. Yet they belonged to different strains as 3B, 2A and SI had 98%, 97% and 98% homologies,respectively with *Pseudomonas aeruginosa, Pseudomonas aeruginosa*MYO6 *Pseudomonas aeruginosa* SWD. The isolate 3A had 99% homology with *Pseudomonas putida* strain NAA. 16S rDNA is being widely used to develop comparative cataloguing (Stackbrandt and Woese, 1981). Sequence information from the conserved regions is useful for studying phylogentic relationships (Woese *et al.*, 1985) as well as for the design of universal oligonucleotide probes and primers used for identification and amplification, respectively (Giovannoni, 1991). Variable regions provide sequence data to develop specific probes and primers for detection of microorganisms by hybridization or with polymerase chain reaction (Ward *et al.*, 1992). Using conserved primers, the 16S rDNA can be easily amplified by PCR not only from pure cultures but also directly from the environmental samples (Olsen *et al.*, 1986; Giovannoni *et al.*, 1990).

Polygenetic analysis of 16SrDNA strongly suggested that the bacterial isolates 3B, SI, 2A and

3A may be members of the genus *Pseudomonas* on the basis of their closest relatedness. The ability of *Pseudomonas* to degrade malathion is well known (Bourquin, 1975; Hashmi *et al.*, 2002; Foster and Bia, 2004; Hashmi *et al.*, 2004; Goda *et al.*, 2010). Rather detoxification potential for other pollutants has also been reported by many workers. For example, Guerin and Boyd (1995), Prijambada *et al.* (1995), Duetz *et al.* (1996) and Mclaughlin *et al.* (2006) have reported detoxification potential of *Pseudomonas* species for naphthalene, nylon oligodimer, toluene 4-chlorophenol and toluene, respectively. In addition to the *Pseudomonas* sp. several other bacterial species capable of malathion degradation have been documented. In this regard a brief overview of malathion degrading bacterial isolates with respect to isolation locality and degradation potential is given in Table II.

The present isolates appear good candidates for rehabilitating malathion and/or other pollutant influenced soils. Surely maximum level of malathion tolerance has to be worked out in the presence of field conditions. Further work will delineate their usefulness for remediational strategies meant to be designed for gearing back the polluted environment.

Chromogenic nature of these malathion degrading *P. aeruginosa* isolates is an attractive attribute for designing water treatment bioremediation strategies where appearance of the pigment may have predictive value of reductions in the levels of a pollutant. Such earmark becomes especially important in field conditions.

References

Abo-Amer, A.E., 2007. *Acta Microbial. Immunol. Hung.*, **54**: 261-277.

Ali, N.S., Munir, M., Ali, S.S. and Shakoori, A.R., 2007. *Pakistan J. Zool.*, **39**: 179-184.

Bhadhade, B.J., Sarnaik, S.S. and Kanekar, P.P., 2002. *Curr. Microbiol.*, **45**: 346-349.

Blain, P.G., 1990. *Adverse Drug React. Acute Poison. Rev.*, **9**: 37-68.

Bourquin, A.W., 1975. U.S. *Environ. Protec. Agency*, Washington. Report No. EPA-660/3-75-035

Butler, P.A., 1963. *U.S. fish Wildl. Serv. Circ.*, **167**: 11-24.

Cisar, J.L. and Synder, G.H., 2000. *ACS Symp. Ser.*, **743**: 106-126.

Culley, D.D. and Applegate, H.G., 1967. *Pestic. Monit. J.*, **1**: 21-28.

De-Schrijver, A. AND De-Mot, R., 1999. *Crit. Rev. Microbiol.*, **25:** 85-119.

Duetz, W.A., Marques, S., Wind, B., Romas, J.L. and Van Andel, J.G., 1996. *Appl. environ. Micorbiol.*, **62**: 601-606.

Elashvili, I., Defrank, J.J. and Culotta, V.C., 1998. *Appl. environ. Microbiol.*, **64**: 2601-2608.

El-Deeb, B.A., Soltan, S.M., Ali, A.M. and Ali, K.A., 2000. *Folia Microbiol. (Praha)*, **45**: 211-216.

Engel, L.S., Hill, D.A., Hoppin, J.A., Lubin, J.H., Lynch, C.F., Pierce, J.J., Samanic, C., Sandler, D.P., Blair, A. and Alavanja, M.C., 2005. *Am. J. Epidemiol.*, **161**: 121-125.

Foster, L.J.R. and Bia, H., 2004. *FEMS Microbiol. Lett.*, **240**: 49-53.

Getenga, Z.M., Jondiko, J.I.O., Wandiga, S.O. and Beck, E., 2000. *Bull. environ. Contam. Toxicol.*, **64**: 359-367.

Ghauri, M.A., Khalid, A.M., Grant, S., Haephy, S. and Grant, W.D., 2003. *Extremophiles*., **7**:341–345.

Gill, I. and Ballesteros, A., 2000. *Biotechnol. Bioeng.*, **70**: 400-410.

Giovannioni, S.J., Britschgi, T.B., Moyer, C. and Field, K.G., 1990. *Nature* (London), **345**: 60-62.

Giovannoni, S., 1991. In: *Nucleic acid techniques in bacterial systematics* (eds. E. Stackebrandt and M. Goodfellow), John Wiley & Sons, New York, pp. 177-203.

Goda, S.K., Elsayed, I.E., Khodair, T.A., El-Sayed, W. and Mohamed, M.E., 2010. *Biodgradation*, **21**: 903-913.

Golec, J., Hanke, W. and Dabrowski, S., 2003. *Med. Pr.*, **54**: 465-472.

Guerin, W.F. and Boyd, S.A., 1995. *Appl. environ. Microbiol.*, **61**: 4061-4068.

Hashmi, I., Khan, M.A. and Jong-Guk, K., 2002. *Pak. J. biol. Sci.*, **5**: 699-703.

Hashmi, I., Khan, M.A. and Kim, J.G., 2004. *Bitotechnology*, **3**: 82-89.

Kannan, V. and Vanitha, V., 2005. *Ind. J. Biotech.*, **4**: 277-283.

Kumari, R., Jeevan, G., Ashlock, CH. M. and Rao, K., Vamsi, K.S.K., 2012. *IOSR J. Pharm.*, **2**: 37-42.

Mallick, K., Bharati, K., Banerji, A., Shaki, N.A. and Sethunathan, N., 1999. *Bull. environ. Contam. Toxicol.*, **62**: 48-54.

Mclaughlin, H., Farrell, A. and Quilty, B., 2006. *J. environ. Sci. Hlth.*, **41**: 763-777.

Mohamed, Z.K., Ahmed, M.A., Fetyan, N.A. and Elnagdy, S.M., 2010. *J. Adv. Res.*, **1**: 145-149.

Ohshiro, K., Kakuta, T., Sakai, T., Hirota, H., Hoshino, T. and Uchiyama, T., 1996. *J. Ferment. Bioeng.*, **82**: 299-305.

Olsen, G.J., Lane, Giovannioni, S.J. and Pace, N.R., 1986. *Ann. Rev. Microbia.*, **40**: 337-365.

Pankaj, G., Divya, B. and Kumar, M.D., 2013. *Res, J. Chem. Environ.*, **17**: 59.

Parekh, N.R., Walker, A. and Roberts, S.J., 1994. *J. Agric. Fd. Chem.*, **36**: 193-199.

Prijambada, I.D., Negoro, S., Yomo, T. and Urabe, I., 1995. *Appl. environ. Microbiol*., **61**: 2020-2022.

Rosenberg, A. and Alexander, M., 1979. *Appl. environ. Microbiol*., **37**: 886-891.

Saleem, M.A. and Shakoori, A.R., 1987. *Archiv. Insect Biochem. Physiol*., **5**: 45–55.

Shakoori, A.R. and Saleem, M.A., 1989. *Archiv. Insect Biochem. Physiol*., **11**: 203–215.

Singh, B., Kaur, J. and Singh, K., 2011. *World J. Microbiol. Biotechnol*., **28**: 1133-1141.

Singh, B., Kaur, J. and Singh, K., 2012. *Biotech. Lett.*, **34**: 863-867.

Stackebrant, E. and Woese, C.R., 1981. *Sym. Soc. Gen. Microbiol.*, **32**: 1-32.

Tagatz, M.E., Borthwick, P.W., Cook, G.H. AND Coppage, D.L., 1974. *Mosq. News*, **34**: 38-42.

Thabit, T.M.A. and El-Naggar, M.A.H., 2012. *World Rur. Observ.*, **4**: 57-65.

Tse, H., Comba, M. and Alaee, M., 2004. *Chemosphere*, **54**: 41-47.

Van, M.F.G. and Willems, J.L., 2003. *Occup. environ. Med.*, **60**: 634-642.

Ward, M.W., Bateson, M.M., Waller, R. and Ruff-Roberts, A.L., 1992. In: *Recent advances in microbial ecology* (ed. K.C. Marshall), Plenum Press, New York, pp. 219-286.

Wester, R.C. and Cashman, J.R., 1989. In: *Sulphur containing drugs and related organic compounds: Chemistry, biochemistry and toxicology* (ed. L.A. Damani). Halsted Press, New York.

Woese, C.R., Stackebrandt, E., Macke, T. and Fox, G.E., 1985. *Appl. Microbial*., **6**: 143-151.

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The Relationship Between Growth and Parasites in Carp (*Cyprinus carpio* L., 1758) Inhabiting Karacaören II Dam Lake

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Abstract.- This study was carried out between January 2010 and December 2010 to determine the parasites and their effect on growth of carp (*Cyprinus carpio* L., 1758) inhabiting Karacaören II Dam Lake. Sixty four samples were captured on monthly basis, their age and parasites were examined. These fish fall in groups I-VIII and 39 (60.9%) of them harboured *Dactylogyrus anchoratus*, *Dactylogyrus minutus*, *Argulus foliaceus*, *Lernaea cyprinacea*, *Caryophyllaeus laticeps* and *Bothriocephalus acheilognathi*. It was determined that among the fish of same sex and age and captured during the same month, the ones without a parasite were longer and heavier than the ones with parasite. It was found that the parasitized carps were shorter and weaker than the ones without a parasite.

Key words: *Cyprinus carpio*, crustacean ectoparasites, monogenean ectoparasite, cestode endoparasites.

Parasites not only reduce the nutritional value of the fish, but they also lead to a significant economic loss by preventing the fish from growing, breeding and feeding. Thus, research must be conducted upon fish diseases and parasites which is a central problem to pisciculture as well as on fishery.

Several studies have been conducted on carps' growth and parasites in this country. (Aydoğdu e*t al.*, 2003; Kır e*t al.*, 2004; Öztürk,

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2005; Öztürk and Bulut, 2006; Buhurcu and Öztürk, 2007; Kır, 2007; Kartal and Öztürk, 2009; Soylu, 2013), but no information is available on parasites of the carp (*Cyprinus carpio* L., 1758) inhabiting Karacaören II Dam Lake. This study aims at examining the ecto and endoparasites of the carp in the reservoir and identify the seasonal infection conditions of these parasites and their impact on growth.

Materials and methods

The coordinates of Karacaören II Dam Lake; 37° 18'N, 30° 48'E; elevation from sea level is 347 m and space of the lake is 2.34 km^2.

Sixty four carps were captured from different parts of the lake by monthly periods during January 2010 to December 2010 and examined parasitologically. Parasites were found and ecto parasites were dyed with glycerine-gelatine and endo parasites with acetocarmine and then their permanent slides were made. For identification of parasites Bykhovskaya-Pavlovskaya (1964), Reinhenbach-Klinke (1966), Cheng (1973) and Bauer (1987) were followed.

Age of each fish was deduced from their scale (Chugunova, 1963). Results achieved have been evaluated seasonally.

Results and discussion

Carps of age groups from I to VIII were collected from Karacaören II Dam Lake. As a result of parasitological examination, two crustacean ecto-parasites, *Argulus foliaceus* and *Lernaea cyprinacea*, two monogenean ecto-parasites, *Dactylogyrus anchoratus* and *Dactylogyrus minutus*, and two cestode endoparasites *viz.*, *Caryophyllaeus laticeps* and *Bothriocephalus acheilognathi* were encountered. Table I shows the prevalence of parasite during different seasons; 39 out of 64 carps (60.9 %) were detected to be infected by those parasites and infected fish had minimum 2 and maximum 180 parasites. Table I also shows comparison of the carps' size and weight with and without parasites at the same age and with the same gender caught at the same time. It was found that carps with parasites grew 8.9% less in terms of mean size and 20.2% less in terms of mean weight.

Table I.- Comparison of parasitised and non-parasitised carps in terms of size and weight.

Season	No. of carps examined / No. of parasitised carps	Sex	Age	Non-parasitized fish		Parasitised fish		Losses	
				Size (mm)	Weight (g)	Size (mm)	Weight (g)	Size (mm)	Weight (g)
Winter	16/7 (44%)	♂♂♂♂♀	VI	351	650	308	443	43 (12.3)	207 (31.9)
Spring	21/13 (62%)		VII	425	1086	399	832	26 (6.2)	254 (23.4)
Summer	11/9 (82%)		VIII	415	1020	412	915	3 (0.8)	105 (10.3)
Autumn	16/10 (60%)		V	316	504	254	414	62 (19.7)	90 (17.9)
Total	**64/39 (61%)**			**376.7**	**815**	**343.2**	**651**	**33.5 (8.9)**	**164 (20.2)**

Kır *et al.* (2004) and Kır (2007) reported heavier and longer sizes of the fish without parasites than those with parasites at the same age and with the same gender caught at the same time. Having compared the carps with and without parasites in terms of size and weight, this study shows that on average the fish with parasites grew less in terms of size (8.9%) and weight (20.2%).

This is the first report on parasites of fish inhabiting Karacaören II Dam Lake though they have been reportedly found on other carps living in other regions across Turkey (Öktener, 2003). Burgu *et al.* (1988) also reported an increased level of parasites in carp during spring months. Kır (2002) and Kır *et al.* (2004) also reported highest level of parasite infection in *Carassius carassius* and *Cyprinus carpio* during spring and summer. The highest level of parasites (81.8%) in the present report was also recorded during summer.

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References

Aydoğdu, A., Kostadinova, A. and Fernandez, M., 2003. *Helminthologia*, **40**: 33-40.

Bauer, O.N., 1987. *Key to the parasites of freshwater fishes in the fauna of the U.S.S.R.* Leningrad, 583 pp.

Buhurcu, H.I. and Öztürk, M.O., 2007. *Türkiye Parazitol. Derg.*, **19**: 109-113.

Burgu, A., Oğuz, T., Körtıng, W. and Güralp, N., 1988. *Etlik Vet. Mikrob. Derg.*, **3**: 143-166.

Bykhovskaya–Pavlovskaya, A.V., 1964. *Key to parasites of freshwater fishes of the U.S.S.R.* II, III., Transl. by Birrow, A., Cale, Z.S., Israel Program for Scientific Translations, Jerusalem, 890 pp.

Cheng, C.T., 1973. *General parasitology*. Academic Press Inc., London, 965 pp.

Chugunova, L.P., 1963. *Age growth studies in fish national science foundation*. Washington, 132 pp.

Kartal, K. and Öztürk, M.O., 2009. *Türkiye Parazitol. Derg.*, **33**: 101-106.

Kır, İ., 2002. *Türkiye Parazitol. Derg.*, **26**: 440-443.

Kır, İ., Ayvaz, Y., Barlas, M. and Tekin-Özan, S., 2004. *Türkiye Parazitol. Derg.*, **28**: 45-49.

Kır, İ., 2007. *Türkiye Parazitol. Derg.*, **31**: 162-164.

Öktener, A., 2003. *Zootaxa*, **394**: 1-28.

Öztürk, M.O., 2005. *Türkiye Parazitol., Derg.*, **29**: 204-210.

Öztürk, M.O. and Bulut, S., 2006. *Fırat Üniv. Fen ve Müh. Bil. Derg.*, **18**: 143-149.

Reichenbach-Klinke, H.H., 1966. *Krankheiten und Schädigungen der Fischer*, Gustav Fischer Verlag. Stuttgart, pp. 389.

Soylu, E., 2013. *Pakistan J. Zool.*, **45**: 47-52.

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Serum Protein Levels in Goat Breeds of Gilgit-Baltistan, Pakistan

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Abstract.- Since serum protein level provides useful information on homeostasis of body, the present study was conducted to determine the serum protein levels of three different breeds of goats (Gojali, Baltistani and Kohai Ghezir breeds) from three regions of Gilgit-Baltistan *viz.*, Ghizer, Baltistan(Skardu and Ghanche districts) and Hunza-Nagar. Blood samples were collected randomly from a total of 508 (191 male and 317 female) goats and then serum protein was determined using Micro-lab 300.The results showed that male goat had significant differences ($p<0.05$) within the regions although female goats did not show significant differences. The study showed that the average blood protein level of male goat was 5.05±0.13 g/dl in Ghizer breed, 5.70±0.67 g/dl in Baltistan breed, 5.68±0.16 g/dl in Hunza-Nagar breed, whereas in female goat the serum protein level was 5.24±0.14 g/dl in Ghizer breed, 5.02±0.09 g/dl in Baltistan breed, and 5.41±0.11 g/dl in Hunza-Nagar breed, respectively.

Key Words: Goats, Serum protein level, Gilgit-Baltistan.

Goats are important domestic animals in the tropical livestock production system. In subsistence sector, pastoralist and agriculturist

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often depend on them for much of their livelihood (Devendra and Meleroy, 1982). Serum proteins are important in osmotic regulation, immunity and transport of several substances in the animal body (Jain, 1986). Pregnancy and lactation are physiological statuses considered to modify metabolism in animals (Krajničáková *et al.,* 2003, Iriadam, 2007). Blood biochemical parameters including total protein, triglycerides, free fatty acids and urea are important indicators of the metabolic activity in lactating animals (Karapehlivan *et al.,* 2007). Individual protein fractions, or blood serum proteins have different functions and their identification is used also as a diagnostic tool (Janků *et al.,* 2011). Serum protein profile, suggests that some fraction alterations in obese individuals are strong indicators of their roles in alteration of body mass (Akram *et al.,* 2011).The parasitic infections in ruminants cause decrease in total proteins and albumin levels (Oliveira and Penha, 1978; Fernandez *et al.,* 2006; Lawal *et al.,* 2007).

Present study was undertaken to determine the level of protein in goat serum while the animals were feeding on pure organic feed at pasture and to make comparison on serum protein level for goat's breeds from three major regions of Gilgit-Baltistan *viz.,* Gojali from Hunza-Nagar, Kohai Ghezir from Ghezir and Baltistani from districts Skardu and Ghanche. The study has also depicted the vegetative potential of these three regions of Gilgit-Baltistan. This study provided an idea of serum protein concentrations in healthy goats in the area, which could be used as basis for correct interpretation of acquired values for diagnosis of diseases.

Materials and methods

A total of 508 goats, 2 years old, including 191 male and 317 female from four districts of Gilgit-Baltistan *viz.,* Ghizer, Hunza-Nagar, Skardu and Ghanche were used in this study.

Five ml blood was taken from the jugular vein in 5 ml disposable syringe from each animal and was used for estimation of serum protein using Micro-lab 300 at 570 nm filter (Weichselbaum, 1946, Josephson *et al.,* 1957).

Data was analyzed by applying one way ANOVA using computer software SPSS (version 16.0). The difference of protein level was statistically determined at $P<0.05$ level.

Results and discussion

Table I shows serum total protein level of male and female goats from Ghizer, Hunza-Nagar and Baltistan. In male goats total proteins estimates were 5.05±0.14, 5.68±0.15, and 5.70±0.66 g/dL in the three sampling areas, respectively. Male goats of Baltistan showed higher values of serum protein level which followed Hunza-Nagar and Ghezir. Serum protein level in male goats of different regions of Gilgit-Baltistan showed significant difference ($p<0.05\%$) when comparing various regions. Average mean values of female goats from Ghizer, Hunza-Nagar and Baltistan were 5.24±0.14, 5.41±0.10 and 5.02±0.09 g/dL, respectively. Female goats of Ghezir showed higher values of serum total protein then male goats and in other two regions male goat has higher serum protein than female. Female goats on comparison with regions did not show significant difference. The serum total proteins levels in total goats were 5.18±0.10, 5.50±0.09 and 5.33±0.07 from the three areas, respectively. Non-significant difference was observed within regions for total goats. Total serum protein was analyzed for gender and total female goats shown 5.23±0.08 and total male goats has 5.36±0.09, respectively. Total proteins were observed non-significantly differed between regions of Gilgit-Baltistan. Normally the blood proteins are 7.5 g/dl with an optimal range of 7.2-8.0 g/dl. However the values obtained from this study showed the low range than the normal range. Gilgit-Baltistan (GB) has rough mountainous terrain and during

winter animals feed on dry food which affect the serum protein level or may be the goats have high metabolic rate of protein. Brought and Lecee (1970) have shown that the total proteins of small ruminant range from 5.4-7.6 g/100ml. Ruston (1981) also reported that normal protein of ruminant ranges between 6.6-7.5 g/100 ml, which is higher than the values reported here.

Table I.- Serum protein levels of goats of Gilgit-Baltistan.

Region	Blood serum proteins (g/dl)		
	Male*	Female	Total goats
Ghezir	5.05±0.14 (n=98)	5.24±0.14 (n=183)	5.18±0.10 (n=281)
Hunza-Nagar	5.68±0.15 (n=36)	5.41±0.10 (n=66)	5.51±0.09 (n=102)
Baltistan	5.70±0.66 (n=57)	5.02±0.09 (n=68)	5.33±0.07 (n=125)
Total	5.36±0.81 (n=191)	5.23±0.08 (n=317)	5.28±0.06 (n=508)
F value	8.36	1.049	2.061
P value	<0.001	3.52	0.13

*The protein values are significantly different between different regions.

Male goats showed highly significant differences between Ghezir, Hunza-Nagar and Baltistan but Hunza-Nagar and Baltistan shown non-significant differences. It may be because Hunza-Nagar and Baltistan areas are situated at karakoram regions of GB and they may have the same vegetation and environment condition therefore, they may have shown non-significant differences with total protein level. Ghezir is situated in Hindu Kush region and has different environmental conditions and vegetation which may have effect on the total serum protein level. Castro *et al*. (1977) suggested that there was no significant difference between genders but various ages were significantly different, with mean values of 7.3±0.7 mg/dl and 7.78±0.20 g/100 ml.

Abdallatif *et al.* (2009) reported mean value of serum protein 6.98±0.89 g/dl goats during dry summer season. The mean values of serum protein level in both male and female during present study had no comparison with findings of Abdallatif *et al*. (2009). The present study was conducted during summer when animals were feed on the pastures and the blood were collected from goats at the end of summer season and particularly the effect of pasture was studied on the blood parameter. Otesile and Kasali (1993) investigate the influence of age and sex on serum concentration of total proteins and reported that males had significantly higher protein concentrations than female. This finding support the finding of this study that goats found in Baltistan and Hunza-Nagar had higher concentration of protein in males than in females. Tanritanir *et al. (*2009) reported the mean value of serum protein 6.82±0.81 g/dl in goats infested with lice, which again deviates from the findings of the present study. Diogenes *et al*. (2010) reported 5.22±1.15 g/dl serum protein in *Haemonchus* infected goats which is comparable with finding of the present study. Sakha *et al*. (2009) have reported 78±6.9 g/L of serum protein in Riani goat.

Conclusion

This study provided an idea of serum protein concentrations in healthy goats in the area, which could be used as basis for correct interpretation of acquired values for diagnosis of diseases.

References

Abdallatif, P., Mariam, Y. and Hassan, Y., 2009. *Middle–East J. scient. Res*., **4**: 168–174

Akram, A.M., Ali, R. and Ilyas, A., 2011. *Pakistan J. Zool.*, **43**: 489-495.

Broughton, C.W. and Lecce, J.C., 1970. J. Nutr., **10**: 445-448.

Castro, A., Dhindus, D.S. and Hoversland, A.S., 1977. *Am. J. Vet.*; **38**:665-7.

Devendra, C. and Meleroy, G.B., 1982. *Goat and sheep production in the topics*. Longman

Diogenes, P.V., Pollastry, V.A., Suassuna, Ana, C.D., Ahid and Silvia, M.M., 2010. *J. Anim. Vet. Adv*., **9**:1603-1606.

Fernandez, S.Y., Jesus, E.E.V., Paule, B.J.A., Uzeda, R.S., Almeida, M.A.O. and Guimaraes, J.E., 2006. *Arq. Bras. Med. Vet. Zootec.*, **58**: 279-282.

Iriadam, M., 2007. *Small Rumin. Res*., **73**, 54-57.

Jain, N.C., 1986. *Schalms Veterinary hematology*. Lea and Febiger, Philadelphia, USA.

Josephson, B., Gyllensward, C. and Scand, J., 1957. *Clin. Lab. Invest*., **9**:29.

Karapehlivan, M., Atakisi, E., Atakisi, O., Yucart, R. and Pancarci, S.M., 2007. *Small Rumin. Res.*, **73**, 267-271.

Krajnicakova, M., Kovac, G., Kostecky, M., Valocky, I., Maracek, I., Šutiakova, I. and Lenhardt, L., 2003. *Bull. Vet. Inst. Pulawy*, **47**: 177-182.

Lawal, I.A.K., Esievo, A.N., Bisalla, M. and Ibrahim, N. D. G., 2007. *J. Anim. Vet . Adv*., **6**: 585-590.

Janků, L., Pavlata, L., Mišurová, L., Filípek, J., Pechová, A. and Dvořák, R., 2011. *Acta Vet. Brno*., **80**: 185–190.

Oliveira, A.R. and Penha, A. M., 1978. *Arq. Inst. Biol*., **45**: 191-196.

Otesile, E.B. and Kasali, B.O., 1993. *Vet. Parasit*., **40**: 207-216.

Ruston, B., 1981. *Veterinary laboratory data.* VA publication. Ineresk international. Edinburgh. Protein 100 Min. EH21 TUB.

Sakha, M., Shamesdini, F. and Mohamad-Zadeh, 2009. *The Internet J. Vet. Med.*, **6**: DOI: **10**.5580/1acf

Tanritanir, P., Ozdal, N., Ragbetli, C., Yoruk, I., Ceylan, E. and Deger, S., 2009. *J. Anim. Vet. Adv*., **8**:590-594

Weichselbaum, T.E., 1946. *Am. J. clin. Path*., **16**: 40-48.

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Helminths and Nematode Infection in Norway Rats (*Rattus norvegicus)* Captured From Northern Punjab, Pakistan

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Abstract.- The present study provides information about the diversity and zoonotic nature of helminths and nematodes parasitizing the Norway rats prevalent in warm climate of the Northern Punjab. Eighty five percent of the rats were found to have helminths infection. The following parasites were identified with their respective prevalence and intensity: Nematodes: *Strongyloides* sp. (65%, 12.53), *Trichinella* sp. (10%, 13.5), *Trichostrongylus* sp. (15%, 18), *Nippostrongylus* sp. (45%, 26) and Cestode: *Hymenolepis* sp. (35%, 18.28). Of all examined rats, 29.4% were found to harbor at least one parasite species, with higher prevalence in male hosts. Multiple infections occurred with two, three and up to four species per rat showing different combinations of parasite infections. The prevalence of helminth infection was not influenced by host sex as they were found statistically non-significant ($p>0.05$) and a significant ($p<0.05$) association was observed for host maturity.

Keywords: *Rattus norvegicus*, helminths.

Apart from the colossal economic losses in agriculture due to their pestiferous nature, rats thrive in close association with humans and may impart lethal infections to them (Benigno and Marges, 1978). Rodents are known to be reservoirs of a large number of infectious organisms, many of which are transmitted to humans including plague, typhus fever, rat-bite fever, leptospirosis, and Salmonella food poisoning. Parasitic zoonosis

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includes leishmaniasis, schistosomiasis, echinococcossis, trichinelliosis, toxoplasmosis and angio strongyliasis (Gratz, 1994). The transmission may occur directly, by contaminating food with their urine or feces, or from their bite and indirectly via fleas. Rats have been considered as worst invasive species which cause a huge amount of environmental degradation when introduced in new area (Anonymous, 2013). These impacts have made rats interesting subjects for research. There are numerous reports on rat parasites worldwide (Behnke *et al.,* 2001) and few of them in southern plains of Punjab. However, knowledge on *Rattus norvegicus* parasites is meagre in northern areas of Punjab. The aim of the present work was to investigate the prevalence and intensity of parasitic fauna of *Rattus norvegicus* collected from Northern Punjab.

Materials and methods

The rats were captured from Rawalpindi and Islamabad during July-March of the years 2006-2007. The twin cities have humid subtropical climate having lengthy and very hot summers and small, mild, wet winters. In summer, the maximum temperature can sometimes rise up to 47°C, while it may drop to a minimum of - 4°C in the winter.

In this study thirty six female and twenty four male rats weighted 48 to 392 g were captured, using metal traps with food bait and brought to the laboratory for parasitological examination. They were identified as *Rattus norvegicus* through the measurements of length of head, body, tail and dark brown coloration pattern. The sex was determined by visual inspection of external sexual organs and ages were assigned according to Delattre and Le Louarn (1981) adult rats (≥130 g), juvenile rats (≤100 g). The rats were euthanized and gastrointestinal tracts (GI tracts) removed. The stomach, small intestine and large intestine were examined for helminths under stereomicroscope.

The helminth parasites were collected and preserved in 70% alcohol. Nematodes were cleared in glycerol and mounted temporarily on slides. Three parts of cestodes mouth (scolex), neck and mature gravid segments were cut with a sharp blade and heavily coiled cestodes were fixed under the pressure of glass slides. They were stained with Semichon's carmine, dehydrated in alcohol, cleared in xylene and mounted in DPX (Cable, 1963). All the helminths were identified by using standard keys, cestodes and nematodes of vertebrates given by Yamaguti (1959, 1961).

The parasitic infection was calculated, including prevalence (P), intensity of infection (I) and association among different parasites. Statistical analysis was performed using the statistical software package SPSS v. 17. The analysis was investigated by Chi-square test to evaluate the relations between prevalence of infection, host sex and maturity. The critical probability was set at $P = 0.05$.

Results and discussion

A total of 5 helminth parasite species were identified from two taxonomic groups. The gastrointestinal tracts of *R. norvegicus* was infected with four nematode parasites *Strongyloides* sp., *Trichinella* sp., *Trichostrongylus* sp. and *Nippostrongylus* sp. and one cestode *Hymenolepis* sp. Flukes (Trematoda) were not recorded during the entire study. The overall helminth infection rate was found to be 85%. A total of 1434 nematodes were collected from 51 infected hosts and 384 cestodes from 21 infested hosts. The prevalence of nematodes and cestodes were 78.87% and 21.12% respectively. Intensity of nematodes and cestodes were 28.11 and 18.28, respectively. The higher occurrence of helminths recorded might be because of poor hygienic conditions at warehouses and bakeries. Furthermore the infestation of cestodes and nematodes is also associated with the availability of intermediate hosts (Flynn, 1973; Khan, 1990).

The highest prevalence (65%) was found in *Strongyloides* sp. and lowest prevalence (10%) was in *Trichinella* sp. The prevalence rate of *Trichostrongylus* sp. was 15%, *Nippostrongylus* sp. 45% and *Hymenolepis* sp. 35%. On the other hand, *Nippostrongylus* sp. showed the highest intensity (26) followed by *Hymenolepis* sp. (18.28), *Trichostrongylus* sp. (18), *Trichinella* sp. (13.5) and the lowest was in *Strongyloides* sp. (12.53) (Table I). The plausible explanation for higher infestation might be because of exceptional development pathway called autoinfection in most of the

helminths, which causes an increase in the number of adult worms inside the intestine. The other reason may be *R. norvegicus* habit to live in burrows, which may become contaminated with infective larvae and thus more prone to infection (Zain *et al.*, 2012; Miyazaki, 1991).

Table I.- Overall Prevalence and intensity of helminths of *Rattus norvegicus* (n=60) collected from Rawalpindi and Islamabad.

Parasite	Number of host Infected	Prevalence (%)*	Number of worm collected	Intensity**
Nematodes				
Strongyloides sp.	39	65	489	12.53
Trichinella sp.	6	10	81	13.5
Trichostrongylus sp.	9	15	162	18
Nippostrongylus sp.	27	45	702	26
Cestode				
Hymenolepis sp.	21	35	384	18.28

*number of host infected/number of host examined
**number of worms collected / number of host infected

The results revealed that 29.4% of the infected rats harbored one species of helminth parasite 47% had two, 17.6% had three and 5.9% had four different helminth parasites (Table II). Such associations can occur for ecological/ behavioral reasons, although the host immune system may also be considered a reason for establishment of such types of associations (Kataranovski *et al.*, 2010).

The overall prevalence of GI helminth infection was investigated in relation to sex and maturity (Table III). The significant results were seen between adults and juveniles ($\chi 2 = 6.779$, $p < 0.05$), though non-significant association was observed between prevalence of helminth infection and the host sex ($\chi 2 = 0.196$, $p > 0.05$). But the infection rate in male rats was found to be higher than female rats. The plausible explanation may be the male rats have bigger territories and home range which could predispose exposure to infection than females (Davis *et al.*, 1948; Pisano and Storer, 1948; Calhoun, 1962). The male hormone testosterone may affect the immune function, which could be considered another reason for high prevalence of infection in male rats (Poulin, 1996; Ferrari *et al.*, 2004; Luong *et al.*, 2009). The larger size of males is also considered as target for parasitic infections (Arneberg, 2002).

Table II.- Prevalence and association among helminths of *Rattus norvegicus*. The values in brackets are percentage prevalence of parasites.

Parasites	No. of infected rats		
	Male (n=21)	Female (n=30)	Total (n=51)
Strongyloides sp.	3	6	9 (17.6)
Trichinella sp.	3	0	3 (5.9)
Nippostrongylus sp.	3	0	3 (5.9)
Strongyloides sp. + *Nippostrongylus* sp.	9	3	12 (23.5)
Strongyloides sp. + *Hymenolepis* sp.	0	6	6 (11.7)
Trichinella sp. + *Trichostrongylus* sp	0	3	3 (5.9)
Nippostrongylus sp. + *Hymenolepis* sp.	0	3	3 (5.9)
Strongyloides sp. + *Trichostrongylus* sp. + *Hymenolepis* sp.	0	3	3 (5.9)
Strongyloides sp. + *Nippostrongylus* sp. + *Hymenolepis* sp	3	3	6 (11.7)
Strongyloides sp. + *Trichostrongylus* sp. + *Nippostrongylus* sp. + *Hymenolepis* sp.	0	3	3 (5.9)

Table III.- Prevalence of helminths in relation to sex and maturity of *Rattus norvegicus*.

Rodents sex / Maturity	No. of examined	No. of positive	%
Sex			
Male	24	21	87.5
Female	36	30	83.3
Maturity			
Juvenile	18	12	66.6
Adult	42	39	92.8
Total	60	51	85

Conclusion

In view of the diversity and zoonotic nature of rat parasites, the penurious conditions prevailing in

communities where rats thrive close to humans may readily facilitate parasitic transmission to them. Therefore, it is recommended that rat control measures should be reviewed by the relevant authorities and also need to improve rat-borne disease surveillance programs.

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All the authors declare that there is no conflict of interests.

References

Anonymous, 2013. *100 of the World's Worst Invasive Alien Species*. Global Invasive Species Data base. http://www.issg.org/database/species/search.asp?st=100ss&fr=1& str=&lang=EN. Retrieved 17 February 2013.

Arneberg, P., 2002. *Ecography*, **25:** 88-94.

Behnke, J. M., Bajer, A., Sinski, E. and Wakelin, D., 2001. *Parasitology*, **122:** 39-49.

Benigno, E. and Marges, B., 1978. *Rats and their control.* UP Science Education Center, UP Diliman, Manila.

Cable, R. M., 1963. *An illustrated laboratory manual for Parasitology.* Burgress Publishing Company, Minneapolis, pp. 169.

Calhoun, J. B., 1962. *Publ. Hlth. Serv. Public.*, **1008:** 1-288.

Davis, D. E., Emlen, J.T. and Stokes, A. W., 1948. *J. Mammal.*, **29:** 207-225.

Dehghani, R., Vazirianzadeh, B., Asadi, M.A., Askbari, H. And Moravvej, S.A., 2012. *Pakistan J. Zool.*, **44**: 1721-1726.

Delattre, P. and Le Louarn, H., 1981. *Mammalia*, **45:** 275-288.

Ferrari, N., Cattadori, I. M., Nespereira, J., Rizzoli, J. and Hudson, P. J., 2004. *Apodemus flavicollis. Ecol. Lett.*, **7:** 88-94.

Flynn, R. J., 1973. *Parasites of laboratory animals.* The Iowa State University Press. Ames, Iowa, USA, pp. 155-157.

Gratz, N. G., 1994. In: *Rodent pests and their control*. CAB International, Bristol, pp. 85-108.

Kataranovski, D., Kataranovski, M. and Deljanin, I., 2010. *Arch. Biol. Sci. Belgrade*, **62:** 1091-1099.

Khan, A. A., 1990. *Population density and reproduction of house rats living in some sweets and grocery shops in Faisalabad city*. M.Sc thesis, Department of Zoology and Fisheries, University of Agriculture, Faisalabad, Pakistan.

Luong, L.T., Grear, D.A. and Hudson, P. J., 2009. *Int. J. Parasitol.*, **39:** 1263-1268.

Miyazaki, I., 1991. *An illustrated book of helminth zoonosis.* SEAMIC Publication No. 62, pp. 494. Tokyo, Japan.

Pisano, R. G. and Storer, T. I., 1948. *J. Mammal.*, **29:** 374-383.

Poulin, R., 1996. *Am. Nat.*, **147:** 287-295.

Yamaguti, S., 1959. *Systema helminthum.* Vol. II. *Cestodes of vertebrates*. InterScience Publisher Inc., New York, pp. 860.

Yamaguti, S., 1961. *Systema helminthum.* Vol. III. *Nematodes of vertebrates*. InterScience Publisher Inc, New York, pp. 1261.

Zain, S. N. M., Behnke, J. M. and Lewis, J. W., 2012. *J. Parasite Vect.*, **5:** 47.

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Effect of Malathion Applications at Different Times on the Alfalfa Weevil Population, Natural Enemies, Forage Yield and Protein Content

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Abstract.- The aim of this study was to determine the effects of insecticides applied at different times against alfalfa weevil, *Hypera postica* Gyllenhal (Coleoptera: Curculionidae) on pests, natural enemies, forage yield and protein. The study was based on five treatments, including four different spray times (4, 11, 18 and 25 May 2008) and a control, each with four replications and conducted in 18 m^2 (3mx6m) plots. A malathion based insecticide (Malathion 65% EC) was used against alfalfa weevil. All of the insecticide treatments reduced the populations of alfalfa weevils, aphids and natural enemies significantly. Compared to the control, the lowest weevil populations were observed in the plots treated on May 4^{th}. Correspondingly, the forage yield and the crude protein content increased 73.88% and 51.59%, respectively. Overall, results indicated that timely insecticide applications were more effective in chemical control of alfalfa weevil and increase of forage yield and quality.

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Key words: Alfalfa, alfalfa weevil, insecticide spray time, malathion, crop yield, protein content of crop.

In order to achieve the desired livestock production, high quality and quantity of forage is needed. Alfalfa with its wide adaptation capacity is the most widely grown forage clover on the Earth (Erişen, 2005). Alfalfa weevil, *Hypera postica* Gyllenhal (Coleoptera: Curculionidae) is one of the most devastating pests of alfalfa, decreasing quality and quantity of forage production. Chemical control is commonly applied to protect alfalfa production from this pest. Many studies have been carried out over the years to find the most effective insecticide and spray techniques. Cothran *et al.* (1967) tested twenty-five experimental and registered insecticides in different combinations and doses against alfalfa weevil in field conditions and found that among these insecticides those applied before spring in which the peak of the larvae occur, only Azinphosmethyl yielded desired results for the timely prevention of the damage from this pest. They also indicated that the insecticides used during this period were effective in reducing the pest populations between 35% and 75%. Wilson and Armbrust (1968) studied combinations of various insecticides in laboratory and field experiments to determine their effects on alfalfa weevil damaging first cut under both good and bad weather conditions. They showed that methyl parathion and a combination of malathion and methoxychlor were effective, however, under good weather conditions Phorate provided better control of alfalfa weevils. Dondale (1972) tested carbofuran against alfalfa weevil and demonstrated that it reduced population of the pest from 46-100/m^2 to 6-31/m^2. Jennings and Nelson (2002) controlled alfalfa weevils by applying chlorpyrifos or carbofuran. Kamangar and Habibi (2006) found that neem and *Bt* based insecticides were not effective, while a quinalphos based insecticide reduced the weevil population.

Our study was aimed to search the rationality of chemical control of alfalfa weevil in a certain period and demonstrate the importance of timely insecticide applications by analyzing their effects on the weevils, pea aphids, *Acyrtosiphon pisum* (Homoptera: Aphididae), some natural enemies, Coccinellidae (Coleoptera) and Nabidae (Hemiptera) that are commonly seen in alfalfa fields, protein content and yield of the forage.

Materials and methods

This study was carried out in 2008 in a part of a commercial field planted in 2006 with Planetary seed variety at the rate of 2.5 kg per decare and 2.5-3 cm depth with a row spacing of 12 cm. The experiment was based on a randomized block design with five treatments each with four replications. The study plots were 18 m^2 (3mx6m) in size with 2 m spacing between each plot. The treatments consisted of four different spray times (May 4, 11, 18 and 25) of the insecticide (malathion 65% EC) and a control that did not receive any insecticide. Only those plots that were specifically associated with treatment date were sprayed, while the others received no insecticide spray.

All the weeds were removed from the plots by hand-weeding throughout the season. The field was scouted frequently for the alfalfa weevil in order to determine the spray time. The first insecticide was applied on May 4 at the rate of 170,8 ml/da, within 2 days after appearance of alfalfa weevil larvae on the plants, while the other three applications were performed at one week intervals. The amount of spray was determined by calibrating with water before each spray and the rate of insecticide was adjusted proportionally to 187,5 ml/da on May 11, 213,8 ml/da on May 18 and 222,8 ml/da on May 25. The insecticide was sprayed using a back pack sprayer (Taşar model of S. 1, 3.5 Bar pump-type) with a 20 l tank capacity.

Alfalfa weevils, *H. postica* Gyllenhal (Coleoptera: Curculionidae), pea aphid, *A. pisum* (Homoptera: Aphididae) and the predators, Coccinellidae (Coleoptera) and Nabidae (Hemiptera) were sampled using a 35 cm wide standard sweep-net (10 sweeps/plot) within two days after each spray and at weekly intervals thereafter until the harvest (first cut) and their numbers were recorded to determine the impacts of the treatments on these groups. The height of 10 plants per plot was measured before each spray and the amount of spray was recorded for each treatment in order to determine the differences between the

amount of spray used as the foliage increased at different periods. The effects of treatments on alfalfa yield was determined by taking wet weight of plant samples cut from 2 m^2 area in each plot and also taking dry weight of 500 gram of these samples, which were dried in an oven at 78^0C for 48 hours. In order to examine the effects of treatments on the protein content of alfalfa, the dry samples were processed through the mill and 1 gram samples were taken from the processed alfalfa and weighed in Kjehldahl tubes. According to the Weende method, a teaspoonful of catalyst and 20 cc H_2SO_4 were added to each sample. Then the samples were burned, distilled and titrated with N/7 sulfuric acid. Spent sulfuric acid was read in digital burette and the crude protein was calculated as % value (Akkılıç and Sürmen, 1979). These parameters were analyzed by F-test using JMP (SAS Institute, 2002) statistical software package.

Results

The results indicated that the amount of spray used increased in parallel to plant growth (Table I). The amounts of water needed at different treatment periods were 138.88 l/da on May 4, 152.77 l/da on May 11, 173.61 l/da on May 18, and 180.55 l/da on May 25, which indicated that the amount of spray needed increased by 10% on May 11 compared to May 4, while this increase appeared to be 25% on May 18, and 30% on May 25.

Table I.- Plant heights and spray amounts at different treatment periods.

Treatment dates	Plant height (cm)	Spray amount (l/da)
May 4	38.65	138.88
May 11	44.25	152.77
May 18	50.27	172.61
May 25	52.47	180.55

The numbers of weevil larvae were significantly different between the treatments (F = 89.84, df= 4, $p< 0.0001$), where all the sprays at different periods led to decreases in the populations of larvae (Table II). The lowest larval population occurred in the plots sprayed on May 4, while the highest larval population was observed in the control plots. Compared with the control, the insecticide treatment on May 4 decreased the number of larvae by 90.19%, however the applications on May 11, 18 and 25 led to decreases by 81.89%, 61.18% and 36.64%, respectively.

The first adult individuals were seen on June 3. The treatments reduced their populations significantly as well (F = 14.16, df= 4, $p = 0.0002$) (Table II). Compared to the other insecticide received plots, the highest adult population was recorded in the plots treated on May 4, where the treatment lowered their numbers only by 33.99%. The adult numbers were 71.95% lower when plots were treated on May 11, 77.51% on May 18, and 80.29% on 25 May.

Pea aphid, *A. pisum* (Homoptera: Aphididae) was one of the most common pest species in the treatment plots and they were significantly affected (F = 7.20, df = 4, $P = 0.0034$) by the insecticides (Table II). Compared with the control, the insecticide treatment on May 4, 11, 18 and 25 reduced the aphid populations by 49.05%, 56.60%, 41.50% and 68.86% respectively.

In general, the populations of natural enemies were low in the treatment plots and they were mostly the members of Coccinellidae (Coleoptera) and Nabidae (Hemiptera). All the insecticide treatments had significant adverse impacts on these predators (F = 24.96, df= 4, $p<0.0001$) (Table II). The effects of early insecticide applications were more severe and reduced the total numbers of Coccinellidae and Nabidae by 84.94% on May 4 and 87.09% on May 11. The later treatments caused 55.91% decrease on May 18 and 31.18% on May 25.

Each of the insecticide application protected the yield from weevil damage significantly (F = 143.15, df = 4, $p <0.0001$) (Table III). Compared to the control, the wet weight of alfalfa increased by 53.41% (1251.85 kg/da) in the plots treated with insecticide on May 4, followed by an increase of 33.92% (1092.85 kg/da) in the plots treated on May 11, 18.35% (965.75 kg/da) on May 18 and 11.31% (908.35 kg/da) on May 25.

Like wet weight, dry weights of the alfalfa were significantly different (F = 220.94, df = 4, $p<0.0001$) (Table III). The amount of yield removed from control plots was 319.21 kg/da. The

Table II.- Seasonal abundances (mean ± SE) of alfalfa weevils, *Hypera postica* Gyllenhal (Coleoptera: Curculionidae) larvae and adults, pea aphid, *Acyrtosiphon pisum* (Homoptera: Aphididae) and the predators, Coccinellidae (Coleoptera) and Nabidae (Hemiptera) collected from the treatment plots within two days after each spray and at weekly intervals thereafter until the harvest (first cut).

Treatment dates	Larvae	Adult	Aphids	Predators
May 4	21.83 ± 6,69 a*	4.99 ± 2,12 a*	5.4 ± 2,03 a*	3.5 ± 3,38 a*
May 11	40.33 ± 13,88 a	2.12 ± 0,23 b	4.6 ± 2,01 a	3.0 ± 2,78 a
May 18	86.45 ± 11,35 b	1.70 ± 0,62 b	6.2 ± 1,99 a	16.0 ± 2,85 b
May 25	141.12 ± 4,55 c	1.49 ± 0,25 b	3.3 ±1,19 a	10.25± 2,22 c
Control	222.74 ± 28,21 d	7.56 ±1,68 c	10.6 ± 1,85 b	23.25 ± 3,88 d

* Within a column, numbers indicated by different letters are significantly different at $p < 0.05$.

Table III.- Yield (kg/da) and protein content (mean ± SE) of alfalfa plants obtained from treatment plots.

Treatment dates	Wet weight (kg/da)	Dry weight (kg/da)	Crude protein (%)
May 4	1251.85 ± 37,13 a*	555.07 ± 17,19 a*	21.8 ± 0,33 a*
May 11	1092.85 ± 18,59 b	478.01 ± 8,63 b	19.8 ± 0,27 b
May 18	965.75 ± 24,28 c	420.10 ± 9,95 c	16.9 ± 0,32 c
May 25	908.35 ± 27,44 d	377.32 ± 9,45 d	15.5 ± 0,18 d
Control	816.00 ± 12,36 e	319.21 ± 6,28 e	14.4 ± 0,47 e

* Within a column, numbers indicated by different letters are significantly different at $p < 0.05$.

insecticides led to increase of yield by 73.88% (555.07 kg/da) when treated on May 4, 49.74% (478.01 kg/da) on May 11, 31.6% (420.10 kg/da) on May 18 and 18.20% (377.32 kg/da) on May 25. The results indicated that the amount of yield lost to the weevil increased as the treatments were delayed.

Eventually, the insecticide treatments affected the crude protein of forage significantly ($F = 274.64$, $df = 4$, $p < 0.0001$). The crude protein content of the forage obtained from plants treated on May 4, 11, 18 and 25 were 51.59%, 37.5%, 17.36% and 7.6% higher than those obtained from the control plants.

Discussion

The use of the insecticide (Malathion) in all of the periods provided significant reduction in the populations of alfalfa weevil. Dorsey (1966) also showed that spring (April) applications of Malathion could control alfalfa weevil. Yardım *et al.* (2001) found that the application of Malathion may cause a 87.4% decrease in the density of alfalfa weevil and emphasized upon the importance of early applications against alfalfa weevil. Risk of major damage increased as delay of spray was realized.

Aphids and the predators were also significantly affected from insecticide applications. Their populations were higher in the control plots than those in the other insecticide received plots. The numbers of predators were lower in the plots treated earlier compared to the others. Early applications of insecticides provided better increases in the forage yield and protein. The leaves of alfalfa contain the highest content of crude protein (Ergun *et al.*, 2002). Probably because alfalfa weevil larvae and adults fed on and damaged the leaves of the plants severely, the crude protein content of the feed decreased significantly as the treatments were delayed.

The results of this study clearly indicated that the timely insecticide application against alfalfa weevil is critical to protect the yield and quality of forage. Also, because less amount of insecticide is needed to cover the foliage and less labor are required at early stage of plant growth, early insecticide applications may cost less than later applications and may cause less environmental pollution.

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References

Akkılıç, M. and Sürmen, S., 1979. *Feed materials and animal feeding*. Ankara Üniv. Vet. Fak., Yay. No: 357, Ankara. (in Turkish)

Cothran, W.R., Armbrust, E.J., Horn, D.J. and Gyrisco, G.G., 1967. *J. econ. Ent.*, **60**: 1151-1154.

Dondale, C. D., 1972. *Can. Entomol.*, **104**: 1433-1437.

Dorsey, C. K., 1966. *J. econ. Ent.*, **59**: 735-738.

Ergün, A., Tuncer, Ş.D., Çolpan, I., Yalçın, S., Yıldız, G., Küçükersan, M. K., Küçükersan, S. and Şehu, A., 2002. *Feeds, feed hygiene and technology*. A.Ü.Veteriner Fak. Hayvan Besleme ve Beslenme Hastalıkları Anabilim Dalı,1-368 Pozitif Matbaacılık, Ankara. (in Turkish)

Erişen, S., 2005. *Tarım Bilimleri Dergisi*, **11**: 311-315. (in Turkish)

Jennings, J.A. and Nelson, C.J., 2002. *Agron. J.*, **94**: 786-791.

Kamangar, S. and Habibi, J., 2006. *J. entomol. Soc. Iran*, **26**: 1-12.

SAS Institute Inc., 2002. *JMP user's guide statistics*, Version 5.0. SAS Inst., Cary, NC.

Wilson, C. M. and Armbrust, E. J., 1968. *J. econ. Ent.*, **61**: 1201-1203.

Yardım, E.N., Özgen, İ. and Kulaz, H., 2001. *Meded. Rijksuniv. Gent. Fak. Landbouwkd. Toegep. Biol. Wet.*, **66**: 518–524.

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Biodiversiy of Ground Beetles (Coleoptera: Carabidae) From District Poonch, Azad Kashmir

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Abstract.- The present study was conducted to explore the fauna and biodiversity of family Carabidae (Coleoptera) from ten different localities of district Poonch, Pakistan during the year 2009- 2010. Diversity, abundance, richness and evenness were calculated. A total of 288 specimens of family Carabidae (Coleoptera) were collected. Five species belonging to 3 sub-families (Licininae, Carabinae, Brachininae) in three genera were identified. *Carabus caschmirensis* was the most abundant species, followed by *Chlaenius quadricolar***,** *Pheropsophus sobrinus***,** *Chlaenius laticollis* and then *Chlaenius hamifer*. Diversity abundance richness and evenness were calculated. The highest abundance, richness and diversity of the family Carabidae was recorded from Rawalakot and the lowest diversity was calculated from Alisojal. The lowest abundance was recorded from Datot. The highest evenness of the family Carabidae was recorded from Rawalakot and lowest was recorded from Alisojal.

Key Words: Biodiversity, abundance, ground beetle, diversity indices.

The family Carabidae is one of the larger groups of beetles with estimated 40,000 species throughout the world (Ball and Erwin, 1969); out of these, 32,500 species have been described (Lorenz, 2005). Ground beetles habitat is a permanent area of vegetated land, field edges, marginal lands or selected areas within a crop field. Members of the family Carabidae are in general of little importance

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as pests, some species are known to feed on seeds of plants but the damage done is usually not significant (Thiele, 1977). The majority of species have been observed as primarily predatory feeding on other insects and related organisms; they capture and consume a wide assortment of soil dwelling insects, including caterpillars, wireworms, maggots, ants, aphids and slug (Kromp, 1999). A few species have also been observed to detect chemical cues from springtails, mollusks and aphids (Lovei and Sunderland, 1996) and to effectively control slugs in greenhouses (Kromp, 1999). Several ground beetle species are phytophagous and feed on the seeds of troublesome weeds species including common ragweed, common lamb's quarters and giant foxtail (Lundgren, 2005) and thus help to regulate weed populations; their weed predation is largely under estimated (Liebman and Gallandt, 1997; Tooley and Brust, 2002). Because of their significance as bioindicators and their role as agents of biological control of agriculture pests, carabids have been extensively used to assess the impact of soil management on ground-dwelling arthropods in many crops (Minarro and Dapena, 2003). In addition, carabid beetles are good indicators of environmental change (Thiele, 1977; Magura *et al.*, 2000; Melnychuk *et al.*, 2003) and are useful for examining geographical changes as they are highly sensitive to the effect of landscape changes, such as fragmentation (Niemela *et al*., 2000; Magura *et al*., 2001). Some recent studies revealed that physiology of carabid beetles was adversely affected by the accumulation of toxic metals (Lagisz *et al*., 2002; Stone *et al*., 2002). Accumulation of copper in carabid species *Poecilus cupreus* at larval stage changed the locomotory behavior due to developmental damage in larval stages (Bayley *et al*., 2001). Kramarz and Laskowski (1997) reported that *Poecilus cupreus* fecundity was adversely affected by zinc treatment.

Carabids are taxonomically well known, with relatively stable systamatics and their ecology has been widely studied due to their sensitivity to environment, and productive role in agriculture (Lovei and Sunderland, 1996). The taxonomy of the family Carabidae is based exclusively on adults (Thompson, 1977), however carabid larvae possess good structural features and could be used to test phylogenetic hypotheses based study of adults (Goulet, 1977).

Carabidae can be very diverse in natural and agricultural environments. Nikolai and Lyubomir (2006) presented the distribution of 348 species of ground beetles belonging to 83 genera in south Dobrudzha, Bulgaria. Ghahari *et al.* (2010) listed 43 species of carabid beetle from apple orchards and alfalfa fields of Iran. Sakine and Martin (2009) recorded 57 species of ground beetles form crops in Turkey.

Beside the work done by Kamaluddin and Hashmi (1999) and Hashmi *et al*. (2005), Carabidae have been largely neglected in Pakistan and Azad Jammu & Kashmir.

Keeping in view a great importance of carabid beetles as bioindicators, this study was designed to explore the fauna, distribution and diversity of the family Carabidae in district Poonch of Azad Kashmir during 2009-2010.

Materials and methods

Azad Kashmir lies between longitude 73˚-75˚and latitude of 33˚–36˚ and it comprises of an area of 13397 Km^2 and total of 13% area is under cultivation. Average rainfall is 1400–1800mm annually. The topography is mainly hilly and mountainous with valleys and plains in some places and rich in diverse fauna and flora. Carabid beetles were collected from ten different localities of district Poonch. The localities were Datot (33.7°N, 73.3°E, 6254ft), Topa (33.7°N, 73.9°E, 6471ft), Singola (33.9°N, 73.8°E, 5950ft), Khai-Gala (33.4°N, 73.9°E, 5761ft), Bunjosa (33.0°N, 73.9°E, 5873ft), Hussainkot (33.8°N, 73.7°E, 6699ft), Rawalakot (33.8°N, 73.8°E, 5345ft), Alisojal (33.0°N, 73.9°E, 5524ft), Hajira (33.6°N, 73.3°E, 3076ft) and Abasspur (33.6°N, 73.0°E, 4261ft). The localities were selected depending on the road links available and were at least 10-15 Km^2 apart from each other. The maximum area of each locality was covered during the sampling from mid April 2009 to mid October 2010. The localities were visited fortnightly from 9:30 pm to 4:30 am.

Carabid beetles were collected with the help of pitfall traps. Five pitfall traps were set up at each locality at appropriate distance but distances was not constant due to hilly topography of area. Each trap

was a plastic cup with 25% ethylene glycol. Traps were set up for 15 days per month for 3 month during 2009 and 2010. Pitfall traps were visited fortnightly. The collection, stretching, pinning, labeling and preservation methods for the study of Carabidae were followed after Richard (1983).

The collected specimens of Carabidae were identified to the species level by using the available keys (Andrews, 1929; Choate, 2001). The relative abundance of the sub-families and species of the family Carabidae was calculated by using the formula;

$$R=n_i/N$$

where, "R" is the relative abundance(%), "n_i" is the number of individuals in "ith" species and "N" is the total number of individuals in the sample.

The diversity was calculated using Shannon-Weiner's diversity index (Shannon and Weiner, 1963). The actual form of the index is:

$$H'= -\sum (p_i) \log 2p_i),$$

where, "p_i" is the proportion within the sample of the number of the individuals of "ith" species But the form of the index used in the present study was:

$$H'=C \{\log_{10}N-1/N\sum (n_r \log_{10} n_r)\}$$

Where "N" is the total number of the individuals, "n_r" is the rank abundance in "ith" species "C" is the conversion factor from $\log_2$ to $\log_{10}$.

The richness was calculated by using Margalef's index (Margalef, 1969).

$$d = S - 1 / \log_e N$$

Where "S" is the total number of species and "N" is the total number of individuals.

The evenness was calculated using RI index (Nakamura and Toshima, 1995).

The form of Nakamura's RI index used in this study was:

$$RI = \sum_{r=i}^{S} R_i / S (M-1)$$

Where "S" is the number of investigated species of insects, "M" is the number of rank of abundance (0, 1, 2, 3,… M – 1) and "R_i" is the rank value of "$_i$th" species in the sample.

Results and discussion

A total of 288 specimens of family Carabidae were collected. Five species belonging to 3 sub-families in 3 genera were identified. These sub-families are Licininae, Carabinae, Brachininae and the species are *Carabus caschmirensis, Chlaenius quadricolar***,** *Pheropsophus sobrinus***,** *Chlaenius laticollis* and *Chlaenius hamifer*. *Carabus cashmirensis* was the most abundant specie. It was followed by *Chlaenius quadricolar***,** *Pheropsophus sobrinus***,** *Chlaenius laticollis***,** and then *Chlenius hamifer*. The maximum abundance of family Carabidae was recorded from Rawalakot and the minimum abundance was recorded from Datot (Table I).

The relative abundance of the sub-families and species of Carabidae from each locality is given in Table I. *Carabus caschmirensis* yielded the highest and *Chlaenius hamifer* yielded the lowest percentage. Calculated values of diversity indices from each locality are given in Table II. The values of Shannon-Wiener's diversity index of the family Carabidae collected from localities of district Poonch ranged between 0.86 (Datot) to 2.25 (Rawalakot) (Table II). The calculated values of the Margalef's richness index of the family Carabidae collected from district Poonch ranged between 0.64 (Alisojal) to 1.29 (Rawalakot) (Table II)

The evenness of the species was measured by Nakamura and Toshima's diversity index (Nakamura and Toshima, 1995). The calculated values ranged between 0 (Alisojal) to 0.833 (Singola, Khai-Gala and Rawalakot) (Table II).

Subfamily Carabinae is distributed through India, Pakistan, Russia, Europe, south and Asia (Andrews, 1929). *Carabus caschmirensis* distributed throughout mountains of Pir Panjal 5000-6000ft, Punjab: Murree, Simla Kulu, and Hamalaya 7000ft (Andrews, 1929). This species is found to be active during night and in the moist habitats. Subfamily Licininae is distributed in Palearctic region (Ghahari *et al*., 2009) and the species *Chlaenius quadricolar, Chlaenius laticollis* and *Chlaenius hamifer* have not been reported from this region (Azad Kashmir) prior to this study. These species are found in soil and on dead decaying matter. Subfamily Brachininae distributed throughout Middle Asia, Russia, Ukraine, Moldova,

Table I.- Species of family Carabidae and their relative abundance (%) collected from different localities of District Poonch.

Name of species	Abundance	Datot	Topa	Singola	Khai-gala	Banjosa	Hussainkot	Rawalakot	Alisojal	Hajira	Abbaspur
Carabus caschmirensis	108(37.5%)	4(1.38%)	12(4.16%)	18(6.25%)	8 (2.77%)	9(3.47%)	13 (4.16%)	12 (4.16%)	14(4.86%)	4(1.38%)	14 (4.86%)
Chlaenius hamifer	24 (8.33%)	4(1.38%)	2 (0.69%)	4(1.38%)	2 (0.69%)	2(0.69%)	2 (0.69%)	-	-	4(1.38%)	4 (1.38%)
Chlaenius laticollis	36(12.25%)	6(2.08%)	4 (1.38%)	-	-	4(1.38%)	4 (1.38%)	4 (1.38%)	4 (1.38%)	4(1.38%)	6
Chlaenius quadricolar	80(27.77%)	4(1.38%)	11(3.47%)	8 (2.77%)	12 (4.16%)	9(3.47%)	4 (1.38%)	18 (6.25%)	4 (1.38%)	6(2.08%)	4 (1.38%)
Pheropsophs sobrinus	40(13.88%)	4(1.38%)	2 (0.69%)	2 (0.69%)	6 (2.08%)	8(2.77%)	4 (1.38%)	6 (2.08%)	-	6(2.08%)	2 (0.69%)
Total no of Individuals	288	22	32	32	28	32	27	40	22	25	30
No of species		5	5	4	4	5	5	4	3	5	5

Caucasia Europe, Mountains of South East Middle Asia, Turkey, Moldova, Ukraine, Armenia, Kazakhstan, Tajikistan and Iran (Ghahari *et al*., 2009) and there is no record of *Pheropsophus sobrinus* from Azad Kashmir. This species is found active during night. Due to granivory and ant predation habit of carabid beetles their richness, diversity, and abundance were found associated with availability of food (Tooley and Brust, 2002; Ellsbury *et al*., 1998). The calculated abundance and diversity values showed some fluctuations probably reflecting because their great concern with environmental changes and anthropogenic activities. The habitat of beetles in the study area is under anthropogenic stress and rapid deforestation is occurring due to which level of abundance and diversity are fluctuating and are not very high.

Table II.- Calculated values of diversity indices from different localities of district Poonch.

Locality	Name of Locality	Shanno-Wiener's Index (H')	Nakamura's Index (RI)
Abbaspur	1.50	1.17	0.75
Alisojal	1.20	0.64	-
Banjosa	1.47	1.15	0.75
Datot	0.86	1.29	0.75
Hajira	0.99	1.25	0.75
Hussainkot	1.21	1.21	0.75
Khai-gala	1.38	0.90	0.83
Rawalakot	2.25	0.81	0.83
Singola	1.86	0.86	0.83
Topa	1.50	1.15	0.75

This type of study was the first study in the district Poonch. It is difficult to make conclusion about the fauna that it is at the brink of annihilation.

Therefore, it is suggested that continues monitoring of the area of the present study should be done in the coming years to perceive the changes in the diversity of the carabid beetles. Continuous monitoring and comparing the data collected of every year can observe the changes in the biodiversity.

References

Agvin, S.S. and Emre, I., 2010. *Pakistan J. Zool.,* **42**: 23-32.

Andrews, H. E., 1929. *The fauna of British India. Coleoptera (Carabidae).* Vol. (i,ii): 431pp.

Ball, G. E. and Erwin, T. L., 1969. *Can. J. Zool*., **47**: 877-907.

Bayley, M., Baatrup, E., Heimbach, U. and Bjerregaard, P., 1995. *Ecotoxicol. Environ. Saf*., **32**: 166-170.

Choate, P. M., 2001. *Manual for the identification of ground beetles (Coleoptera: Carabidae) (including tiger beetles) of Florida*. Depart. Entomol., pp. 1-40.

Ellsbury, M. M., Janine, E. P., Frank, F., David, W.W., Sharon, A. C. and Walter, E. R. 1998. *Annl. entomol. Soc. Am*., **91**: 619 -625.

Ghahari, H., Memis, K., Najmeh, S., Hadi, O., Mohammad, H. and Sohrab, I., 2009. *Mun. Ent. Zool*., **4**: 436-450.

Ghahari, H., Avgin, S.S. and Ostovan, H., 2010. *Turke Entomol. Derg*., **34**: 179-195.

Goulet, H., 1977. *Contributions of characters of larvae to systematics of Carabidae. Carabid beetles, their evolution, natural history, and classification. Junk*, The Hague, pp. 205-208.

Hance, T., 2002. In: *The agroecology of carabid beetles*, Intercept Ltd., Andover, pp. 231-249.

Hashmi, S.N., Kamaluddin, S. and Hussain, S. Z., 2005. *Int. J. Biol. Biotechnol*., **2**:259-272.

Kamaluddin, S. and Hashmi, S.N., 1997. *Proc. Pakistan Congr. Zool*., **17**: 73-79.

Khan, D. A., 2011. *Agriculture biodiversity a dilemma in future crop production.* www.agrilive.com.pk.

Kramarz, P. and Laskowski, R., 1997. *Bull. environ.Contam. Toxicol*., **59**: 525–530.

Kromp, B., 1999. *Agric. Ecosyst. Environ*., **74**: 187-228.

Lagisz, M., Kramarz, P., Laskowski, R. and Tobor, M., 2002. *Bull. environ. Contam. Toxicol*., **69**: 243–249.

Landres, P.B., Verner, J. and Thomas, J. W., 1988. *Conserv. Biol*., **2**: 316–328.

Liebman, M. and Gallandt, E.R., 1997. In: *Ecology in agriculture.* Academic Press, San Diego.

Lorenz, W., 2005. *Nomina Carabidarum- A directory of scientific names of the ground beetles (Insecta, Coleoptera "Geadephaga": Trachypachidae and Carabidae incl. Paussinae, Cicindellidae, Rhysodinae).* Lornez Publishing, Cornell University, 2nd ed. 993 pp.

Lovei, G.L. and Sunderland, K.D., 1996. *Annu. Rev. Ent.*, **41**: 231–256.

Lundgren, J. G., 2005. *Am. Entomol*., **51**:224-226.

Magura, T., Tothmeresz, B. and Bordan, Z., 2000. *Biol. Conserv*., **93**: 95-102.

Magura, T., Kodobocz, V. and Tothmeresz, B., 2001. *J. Biogeog*., **28**: 129-138.

Margalef, S. N., 1969. *Brookhaven Symp. Biol*., **22**: 25-37.

Melnychuk, N.A., Olfert, O., Youngs, B. and Gillott, C., 2003. *Agric. Ecosyst. Environ.*, **95**: 69-72.

Minarro, M. and Dapena, E., 2003. *Appl. Soil Ecol*., 23: 111–117.

Nakamura, H. and Toshima, H., 1995. *Environ. Ent. Zool*., **10**:143-159.

Niemela, J., Kotze, J., Ashworth, A., Brandmayr, P., Desender, K., New, T., Penev, L., Samways, M. and Spence, J., 2000. *J. Insect Conserv*., **4**: 3-9.

Nikolai, D.K. and Lyubomir, P.D., 2006. *Acta zool. Bulg*., **58**: 147-180.

Pielou, E. C., 1977. *J. theor. Biol*., **10**: 370-380.

Popovic, A. and Strbac, P., 2010. *J. Cent. Eur. Agric*., **11**: 423-432.

Rainio, J. and Niemela, J., 2003. *Biodivers. Conserv*., 12: 487–506.

Richard, E. W., 1983. *Peterson: a field guide to beetles*. Houhton Mifflin Company, New York, pp. 433.

Stone, D., Jepson, P. And Laskowski, R., 2002. *Comp. Biochem. Physiol*., **132**: 105–112.

Sakine, S. and Martin, L. L., 2009. *Proc. entomol. Soc. Washington*, **111**: 326-334.

Shannon, E.R. and Wiener, W., 1963. *The mathematical theory of communication.* University of Illinois Press, Urbana, Illinois, pp. 117.

Thiele, H.U., 1977. *Carabid beetles in their environments. A study on habitat selection by adaptation in physiology and behaviour.* Springer-Verlag, Berlin, Germany, pp. 369.

Thompson, R.G., 1977. *Proc. biol. Soc. Washington*, **90**: 99-107.

Tooley, J. and Brust, G.E., 2002. In: *The agroecology of carabid beetles.* Intercept Ltd. Andover, pp. 12.

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Motorway (M 2): A Threat to the Wild Animals

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Abstract.- Present study was aimed at recording the number of animals killed on the Lahore-Islamabad Motorway (M 2) due to accidents on its section from Sheikupura interchange to Sial Morr interchange. Data for a period of two years were collected from May 2010 to April 2012. During the study period 392 animals were found killed on this section of motorway, out of which 71.94% were mammals (dogs, jackals, wild cats and wolves). Highest mortalities were observed during the months of December, January and February in each year. Month-wise comparison of two years data revealed no difference. Mortality data were negatively correlated with the temperature. It is recommended that wildlife protection agencies and media should play their role to highlight the issue to save wildlife animals. Authorities concerning the motorways should also ensure that fences on both sides of the motorway were properly managed to restrict the entry of animals on the motorway.

Keywords: Wildlife, Motorway, temperature and mortality

We (authors) are working at the Department of Biological Sciences, University of Sargodha, Pakistan. Permanent residence of authors is in Lahore. Every Monday morning (06:30-07:30 am) we start our journey from Lahore to Sargodha via Motorway on our personal car. We come back to Lahore through the same route every Friday evening (after 05:00 pm). During our journey we observed that large number of wild animals is being killed due to collision with vehicles moving on the motorway (Sheikupura interchange to Sial Morr interchange, Sargodha). We started collecting mortality data during our each trip from Lahore to Sargodha.

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Data collection from Sargodha to Lahore was not possible as we would come back at night.

Lahore-Islamabad motorway is one of the major means of transportation between these two cities in Pakistan. Being a permanent physical infrastructure it acts as a barrier for the animals and affects the wildlife and its habitat adversely (Baskaran and Boominathan, 2010). Motorways not only act as movement barrier for wild animals but they also are recognized as major contributors to the global biodiversity crisis for many taxa (Coffin, 2007; Jaeger *et al.*, 2005). Large number of animals is killed every year due their collisions with motor vehicles on motorways (Eigenbroad *et al.*, 2009). Although there are compensatory measures such as protective fences to prevent the entry of bigger animals on the motorways there is no proper management to stop the small animals. The road traffic along with other factors has a strong impact on the population of small sized animals (Barbara and Jones, 2009; Rais *et al.*, 2011).

Although mortality of wild animals on the motorways is a concern but few studies have been conducted to highlight this issue. Present study was designed to record the data of vertebrate mortality caused by the direct collision with the vehicles on 92 km motorway section (from Sheikupura interchange to Sial Morr interchange, Sargodha). The outcome of the study would be helpful for the wildlife protection agencies in the area. Furthermore, the information will also be valued for the motorway authorities to make necessary arrangements to restrict the entry of wild animals on the motorway.

Methodology

Study was conducted from May, 2010 through April, 2012. Data on animal mortality caused by automobile traffic on 92 km section of the motorway (Skeikupura interchange to Sial Morr interchange) were documented. Data on dead animals were collected four times in a month. Only the data on mammals, reptiles and birds were recorded. We were not able to collect the data on amphibians. Kolmogorov-Smirnov test was used to analyze the normal distribution of the data. Monthly data for two years were compared using Mann-Whitney U test. Data of temperature and humidity

were collected using environmental data recorder Kestrel 4500 installed at 45 km and 85Km from Lahore to Sial Morr on Motorway M2. Pearson's correlation was used to find out the relationship of mortality data with temperature. All statistical analyses were performed using SPSS (version 16) and Minitab (version 13.2).

Results

In total, 392 animals were found killed during the study period of two years (Table I). Of these, the number of mammals, reptiles and birds was 282 (71.94%), 74 (18.87%) and 36 (9.18%), respectively. Among mammals highest number was represented by stray dogs (61.70%), followed by jackals *Canis aureus* (17.02%), wild cats *Felis lybica* (8.86%) and wolves *Canis lupus* (6.02%). Other mammals were not identifiable. Among reptiles, monitor lizard (*Varanus monitor*) constituted the largest category followed by tortoises of unidentified species, while among birds the dominant bird species was common Myna (*Acridotheres tristis*). There was variation in the mortality data but during the months of December, January and February the rate of mortality was higher (Fig. 1). Month-wise comparison of two-year data revealed no significant difference (Mann-Whitney U test, Z = 0.00; P = 1.00). Negative correlation was observed between temperature and mortality data in both years (r = - 0.95; P = 0.003 for 2010-2011 and r = - 0.93, P < 0.01 for 2011-2012).

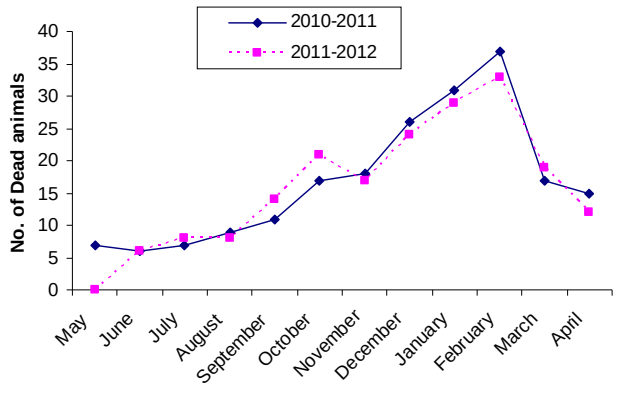

Fig. 1. Mortality data of wild animals collected from motorway (M2) section between Sheikupura to Sial Morr.

Table I.- Number of reptiles, birds and mammals found killed on the motorway during study period of two years

Animals	2010-2011	2011-2012	Total
Mammals			
Stray dogs	92	82	174
Jackals	21	27	48
Wild cats	14	11	25
Wolves	8	9	17
Unidentified	8	10	18
Total	143	139	282
Reptiles			
Monitor lizards	16	12	28
Snakes	9	11	20
Tortoises	5	4	9
Unidentified	9	8	17
Total	39	35	74
Birds			
Common Myna	8	9	17
Crows	4	5	9
Unidentified	7	3	10
Total	19	17	36

Discussion

The results clearly showed that large number of animals is killed on motorway due to their collision with vehicles. The actual number of animals killed could be many times higher than represented in the Table 1. We were not able to record all the data as motorway authorities ensured the regular removal of dead animals from the motorway. Out of the total animals 71.94% were mammals, mostly dogs, jackals, wild cats and wolves especially during the months of December, January and February. In the present study we observed that mating frequency in dogs varied with seasonal variation. Gavrilovic *et al.* (2008) reported that mostly mating in dogs would take place during the winter and the fewest during summer. During winter months animals might try to cross motorway more in search of mates. Moreover, in winter there may also be possibility of less food availability therefore, in order to search food they might need to cross the road. They can easily enter the motorway section as fences on the both sides of the road are not animal proof/properly managed. Once they

entered the road they could hardly cross the road due to height of the divider erected between roads of two sides. When they try to go back they strike with high speed vehicles and die. According to Gelder (1973) the mortality rate of animals on the motorway increased with the increasing flow rate/hour of vehicles.

During winter season heat is comparatively high on the motorway due to plying vehicles compared to the surroundings. It may be another reason of movement of animals from surrounding to the motorway and high death rate. On both sides of the motorway from Sheikupura interchange to Sial Morr interchange (Sargodha) there is agricultural land. During the winter months the moisture level is very high in the crops. Thus there is a possibility that animals may not feel comfort and try to cross the road in search of suitable habitat. Another possible reason of high mortality during December, January and February could be the fog which reduces the visibility of both animals and drivers of vehicles.

We are not sure which one of the above explanations is true, fact remains that a large number of wild animals are killed on the motorway from Sheikupura interchange to Sial Morr interchange. There is a possibility of similar mortality on the other motorway sections.

It is recommended that wildlife protection agencies and media come into action and highlight the situation in order to save the animals from being killed accidentally. Furthermore, it is the responsibility of Motorway Authorities to ensure that fences on both sides of the motorway were properly secured to block the entry of small sized stray wild, feral or domesticated animals. It is also recommend that safe passages for animals should be constructed over or underneath the motorway so that animals from both sides of the motorway could interact.

References

Barbara, C. and Jones, J., 2009. Traffic Volume as a primary road characteristic impacting wildlife: A tool for land use and transportation planning. Proceedings of the 2009 International Conference on Ecology and Transportation. Cf. http://escholarship.org/uc/item/4fx6c79t.

Baskaran, N. and Boominathan, D., 2010. *JoTT Commun.*, **2**: 753-759.

Coffin, A.W., 2007. *J. Trans. Geogr.*, **15**:369-406.

Eigenbrod, F., Hecnar, S.T. and Fahrig, L., 2009. *Ecol. Soc.*, **14**: 24. http://www.ecologyandsocity.org/vol14/iss1/art24/

Gavrilovic, B.B., Andersson, K. and Forsberg, C.L., 2008. *Theriogenology*, **70**: 783–794.

Gelder, Van J.J., 1973. *Oecologia*, **13**: 93-95.

Jaeger, J.A.G., Bowman, J., brennan, J., fahrig, L., D. Bert, D., Bouchard, J., Charbonneau, N., Frank, K., Gruber, B. and Toschanowitz, K. T.V., 2005. *Ecol. Modl.*, **185**:329-348.

Rais, M., Khan, M.Z., Abbass, D., Ghulam Akber, G., Nawaz, R. and Islam, S., 2011. A Qualitative Study on Wildlife of Chotiari Reservoir, Sanghar, Sindh. Pakistan. *Pakistan J. Zool.*, **43**: 237-247.

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A Rapid DNA Extraction Method for *Bacillus thuringiensis*

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Abstract.- The DNA extraction method must be simple, quick and efficient. Safety, cost and DNA quality must also be considered. *Bacillus thuringiensis* produces endospores that resist the lysis by usual methods used for other bacteria. SDS - NaOH is mostly used in lysis buffer along with incubations for lengthy time periods. Here we devise a rapid method for DNA extraction from *Bacillus thuringiensis* using triton X-100 in lysis buffer. After incubation at 60°C for 30 min, the lysate is phenol-chloroform extracted. DNA was precipitated with absolute ethanol, washed with 70% ethanol and dissolved in TE buffer. The entire method did not take more than 1 hour. The DNA extracted by this method was utilized in enzymatic reactions including PCR, restriction analysis and produced good results. This method can also be used for DNA extraction from other species including Gram negative as well as Gram positive isolates.

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Key words: *Bacillus thuringiensis, Bt.* DNA isolation.

The development of methodologies for the analysis of microorganisms and microbial ecology, at the molecular level (*i.e.*, nucleic acids, proteins, lipids, and their genes) has progressed phenomenally in recent years. The advances in PCR, cloning, gene probing, sequencing and fingerprinting have enabled techniques exploiting nucleic acids to be utilized extensively for the analysis of microorganisms. Many different methods and technologies are available for the isolation of genomic DNA. These approaches often involve combinations of detergents, physical disruption, solvent extraction, and enzymatic lysis to obtain crude extracts of nucleic acid. The choice of a method depends on many factors: the required quantity and molecular weight of the DNA, the purity required for downstream applications, and the time and expense. An ideal protocol requires that nucleic acids be extracted in a form which can be employed for further analysis, produce sufficient amount of DNA, requires minimum use of chemicals and time. Subsequent protocols have usually involved some modification of one or more of these general steps. *Bacillus* is a genus of rod-shaped bacteria that produce not more than one endospore per cell, sporulation is not repressed by exposure to air, are Gram-positive aerobes or facultatively anaerobic. *Bacillus thuringiensis* (*Bt)*, is distinguished by the production of one or more protein parasporal crystals in parallel with spore formation. The parasporal protein crystals are delta endotoxins (Cry and Cyt proteins) that are generally toxic to a variety of insects. More than 250 Cry proteins based on *cry* gene nucleotide sequences and amino acid homologies have been described (Ben-Dov *et al.*, 1996; Crickmore *et al.*, 1998). *Bt.* strains have a genome size of 2.4 to 5.7 million bp (Carlson and Kolstø, 1993). Physical maps have been constructed for two *Bt* strains (Carlson *et al.*, 1996). Most *Bt* isolates have several extrachromosomal elements, some of them circular and others linear (Carlson *et al.*, 1994).

Present protocol was developed to extract *Bt* DNA rapidly, which is more efficient than that of Kronstad *et al.* (1983).

Materials and methods

Bt cells were grown in LB broth with shaking at 37°C as described previously (Saleem, and Shakoori, 2010).

Results and discussion

The 3 ml overnight culture was harvested by centrifugation. Cell walls and membranes must be broken to release the DNA and other intracellular components. This was accomplished with an appropriate combination of enzyme to digest the cell wall and detergents to disrupt membranes The sedimented cells were resuspended in 200µl of lysis buffer (2% triton X-100, 150 mM Tris-Cl, 3mM $MgCl_2$, 40 mM $(NH_4)_2$ SO_4) and 12µl of proteinase K (5µg/ml). The preparation was mixed gently by inverting the tube four or five times (not vortexing), and mixture was incubated at 50°C for 30 min to lyse the cells and then at 95°C for 10 min to inactivate proteinase K. The protein removal was done by denaturation and extraction into an organic phase consisting of phenol and chloroform. Therefore lysate was then extracted with 400µl phenol (equilibrated) and chloroform (1:1) and mixed. Then centrifuged at 12000 rpm for 2 min and aqueous phase was removed after extraction. Then 400µl of chloroform was added in aqueous phase and mixed. The mixture was centrifuged at 12000 rpm for 2min (Note: Phenol, chloroform extraction step is optional). Afterwards, cold ethanol was added, kept at room temperature for 5 min and centrifuged at 12,000 rpm for 5min. Alternatively the DNA could be spooled out. The pellet was washed with 70% ethanol, air dried and dissolved in TE buffer.

This DNA extraction method is simple, quick and efficient. Safety, cost and DNA quality is also considered. The DNA extracted by this method produced good results on agarose gel electrophoresis (Fig. 1) and also when utilized in enzymatic reactions including PCR. It is a simple, low-cost, high-throughput method to prepare genomic DNA for PCR amplification as DNA quality is critical because the efficiency of PCR amplification can be reduced by inhibitors from the matrix. *Bt* produces endospores that resist the lysis by usual methods used for other bacteria. However, no difference was evident between DNA extracted

from vegetative cells and spore, since DNA of the spore is derived from DNA of the vegetative cells (Fitz-James and Young, 1959). SDS - NaOH is mostly used in lysis buffer along with incubations for lengthy time periods. Incubation period devised by Kronstad *et al.* (1983) takes almost one and a half hour (20 min at 37°C for lysozyme treatment and 50-60 min at 60°C for lysis solution and phenol:chloroform extractions). Our method devised incubation time period is 30 min at 50°C for lysis buffer and proteinase treatment. The procedure will help rapid genomic DNA extraction method of *Bt* with increased efficiency and reduced cost. The entire method did not take more than one hour. This method can also be used for DNA extraction from other Gram positive as well as Gram negative bacteria. Thus we report a very simple, rapid, and high-throughput protocol for extracting of high-quality DNA from Gram positive as well as Gram negative.

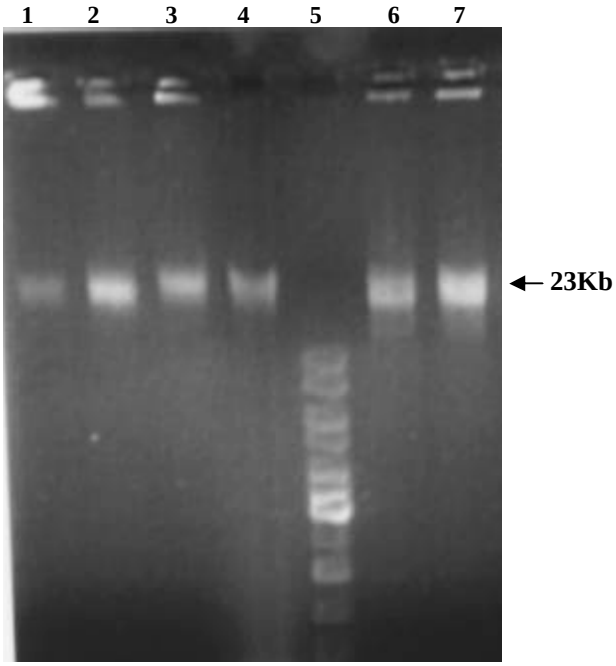

Fig. 1. Total DNA extracted from various strains of *Bacillus thuringiensis.* HD29 (lane1), SBS-BT1 (lane 2), SBS-BT2 (lane 3), SBS-BT3 (lane 4), DNA ladder (lane 5), SBS-BT4 (lane 6), SBS-BT5 (lane 7)**.**

References

Ben-Dov, E., Einav, M., Peleg, N., Boussiba, S. and Zaritsky, A., 1996. *Appl. environ. Microbiol.*, **62**: 3140-3145.

Carlson, C.R., Caugant, D.A. and Kolstø, A.B., 1994. *Appl. environ. Microbiol*., **60**: 1719-1725.

Carlson, C.R., Johansen, T., Lecadet, M.-M. and Kolsto, A.-B., 1996. *Microbiology*, **142**: 1625-1634.

Carlson, C.R. and Kolstø, A., 1993. *J. Bacteriol*., **175**: 1053-1060.

Crickmore, N., Zeigler, D., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J. and Dean D., 1998. *Microbiol. Mol. Biol. Rev.*, **62**: 807-813.

Fitz-James, P.C. and Young, I.E., 1959. *J. Bacteriol.*, **78**: 755.

Kronstad, J., Schnepf, H. and Whiteley, H., 1983. *J. Bact*., **154**: 419-428.

Miller, J.H., 1972. *Experiments in molecular genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Saleem, F. and Shakoori, A.R., 2010. *Pakistan J. Zool.*, 42: 181-193.

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Report of Imposex Syndrome in *Thais tissoti* (Neogastropoda) from Vicinity of Karachi Port, Pakistan

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Abstract.- Wild populations of muricid gastropod *Thais tissoti* were surveyed to observe morphological response of the species against imposex syndrome. Imposex syndrome was encountered only in the vicinity of Naval and merchant shipping activities at Karachi Port. Slightest frequency and intensity of imposex syndrome revealed in morphological examination was 10.60% only. Whereas at other two localities namely Buleji and Sonmiani where only local made small boats for fishing been practiced, there was no sign of imposex syndrome present.

Key words: Thais tissoti, Muricid, imposex syndrome, endocrine disorder.

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Term "imposex" has been widely used for the syndrome cause of endocrine disorder due to chronic exposure to organotin contaminants was first introduced by Smith (1971). Syndrome is well reported particularly in muricid gastropod species belonging to family muricoidae due to their sensitivity and morphological response against the contaminants as described by many authors (Gibbs *et al*., 1988; Oehlmann *et al*., 1991; Tan 1997; Castro and Fillmann**,** 2012). It is also agreed by certain authors that best customary example of endocrine disruption in wild life is the musculanization of female gastropods by the endocrine disrupting chemicals (Matthissen and Gibbs, 1998; Santos *et al*., 2005; LeBlance *et al*., 2005; Nakanishi, 2008). Komaran *et al*. (2011) reported the screening test for antimicrobial activities of marine molluscs *T. tissoti* and Babylonia spirata, being used against human and fish biofilm pathogenic microorganisms. Test showed the human bacterial pathogen Kiebsiella pneumonia and fungal pathogen *Aspergillus niger* most sensitive to ethyl ester extracts of *T. tissoti.* Gastropods specially Muricids: Chicoreus, Thais, Murex etc. are commercially important as of regular diet and other products (Tewari *et al*., 2002; Afsar *et al*., 2012c, 2013). Modern technologies have unveiled new dimensions of research in the shape of natural products from ocean and sea to treat deadly diseases as of many classes of natural products exhibit antitumour, antilukemia, antibacterial and antiviral activities (Kumaran *et al*., 2011; Afsar *et al*., 2012c).

The detrimental effect on food web led to restrictions to use organotin containing paints on ship hulls and other maritime objects which have significant effect to lower the concentrations in natural environment of ports and marinas and adjacent ambient waters world around (Fernandez *et al*., 2002). Pakistan is a maritime country, although restrictions put a positive posture in port areas in lowering the contamination at Karachi and Bin-Qasim port areas but unrestricted leaching to the natural environment at Gaddani beach, Balochistan due to ship breaking industry is alarming where higher imposex syndrome indices are in contemporary evident (unpublished data).

Materials and methods

Snails were collected during ebb tides by hand picking method. Collections were made during October 2005 to June 2006 from Manora Channel the vicinity and formal and eminent passage for Naval and merchant shipping activities at Karachi Port as described by Afsar *et al*. (2013) other than that sampling was made from Buleji of Sindh coast and Sonmiani coast Balochistan. A total of 98 individuals procured from Sonmiani followed by 93 from Manora Channel the and only 8 from Buleji. Samples were procured randomly according to their population size and availability at different sites over a time period in different spans as detailed in Table I. Individuals were examined morphologically to observe morphological response against imposex syndrome by following the method as detailed previously (Afsar *et al.*, 2012b, 2012c).

Table I.- *Thais tissoti*:Biometric data from three (3) sites.

Site	Manora channel	Buleji	Sonmiani
Sampling Period	October 2005, November 2005 & February 2006 to June 2006	February & March 2006	December 2005
Male shell length ± SD (mm)	20.96±3.38	16.50±3.69	22.88±2.61
Female shell length ± SD (mm)	21.87±3.18	20.75±0.95	22.27±2.35
Male penis length ± SD (mm)	8.55±2.45	7.75±3.59	7.11±1.84
Female penis length ± SD (mm)	0.31±1.64	0.00±0.00	0.00±0.00
RPLI	8.35	-	-
RPSI	0.06	-	-
VDSI	0.14	-	-

Results and discussion

Though the specimens of this species were also found at Buleji and Sonmiani, the imposex females were only observed in the samples collected from Manora Channel during October 2005 to June 2006. This species was found to be least sensitive to organotin contamination and the incidence of imposex was significantly low (10.60%) when compared to other muricid gastropods examined from the same locality as previously reported by Afsar *et al*. (2012a,c, 2013).

There was no pronounced development of

penis in the imposex females; however, small bud-like structures or tiny pseudo-penes were evident with an average size of 0.32±0.14mm. Uncubed relative penis length index (RPLI) cubed relative penis length index (RPSI) and vas deference index (VDSI) were calculated as described previously (Afsar *et al.*, 2012a,c) The RPLI was 8.35 and RPSI 0.06. The VDSI in this species was 0.14 (Tables I, II). Stroben *et al.* (1992) demonstrated that RPS (cubed and un-cubed) relates the more or less constant average female penis length to the inconstant mean male penis size.

Table II.- Sex-ratios and chi-square distribution of *Thais tissoti* at different sites.

Site	n	Males	Females	Proportion of males	Chi-square
Manora Channel	93	27	66	0.29	16.35*
Buleji	8	4	4	0.50	0.00
Sonmiani	98	18	80	0.18	39.22*

*Significance at 5% level.

At Manora Channel and Sonmiani the sex-ratio ($P<0.05$) was significantly in favor of females, whereas, from Buleji only 4 male and 4 female specimens were procured (Table II). Only a few samples were examined histologically to observe any shift in gonocycle or altered gonadal cycle like initiation of spermatogenesis in females or imposex females and in comparison few samples were also tested histologically from contamination free sites Buleji and Sonmiani where morphological impression of imposex syndrome was not present. From Manora Channel only 10 females were histologically examined comprised of 8 morphologically normal and 2 imposex females. These specimens were obtained in March and June (2006). Both normal and imposex females found with normal oogenesis with pre-vitellogenic and post- vitellogenic oocytes in the histological sections. No sign of gonadal spermatogenesis or ovo-testis was present. Whereas female specimens (n=3) histologically examined from Sonmiani showed ripe and spawning gonadal condition with normal oogenesis. Similarly females (n=3) from Buleji also showed normal oogenesis with clearly evident vitellogenic and post-vitellogenic oocytes in ripe gonads. Analogous findings accounted by Ramasamy and Murugan (2002) in *Thais biserialis* from Tuticorin harbour, southeast coast of India. VDS stages 1a and 2a were found in imposex affected individuals. Variability in the incidence of imposex has been reported in species of Thais from Gujarat coast, India by Tewari *et al.* (2002) where lower (10.29%) incidence of imposex in *T. tissoti* was found in contrast to *T. rudolphi* (46.55%) and *T. bufo* (44.80%). Further Tewari *et al.* (2002) added that causative agent in sea water is neither toxic nor growth inhibitory but it interferes with the reproductive mechanisms and morphogenesis.

Tan (1999) also observed difference in extent of imposex in three species of gastropods, *T. clavigera, T. gradate* and *Chicoreus capucinus* and attributed this to the difference in sensitivity of species to the pollutant though the species co-existed in the same habitat. He also suggested that in addition to this the difference in diet and physiology could be the factors determining species response to TBT contamination. Leblance *et al.* (2005) avowed that elevated levels of testosterone in gastropods exposed to tributyltin (TBT) are main escort to put forward this endocrine dysfunction that is responsible for imposex. During the presented study only cursory observations were obtained on gonadal development. It is recommended that complete reproductive cycle study of *T. tissoti* could be taken up to understand the correlation between ecological hazard effects on life span of an individual and effect of seasonal variability dilemma on reproduction efforts.

References

Afsar, N., Siddiqui, G. and Ayub, Z., 2012a. *Pakistan J. Zool.*, **44**:572-576.

Afsar, N., Siddiqui, G. and Ayub, Z., 2013. *Pakistan J. Zool.*, **45**: 459-467.

Afsar, N., Siddiqui, G., Rasheed, M., Ahmed, V. and Khan, A., 2012b. *J. chem. Soc. Pak.*, **34**:565-569.

Afsar, N., Siddiqui, G. and Ayub, Z., 2012c. *Indian J. mar. Sci.*, **41**:418-424.

Castro, B. I. and Fillmann, G., 2012. *Environ. Toxicol. Chem.*, **31**:955–960.

Fernandez, M. A., Limaverda, A. M., de Castro, I. B., lmeida, A.C.M. and Wagener, A., de L, R., 2002. *Cad. Saude Publica, Rio de Janeiro*, **18**: 463-476.

Gibbs, P. E., Pascoe, P.L. and Burt, G. R., 1988. *J. mar. Biol. Assoc. U.K.*, **68**: 715–732.

LeBlance, G. A., Gooding, M.P. and Sternberg, R.M., 2005. *Integr. Comp. Biol*., **45**: 81-87.

Matthissen, P. and Gibbs, P., 1998. *Environ. Toxicol. Chem*., **17**: 37-43.

Nakanishi, T., 2008. *J. Toxicol. Sci*., **33**: 269-276.

Oehlmann, J., Stroben, E. and Fioroni, P., 1991. *J. Moll. Stud*.**,** **57**: 375–390.

Ramasamy, M.S. and Murugan, A., 2002. *Indian J. Mar. Sci*., **31**: 243-245.

Santos, M. M., Reis-Henriques, M. A., Guillot, R., Lima, D., Franco-Duarte, R., Mendes, I., Queiros. S., Filipe. L. and Castro, C., 2005. *Comp. Biochem. Physiol. Part C.* **148**:87-93.

Smith, B.S., 1971. In: *Proc. Malacol. Soc. London,* **39**: 377-388.

Sri Kumaran, N., Bragadeeswaran, S. and Thangaraj, S., 2011. *Afr. J. Microbiol. Res*., **5**: 4155-4161.

Stroben, E., Oehlmann, J. and Fioroni, P., 1992. *Mar. Biol*., **113**: 625-636.

Tan, K. S., 1997. *Mar. Poll. Bull*., **34**: 577-581.

Tan, K. S., 1999. *Mar. Poll. Bull*., **39**: 295-303.

Tewari, A., Raghunathan, C., Joshi, H.V. and Khambhaty, Y., 2002. *Indian J. mar. Sci*., **31**:321-328.

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