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Acute Toxic Effects of Organophosphate Pesticides on Killifish Fish (*Aphanius dispar)* Juveniles

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Abstract.- In the present study an attempt has been made to investigate the effect of organophosphate pesticide on *Aphanius dispar* (killifish) through acute toxicity bioassay (24 h). The results showed that the LC_{50} values for two pesticides tested were low 0.0025 ppm for chlorpyrifos and 0.039 ppm for methyl parathion indicating high sensitivity of fish juveniles to different pesticides. The treatment with pesticides also resulted in decreased total tissue proteins in *A. dispar* which reduces the nutritive quality of fish and hence producing negative impact on the health and safety of fisheries products.

Key words: Methyl parathion, Chlorpyrifos, pollution, pesticides, fish, *Aphanius dispar*.

Variety of pesticides used in agriculture to control pests has lead to environmental contamination. Approximately 90% of agricultural pesticide application never reach its target organisms but is, instead, dispersed through the air, soil and water (Moses *et al.,* 1993). There is growing concern that pesticides pose a serious threat to coastal areas receiving direct pesticide applications or run-off from agricultural land (Clark *et al*., 1993). Laboratory studies have shown that pesticides can be acutely toxic to fish (Tooby *et al*. 1975; Linden *et al*. 1979; Mayer and Ellersieck, 1986). Acute toxicity bioassays are a convenient tool used extensively to assess the toxicity of physiologically active substances and also to evaluate the potential of chemical contamination on ecologically important species (Ahsanullah and Arnott, 1978).

In the present study an attempt has been made to investigate the acute toxicity (24 hour LC_{50}) of organophosphate (OP) pesticides on killifish and affect of pesticides on total protein content. The Total protein content in fishes exposed to pesticides have been reported by Khattak and Hafeez (1996), Sancho *et al.* (1997) and Tilak *et al.* (2005).

Materials and methods

Preparation of chemicals

Pesticides, methyl parathion 5% EC, chlorpyrifos 40% EC were procured from Pakistan Agricultural Research Center. Stock solution of 100 ppm and appropriate working concentrations were prepared in filtered seawater.

Test organism

The juveniles of killi fish (*Aphanius dispar*) were collected at low tide from Sandspit (mangrove area) using a hand net. The juveniles were transported in clean aerated seawater to the laboratory ensuring minimum stress. The fishes measuring 1.7cm ± 2cm in length and 112mg ± 1mg in weight were acclimatized to the laboratory conditions for 48 hours prior to experiments. The fishes were kept in clean aerated seawater in glass aquaria (60cm length x 30cm width x 29.5 cm height) at temperature (23±1°C), with photoperiod of 16 hour light and 8 hour dark. Seawater in each aquarium was replenished everyday in order to remove faeces and remaining food and to maintain the water quality. Fishes were fed *ad libitum* and commercial diet two times a day.

Bioassay

Standard bioassay methods (APHA, 1971) were followed to evaluate toxicity of pesticide using static bioassay system (Doudoroff *et al*., 1951). Bioassays to evaluate LC50 were carried out in glass jars (20.5cm length x 13.5cm width) of two liters capacity for fish juveniles. All glassware were acid cleaned prior to the tests. Initially test organism was treated with wide range of pesticide concentration in filtered seawater to evaluate the concentration at which mortality around 50% occurs. The experiment was repeated with five or

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more concentrations of two pesticides for test organism ranging between 0.001-0.08 ppm. All concentrations were prepared with filtered seawater. The tests and controls for each experiment were in triplicate and the controls had only seawater. The other experimental conditions, such as, temperature 23-28°C, Salinity 30‰, pH 7.6, photoperiod (16 h light and 8 h dark), were maintained throughout the experiment. Acute toxicity, measured as mortality of organism exposed to two pesticides, was estimated by 24 h LC_{50} (the concentration of the pesticides which kills 50% of the test animals after 24 hour exposure). Organisms were considered dead if they did not exhibit any internal or external movement. At the end of exposure period (24h) fish were removed from jars and frozen for tissue analysis. The LC_{50} values were determined by using computer programme (Biostat, 2009) based on Finney Method 1952 (Probit analysis).

Total protein analysis

Total protein content in tissues of fish exposed to OP pesticides was analyzed to determine degree of impact according to procedures described earlier (Ahmad *et al*., 2000). Known weight (0.5 mg) of fish (six replicates; both test and control) tissue was homogenized with distilled water (2ml) using mortar and pestle. Content was centrifuged at 15000 rpm for 15 min. Supernatant was immediately analyzed for total protein using analysis kits which employs Biuret method (Tietz, 1995; Randox kit (TP 245). Protein concentration in tissues was calculated as follows:

Total Protein Conc. = $[A_{Sample} / A_{Standard}]$ x Standard concentration

where, A_{Sample} = Absorbance of sample; $A_{Standard}$ = Absorbance of standard

Results and discussion

Figure 1 shows LC_{50} of methyl parathion (0.039 ppm) and chlorpyrifos (0.0025 ppm) for the killifish juveniles. This reflects that fish juveniles showed significantly different degrees of response variations against the two pesticides tested.

The total protein levels decreased significantly in fish tissues treated with both pesticides. Marked decrease was observed in methyl parathion treated group (82%). Reduction in protein level in chlorpyrifos treated group was low (7.6% of control) (Table I).

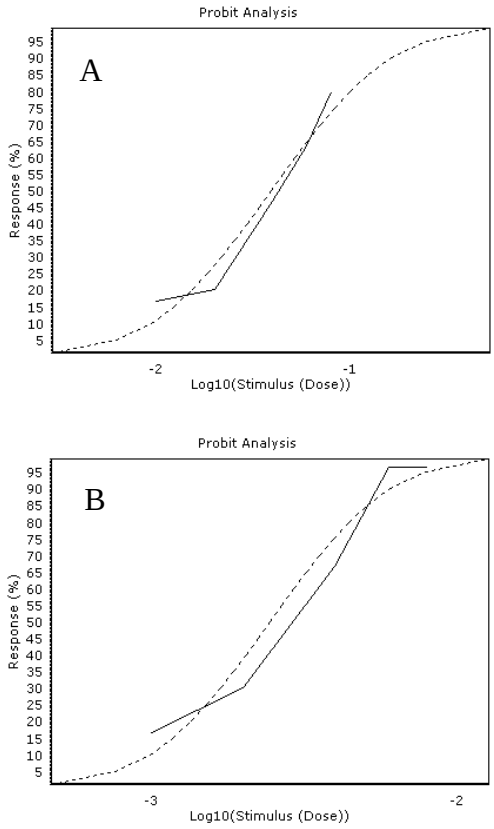

Fig. 1. Probit analysis curve showing response of fish (*Aphanius dispar*) exposed to different concentrations (dose) of pesticides; A: Methyl parathion and B: Chlorpyrifos.

Table I.- Total protein content in tissue of fish *Aphanius dispar* exposed to lethal concentrations of organophosphate pesticides.

Pesticide	Mean±SD (U/L)	
	Control	Treated
Methyl parathion	22.02±2.85	3.84±0.63
Chlorpyrifos	19.66±5.54	181.15±12.46

* values are significantly different from control ($p<0.05$).

Despite the fact that OPs are short-lived, these pesticides are highly toxic compared to a number of other pesticides, for example, DDT (Scott and Matsumura, 1983; Babu *et al*., 1987),

aldrin (Sanders, 1969) and aroclor 1254 (Nimmo *et al*. 1971). Result of the present study suggests that fish is highly sensitive to the OP insecticides. These results are in agreement with the previous reports (Bansal *et al*. 1980; Mayer and Ellersieck, 1986; Takimoto *et al*. 1987; Vittozzi and Angelis, 1991; DOSE, 1997; Chindah *et al.*, 2004; Srivastava *et al.*, 2010).

Killifish responded differently, in the present study, to the two OPs tested, thus indicating variability in sensitivity of test organisms. The comparison of LC_{50} values for different organisms provides only a rough indication of differences in specific tolerance as a number of factors influence the bioassay results, such as, purity (Chambers and Yarbrough, 1974), temperature (Macek *et al*., 1969), and degree of susceptibility of test organisms (Macek and McAllister, 1970). The existence of an interrelationship between temperature and susceptibility of fish to toxicants appears to be a common feature. A wide range of pesticides have been found to increase the toxicity at higher temperature (Macek *et al.*, 1969; Muirhead-Thomson, 1971). An increase in water temperature reduces the solubility of oxygen which would increase the metabolic rate of fish (Davis, 1975) and limit the blood oxygenation, which would result in low dissolved oxygen levels, greater accumulation of waste products and decrease in the resistance of fish to environmental stresses (Srivastava *et al.*, 2010). Reduced solubility of oxygen in water at higher temperatures could also increase the ventilation at gills and respiration rate (Jones *et al.*, 1970). Increased circulation of blood in gills may increase the possibility of greater uptake of contaminants from the medium and intensifying the stress Srivastava *et al.* (2010).

Reduction in protein levels in tissues of fish exposed to OPs, as indicated in the present study, is in conformity with observation made earlier on fish exposed to fenvalerate (Reddy and Bashamohideen, 1988), malathion (Hassanein, 1991; Khattak and Hafeez, 1996), cypermethrin, permethrin and fenvalerate (Singh and Agarwal, 1994), fenitrothion (Sancho *et al.*, 1997), and chloropyrifos (Tilak, 2005; Jaroli and Sharma, 2005). According to Sathyanarayana (2005) the physiological condition of animal is usually indicated by the metabolic status of protein. The reduction of total tissue protein may be due to the effect of pesticides on the following which would lead to the impaired protein synthesis: i) hormonal balance (Murthy and Priyamvada, 1982; Khilare and Wagh, 1988), ii) cellular metabolism (David *et al*., 2004, Joshi and Kulkarni, 2011) and iii) DNA damage, destruction or necrosis of cells (Bradbury *et al.*, 1987; Singh *et al.*, 1996).

In summary the present study reflects that OP pesticides have a dose dependent effect on marine fish with low LC_{50} values. The acute toxicity of pesticide caused reduction in total tissue protein in fish which may have a considerable effect on fish nutritive quality of fisheries product.

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A Record of Imposex in *Morula granulata* (Mollusca: Gastropoda: Muricidae) from Pakistan

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Abstract.- Imposex has been recorded for the first time in the neogastropod *Morula granulata* (Duclos, 1832) from Manora Channel, which is one of the largest shipping hubs in Pakistan. No previous record of imposex is available for this marine intertidal species from Pakistan. Although the degree of imposex was not pronounced. 8% imposex and 16% imposex incidence was recorded in *M. granulata* at Manora Channel site. Histologically examined sections of female gonads from *M. granulata* revealed normal oogenesis with no evidence of development of male characteristics.

Key word: Neogastropod, mesogastropod, imposex, *Morula granulata*, tribulyltin, triphenyltin, Karachi.

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The development of male characteristics, including a penis and vas deferens, in females of prosobranch gastropods is called "imposex" (Smith, 1971) which is mainly caused by organotin compounds tributyltin (TBT) and triphenyltin (TPhT). Imposex has been observed in approximately 170 species of gastropods across the globe particularly in areas experiencing boating activity (Da Costa *et al*., 2008). In general the phenomenon of imposex is common in neogastropods, and best documented in species belonging to the family Muricidae. A number of gastropod species have been studied extensively for imposex and are known to serve as a useful bioindicator of organotin contamination across the globe such as *Nucella lapillus, Ocenebra erinacea, Morula granulata, Thais clavigera, T. bronni* and *T. orbita* (Gibbs *et al*., 1987; Oehlmann *et al*., 1991, 1992; Reitsema and Spickett, 1999; Gibson and Wilson, 2003; Ellis and Pattisina, 1990; Evans *et al*., 1996; Bech, 2002a,b; Meng *et al*., 2005; Swennen *et al*., 2009). The phenomenon of imposex was also documented in *Morula* species from different areas of the world, *e.g.*, in Australia (*M. marginalba* and *M. granulata*; Wilson *et al*., 1993; Reitsema and Spickett, 1999); in Thailand (*M. musiva, M. granulata* and *M. margariticola*; Bech, 2002b); in Malaysia and Thailand (*M. musiva*; Ellis and Pattisina 1990; Swennen *et al*., 2009). Imposex is also known to occur in a few species of mesogastropods such as in *Trivia arctica* and *T. monacha* (Stroben *et al*., 1992a,b) *Littorina littorea* (Bauer *et al*., 1995; Deutsch and Fioroni, 1996) and *Gyrineum natator* (Vishwakiran *et al*., 2006).

In Pakistan there is no published information available on the occurrence of imposex in any gastropod species. In the neighboring waters of India imposex is known to occur in the muricids *T. bufo, T. rudolphi, T. tissoti, Cronia konkanensis* and in the cymatiid *Gyrineum natator* in areas with vessel activity (Vishwakiran and Anil, 1999; Tewari *et al*., 2002; Vishwakiran *et al*., 2006). The present report provides morphological evidence of imposex for the first time in the neogastropod *Morula granulata* in Pakistan.

Material and methods

Specimens of *Morula granulata* were collected from Manora Channel. Specimens of *M. granulata* were also collected from Buleji along the Sindh coast. Buleji is a cleaner site, whereas Manora Channel is a vessel route for ships visiting the Karachi port, which is the largest port of Pakistan. The specimens of these species were collected randomly in different months during the period 2003 to 2006 from the sampling sites.

Animals were hand picked at low tide from the intertidal zone and kept frozen until analysis. Shell length was measured to the nearest 0.01mm using a vernier caliper. Animals were extracted by breaking the shell. Morphological observations were made of the male and female penis/pseudopenis lengths (mm), which were measured from its tip to junction as described by Gibbs *et al.* (1987). Imposex stages were noted by following the scheme given by Oehlmann *et al*. (1992). The frequency and intensity of imposex in various species of gastropods were expressed according to (Fioroni *et al*., 1991; Gibbs and Bryan, 1994; Vishwakiran and Anil, 1999) as follow: (i) uncubed relative penis length index (RPLI); (ii) cubed relative penis size index (RPSI)**; (**iii) vas deferens sequence index (VDSI). VDSI was calculated as the average imposex stage as described by Oehlmann *et al*. (1996). The RPLI and RPSI were calculated by the following formula:

RPLI= (mean female penis length) / (mean male penis length) × 100

RPSI= (mean female penis length) ^{3} / (mean male penis length) ^{3} × 100

Results and discussion

A total of 120 specimens of *M. granulata* were studied from Manora Channel. They comprised of male, female and imposex specimens, constituted 47%, 45% and 8% respectively. The number of females was slightly higher than males in the population but it did not show a significant departure from a 1:1 relationship (X^2 = 0.30; P>0.05). The incidence of imposex was 16% and penis development was limited to small protrusion or bud-like structure less than 1mm in size. The

RPLI was 6.46, RPSI was 0.03 and VDSI was found to be 0.10. Only VDS stage 1a was observed with 1 mm pseudopenis size among females showing imposex. Slightly larger males and females were found at Manora Channel (Table I). In specimens of *M. granulata* (n= 47) from Buleji no incidence of imposex was observed. Here, the sex ratio was close to unity (X^2 = 0.02; P>0.05) and the respective percentages of males and females were 49% and 51% (Tables I, II).

Table I.- Field collection data of prosobranch gastropod species.

Site	Species	Date of sampling	Sample size	% Females	% Imposex Females
Manora Channel	*Morula*	05/ 10/ 05	5	60	0
	granulata	01/ 11/ 05	3	67	0
		12/ 12/ 05	10	50	0
		04/ 02/ 06	17	47	0
		22/ 03/ 06	15	53	7
		20/ 04/ 06	28	29	7
		18/ 05/ 06	12	58	8
		16/ 06/ 06	18	44	22
		17/ 07/ 06	12	42	17
	Bursa	27/ 10/ 03	1	100	0
	granularis	20/ 01/ 04	2	0	50
	Turricula javana	06/ 03/ 04	1	0	100
Buleji	*Morula*	19/ 04/ 04	11	64	0
	granulata	24/ 04/ 05	4	75	0
		21/ 12 05	2	50	0
		04/ 02/ 06	9	33	0
		22/ 03/ 06	14	36	0
		20/ 04/ 06	7	71	0

Table II.- Biometric pooled data of *Morula granulata* at two sites.

Indices	Manora Channel	Buleji
Sample size	120	47
Male shell length ± SD (mm)	20.28±1.90	17.69±1.14
Female shell length ± SD (mm)	20.61±2.01	18.37±2.58
Male penis length ± SD (mm)	8.50±3.39	9.17±1.40
Female penis length ± SD (mm)	0.55±0.16	0
Imposex incidence (%)	15.63	-
Imposex (%)	8.33	-
RPLI	6.46%	-
RPSI	0.03%	-
VDSI	0.10	-

*RPLI, relative penis length index; RPSI, relative penis size index; VDSI, vas deferens sequence index.

A total of 12 female specimens of *M. granulata* were histologically examined from Manora Channel comprising 11 morphologically normal females and one female with imposex. All were mature and in spawning condition with pre- and post-vitellogenic oocytes as observed in histological sections. No sign of an ovo-testis was evident in this species. In histologically examined specimens of females (n = 10) from Buleji normal oogenesis with ripe and spawning condition was evident in all individuals.

In *M. granulata* the sex ratio was found close to unity (1:1) at Manora Channel and Buleji, and incidence of imposex (16%) was also found to be comparatively low, in contrast to other muricid species which exhibited the imposex incidence from the same sites (Afsar, 2009; Afsar *et al*., 2010). In few months imposex was not observed in *M. granulate*, that was only due to disparity in number of collected specimens. *M. granulata* was found to be less sensitive as compared to other muricid, buccinid and bursid mesogastropod species investigated from Manora Channel (Afsar, 2009). Similarly, Reitsema and Spickett (1999) did not observe significant deviation from 1:1 sex ratio in *M. granulata* from Western Australia as a consequence of lower imposex indices observed in the species.

According to the general scheme of imposex stages (Oehlmann *et al.,* 1992) morphologically normal females without any male characteristics are attributed as stage 0 whereas presence of a small penis is defined as stage 1a. Development of a small penis in imposex females is a common character in all muricid species but in case of *Morula* species it is generally limited to a small bud-like structure (less than 1 mm). In species of *Morula* the development of imposex is unique in that development of vas deferens takes place before the formation of penis in certain individuals and more commonly the blistering and folding of the tissue epithelium behind the right tentacle can be observed, with the penis being generally limited to a small bud-like structure less than 1 mm in size (Reitsema and Spickett, 1999). This condition was observed in *M. granulata* from Western Australia (Reitsema and Spickett, 1999), in *M. musiva*, *M. granulata* and *M. margariticola* from Thailand

(Bech, 2002b), and also in *M. granulata* from the present study. Ellis and Pattisina (1990) attributed this to a lower sensitivity of *M. musiva* to TBT contamination in Port Dickson, Malaysia. However one specimen of *M. granulata* at Manora Channel was found with a penis size of 1 mm and with 1a imposex stage. The same condition was observed in *T. tissoti* at this site, with 11% imposex incidence (Afsar, 2009). Reitsema and Spickett (1999) also observed females of *M. granulata* with approximately 2 mm length pseudopenis in the advance stages of imposex including vas deferens formation amongst samples from the King Bay east site in Western Australia.

In the present study the imposex specimens were only found in environments with ship related activities. Imposex is known to be induced by organotin compounds and it has been proved by both laboratory and field experiments (Bryan *et al*., 1986, 1987; Gibbs *et al*., 1988). In Pakistan there are no aquaculture related facilities or marinas present along the coast so the use of toxic biocides is not common. The only likely source of organotin contamination are commercial vessels which enter the Channel to Harbor at the Karachi Port and Karachi Fish Harbor, which is the hub of commercial shipping activity. Approximately 250-300 ships visiting the port each month from all over the world (Afsar, 2009) and it appears to be the localized area of organotin contamination. Despite the fact that the International Maritime Organization has recommended the ban on the use of TBT based coatings on vessels and prohibited their use from year 2003 onwards, TBT based paints are still being extensively used in many Southeast Asian countries (Evans, 2000; Gibson and Wilson, 2003; Liu *et al*., 2011).

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Antimicrobial Activity of Biocides against Different Microorganisms Isolated from Biodeteriorated Paints

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Abstract.- The present study was conducted to analyse the antimicrobial activity of different biocides against the microorganisms isolated from the biodeteriorated paints. Two fungal (MSF1 and MSF2) and two bacterial strains (MSB1 and MSB2) were isolated from the biodeteriorated paints and identified on the basis of morphological characteristics and biochemical tests respectively. Sensitivity of the isolated bacterial and fungal strains against the biocides; $HgCl_2$, $Cu(CH_3COO)_2$, $CdCl_2$ and $(NH_2)_2CO$ at different concentrations was tested in terms of inhibitory zones. Inorganic salts of heavy metals such as $HgCl_2$ and $CdCl_2$ were highly effective against these microorganisms, on the other hand $Cu(CH_3COO)_2$ and $(NH_2)_2CO$ were relatively less effective. Fungal strains showed more sensitivity against different biocides as compared to bacteria. MSF2 showed the highest sensitivity at 300 µg/ml of $HgCl_2$ with 1.9cm of inhibition zone. Overall, $HgCl_2$ was found to be the most effective to kill the microorganisms isolated from biodeteriorated paints.

Key words: Biocides, biodeteriorated paints, zone of inhibition, sensitivity.

Biodeterioration essentially involves the negative aspects of microbial activities (Waites *et al.*, 2001). Paintings whether easel or mural, contain a wide range of organic and inorganic constituents which can be exploited by a large variety of microbial species, which may cause aesthetic and structural damage to paints. Many components of the additives (glues, emulsifiers, thickeners, etc.) which facilitate drawing or enhance the aesthetic quality of finished product are also biodegradable (Inoue *et al.*, 1991). In easel paintings, the supporting materials such as cellulose of paper, canvas, wood, proteins of parchment, silk, and wool may be easily degraded by microorganisms. In mural paintings, pigments are suspended in water or oil, often in the presence of binders such as casein and milk which are also biodegradable (Ciferri, 1999).

During the fungal colonization of mural paintings, Saiz-Jimenez and Samson (1981) have shown that, at the beginning, growth of fungi on a mural's surface caused only aesthetic damage but later on, fungal growth in depth occurred. Hyphae penetrated the painted layer, degrading some of its components especially glues and binders, which resulted in a decrease in cohesion of the painted layers, thus giving rise to exfoliations, cracking, and loss of the paint (Gorbushina and Petersen, 2000; Guglielminetti *et al*., 1994). Similarly, heterotrophic bacteria can use organic components of paints as substrate and produce metabolites, often acidic in nature which causes aesthetic damage to the paints (Heyrman and Swings, 2001). The most common soil inhabitants, both fungi (species of *Penicillium*, *Aspergillus*, *Cladosporium*, *Chaetomium*, and *Alternaria*) and bacteria (species of *Pseudomonas*, *Arthrobacter* and *Streptomyces*), are present in many of the paint samples analyzed (Altenburger *et al.*, 1996).

The major objectives of present study were to isolate, identify and characterize the micro-organisms involved in the biodeterioration of the paints and to check the sensitivity of these microorganisms against different biocides.

Materials and methods

Microorganisms and growth conditions

The samples were obtained from the local biodeteriorated paints which have a clear growth of the fungi deteriorating the paints. The samples were

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serially diluted in sterile water and spread on Potato Dextrose Agar (Sigma-Aldrich) at 25°C for 4 days and nutrient agar (Sigma-Aldrich) at 37°C for 48 hours, to isolate fungal and bacterial strains respectively.

Identification of microorganisms

Two strains of the fungi MSF1 and MSF2 were identified under the microscope on the basis of morphological characteristics (Domsch *et al.*, 1980). Two strains of bacteria MSB1 and MSB2 were isolated and observed under the microscope after gram staining and identified on the basis of biochemical tests (Buchanan and Gibbons, 1974). The identification of the MSB1 was confirmed by 16S rRNA ribotyping.

16S rRNA gene sequencing

Genomic DNA was extracted from bacterial cultures grown overnight at 37°C in Luria-Bertani broth on shaker at 120 rpm. DNA extraction was carried out by using a DNA extraction kit (Fermentas). 16S rDNA amplification was performed according to the method described by Hasnain and Thomas (1996). The 1.5-kb DNA fragment encoding the 16s rRNA gene was amplified using forward primer 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1522r (5'- AAGGAGGTGATCCA (AG)CCGCA- 3') (Johnson, 1994). The product was purified using an Aqua pure DNA extraction kit (Bio-Rad) and sequenced using the 27f primer by ABI PRISM-3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Bioinformatics tools

The sequences of 16S rRNA gene fragment analyzed by BLAST at the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/) and submitted to Gene Bank under the accession number EU409313.

Antimicrobial activity of biocides

Sensitivity of the isolated microbes was checked against four different types of biocides; $HgCl_2$, $Cu(CH_3COO)_2$, $(NH_2)_2CO$ and $CdCl_2$ at various concentrations; 100 µg/ml, 200 µg/ml and 300 µg/ml, by punch hole method (Tagg and McGiven, 1971). 100µl of fungal and bacterial overnight cultures were spread on PDA and nutrient agar respectively. Different concentrations of the antimicrobial agents were added into the wells then the fungi were incubated at 25°C for 4-5 days and the bacteria were incubated at 37°C for 48 hours. After incubation the zone of inhibition was measured in cm (Tagg and McGiven, 1971).

Statistical analysis

Antimicrobial activities of the biocides were checked in triplicates and standard errors of the mean were calculated.

Results and discussion

Two strains of the fungi *Penicilium* (MSF1) and *Aspergillus* (MSF2) were identified under the microscope on the basis of morphological characteristics (Domsch *et al.*, 1980). On the basis of biochemical characterization MSB1 and MSB2 were identified as *Bacillus* and *Pseudomonas* respectively (Table I). The identification of MSB1 was confirmed by sequencing a portion of DNA encoding the 16S rRNA gene, which was found to be *Bacillus cereus* (Genbank accession no. EU409313).

Table I.- Biochemical characterization of bacteria isolated from biodeteriorated paints.

Strain	MSB1	MSB2
Growth	LF[4]	NLF[5]
TSI[1]	A/A/-/+	K/K/-/-
Oxidase	-ve	+ve
Catalase	+ve	+ve
Citrate	+ve	+ve
Indole	-ve	-ve
MR[2]/VP[3]	-ve/-ve	-ve/-ve
Urease	-ve	-ve
Sugar Fermentation		
Glucose	+ve	-ve
Sucrose	-ve	-ve
Lactose	+ve	-ve
Maltose	-ve	-ve
Probable genera	*Bacillus*	*Pseudomonas*

[1]Tipple Sugar Iron test; [2]Methyl Red test; [3]Voges-Proskauer test; [4]Lactose Fermenter; [5]Non Lactose Fermenter.

Sensitivity of the isolated bacterial and fungal strains against the biocides such as $HgCl_2$, $CdCl_2$, $Cu(CH_3COO)_2$ and $(NH_2)_2CO$ at different concentrations was tested by measuring the inhibitory zones (Table II).

$HgCl_2$ was found to be the most active to kill the microorganisms isolated from the biodeteriorated paints and fungal strains showed more sensitivity at various concentrations of $HgCl_2$ as compared to bacteria. MSF2 showed the highest sensitivity at 300 $\mu g/ml^{-1}$ of $HgCl_2$ with the zone of inhibition of diameter 1.9 cm. Against the $CdCl_2$, MSF1 and MSB1 showed the highest sensitivity at 300 $\mu g/ml^{-1}$ with the zone of inhibition of diameters 1.0 and 0.9 cm respectively.

Inorganic salts of the heavy metals such as $HgCl_2$ and $CdCl_2$ were found highly effective against the microorganisms because salts of the heavy metals act by binding to key functional groups of fungal enzymes (Lukens, 1983). $HgCl_2$ was found relatively more effective because metal ions such as mercury (Hg^{2+}) and silver (Ag^{+}) interact strongly with thiol groups in bacterial and fungal enzymes and proteins which ultimately results in the killing of these microorganisms (Slawson *et al.*, 1990). Mercuric salts are biocidal as these salts have also been shown to interfere with translation by reacting with ribosomes (Gilbert and McBain, 2001).

$Cu(CH_3COO)_2$ and $(NH_2)_2CO$ were found less effective as compared to the inorganic salts of the heavy metals. $Cu(CH_3COO)_2$ and $(NH_2)_2CO$ were effective at 300 µg/ml concentrations to kill the microorganisms while at the lower concentrations such as 100 µg/ml and 200 µg/ml the sensitivity of microbes was quite low.

The results obtained in this study showed that mercuric chloride is an effective biocide to kill the microbes involved in the biodeterioration of paints.

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Effectiveness of Groundnut–Maize Bait as Carrier of Coumatetralyl Against Indian Crested Porcupine, *Hystrix indica* Kerr

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Abstract.- Groundnut–maize grain bait was tested for its effectiveness in delivering the lethal quantities of coumatetralyl for controlling Indian crested porcupine. Field trials, conducted in Abbottabad - Balakot tract, revealed that the average consumption of groundnut – maize (1:1) impregnated with 0.0375% coumatetralyl and supplemented with 5% saccharin increased up to 6th night, then steadily decreased until a negligible amount of bait was consumed on the 12th night. Consumption of similar bait formulation but without saccharin increased up to 7th night and then declined steadily until a negligible amount of bait was consumed on the 12th night. Saccharin supplemented coumatetralyl bait caused 80% reduction in porcupine burrow activity, while 70% reduction was recorded for bait without saccharin supplementation.

Key words: Indian crested porcupine, *Hystrix indica*, coumatetralyl, grain baits, groundnut, maize; saccharin.

Indian crested porcupine (*Hystrix indica*) is a large nocturnal rodent (11-18 kg) which lives in extensive burrow systems. It is fairly abundant (Roberts, 1997) and is a serious pest of forest plantations and agricultural crops in Pakistan and many other countries (Ahmed *et al*., 2003; Siddique and Arshad, 2004). Maize, potato and groundnut are the most susceptible crops to porcupine infestation, while newly planted *Pinus roxburghii* (1-6 years) is also seriously affected by porcupine (Khan *et al.*, 2000). Reforestation efforts and food security of the nation demands that such losses be minimized through controlling the porcupine population.

Among the management strategies, chemical control is the most suitable option (Hadler and Buckle, 1992). Acute rodenticides give a quick down, yet have a limited value in porcupine control because of bait shyness (Mushtaq *et al*., 2010). Burrow fumigation is successful only in the loamy soils (Mushtaq *et al*., 2008). Anticoagulant rodenticides do not induce bait shyness and are generally most effective in rodent control because the delayed onset of toxicosis prevents bait shyness (Buckle, 1994). Maize grains in whole form are generally used as base for different bait formulations against rodents, while groundnut has never been tested. In the current study, groundnut - maize (1:1), which is the most favored bait combination against Indian crested porcupine (Mushtaq *et al*., 2009), was used as bait base for delivering the lethal quantities of the anticoagulant

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coumatetralyl (3-α-tetralyl-4-hydroxycoumarin), which is economical and easily available in Pakistan. In addition, a sweetener (saccharin) was also tested to assess possible additive effects.

Materials and Methods

The present study was conducted during winter season (2006-07), in Abbottabad- Balakot area (34° NL, 73° E), when wild grasses and herbs were generally dry, and while potato and maize fields were scattered in suitable patches. Porcupine burrows were located and their active status was confirmed by observing the footprints on the three consecutive mornings on tracking patches laid in front of the burrow opening in the previous evening. Bait was prepared (w/w) by mixing cracked groundnut and maize grains, saccharin (Saccharin soluble, Choheung Chemical Ind. Co. Ltd., Seoul, Korea) and coumatetralyl (0.0375% - supplied under the trade name, Racumin, as 0.75% powder concentrate; Bayer, Germany) in the ratio of 9:9:1:1 and groundnut, maize and coumatetralyl in ratio of 9.5:9.5:1 while the control bait was prepared by mixing groundnut, maize and saccharin (9.5:9.5:1).

One kg bait (using Pesola spring balance, with a minimum count of 1 g) was offered in earthen bowls (20 cm diameter and 6 cm deep), placed deep at the opening of the burrow, in the evening, when the human and/ or livestock activity subsided. Each burrow was visited in the next morning to record the bait consumption by porcupine during the night. The next morning, bowls were replenished with fresh bait materials and the process continued for twelve consecutive nights.

Sixty active porcupine burrows were randomly selected and divided into three sets of 20 burrows each. At set I, poison baiting impregnated with coumatetralyl and supplemented with saccharin was tested; in set II, poison baiting without saccharin was applied; and in set III, control bait was offered. Reduction in burrow activity was judged by recording the porcupine foot prints on freshly sprayed tracking patches (1 x 1 m) at the burrow openings. The burrows having foot prints were considered as active. The burrow activity was monitored, daily, starting from first baiting day until fourteen days after the last poison baiting day. Number of porcupines using the burrow system at different times could not be ascertained.

Mean consumption and standard deviation of each parameter were calculated, using Microsoft Excel. Student's 't' test was applied for comparison of different treatments, using a 5% level of significance (Steel and Torrie, 1980).

Results and Discussion

Average consumption of control bait was significantly higher than the coumatetralyl impregnated bait supplemented with 5% saccharin ($t_{(476)} = 5.35$, $P < 0.05$) and without saccharin ($t_{(476)} = 6.77$, $P < 0.05$) supplementation (Fig. 1). The average consumption of saccharin supplemented poison bait did not differ from consumption of bait without saccharin supplementation ($t_{(476)} = 1.41$, $P > 0.05$). Average daily bait consumption revealed a steady increase in case of control bait, till the 12th night, while an increasing trend was recorded through the 6th night, for saccharin supplemented poison bait and through the 7th night for poison baiting without saccharin bait. The consumption of the coumatetralyl treated bait started decreasing gradually in the subsequent test nights and a negligible amount of consumption was recorded on the 12th test night.

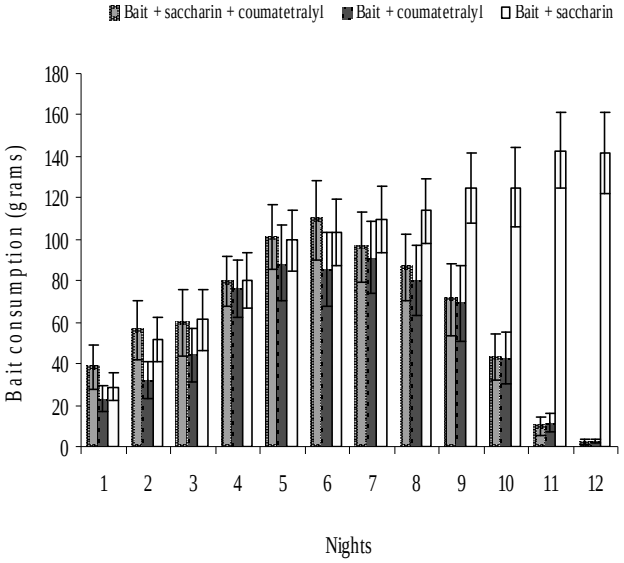

Fig. 1. Relative consumption pattern of groundnut – maize grain bait impregnated with coumatetralyl (0.0375%) by the Indian crested porcupine, *Hstrix indica*.

Current results suggested that porcupine did not exhibit the bait aversion with this rodenticide, and the species continued consuming poison bait which gradually resulted in accumulation of the poison in the body of the animal (s), leading to the lethal effects. General field observations on the fresh faecal pellets revealed that on the 6th and 7th nights of the baiting trial, loose bluish faecal pellets, appeared in the vicinity of the burrows, which led to the conclusion that the pathological symptoms started appearing among the porcupine living in the burrows. On the 10th and 11th day of the baiting trial, two dead porcupines, showing symptoms of bleeding from nose and eyes, were also observed in the territory. Since bleeding is a characteristic symptom of anticoagulants (Brooks *et al*., 1990), this could be because of the rodenticide intake. The inactive burrows were physically visited as a follow up of the coumatetralyl baiting and the smell of dead animals was recorded. Coumatetralyl poisoned baiting caused 80% reduction in burrow activity. When the bait contained 5% saccharin, burrow activity was reduced 70%. We assume that reduced burrow activity was due to the death of the animals. Almost similar results were also recorded against field rats by Hussain and Prescott (2006), and Indian crested porcupine by Khan and Mian (2008). Anticoagulant baits against Indian crested porcupine have not properly studied. Ahmed *et al*. (2003) used 0.005% brodifacoum wax blocks and Khan *et al*. (2006) used whole grain maize bait of coumatetralyl (0.0375%) but did not record the daily consumption, similarly Khan and Mian (2008) tested the coumatetralyl bait by applying bait stations and concluded that anticoagulants are highly effective against Indian crested porcupine. The present study is first of its kind, wherein burrow baiting has been practiced and also testing the efficacy of a sweetener and the control bait. Burrow baiting has proved more effective and safer than the surface baiting against field rodents (Khan *et al*., 1998). We conclude that groundnut – maize (1:1) is an effective and economical bait formulation for delivering the lethal quantities of coumatetralyl against porcupine in the burrow baiting and saccharin further improves the bait uptake. Further studies using the current bait with the second generation anticoagulants are suggested.

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Water Quality Assessment and Mapping for Water Supply System of Abbottabad's Urban Settlements

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Abstract.- This study was intended to assess the qualitative aspect of the water supplied to consumer through water supply network in selective urban settlements of Abbottabad city to figure out the locations

prone to contamination and then mapping out the components of water supply system (WSS). The overall 53 water samples were collected from various water supply components and subjected to physiochemical and bacteriological analyses. Target points of WSS for water sampling were positioned by using Global Positioning System (GPS) device and water quality data visualization of every assessed sampling point was achieved by using ArcView 3.1 software, a tool of Geographic Information System (GIS). The results indicate that although the water source and storage point are at their cleaner level, the consumer end is still suffering from high magnitude of total and fecal coliform contamination. High lead and cadmium indication in almost all household tap water samples predicts the deterioration of the water supply pipelines. It is concluded that developing a database after the integration of the water quality data with the water supply infrastructure will be useful in planning improved water schemes. This approach may also help in the rehabilitation as well as improvement of older WSS both in terms of quality and quantity for the community to meet the core water quality standard.

Keywords: Mapping, urban settlements, water supply; water quality.

Consumption of poor quality water has major socio-economic consequences for Pakistan (Giorgio and Gaspare, 2010). It results in increased morbidity and mortality rates and poses threat to children life and development in Pakistan (Hualou *et al*., 2009).

Fecal contamination of water source, treated water and water supply system (WSS) is a persistent problem, mainly due to increasing populations, urban growth and expansion, peri-urban settlement and increasing pollutant transport into ground and surface water. The WSS suffers a dilemma, first, when the source (ground water or surface water) is fecally contaminated and microbially unsafe water is delivered without any adequate treatment. Second, the contamination of water during distribution through WSS. In some urban settlements, the inadequate water supply infrastructure for water distribution leads to intermittent changes in water pressure, deterioration of pipelines and leakages which may results into intrusion of contaminated water and increased waterborne disease risks. The end result of such deficiencies is the delivery of unsafe water to consumers, even though the water may have been attained from the high quality or protected (physically and chemically treated) source (Brian *et al*., 2005). In view of that, this study was designed to assess the water quality of WSS for selective urban settlements of Abbottabad and came up with digitized map of the selective components of prevailing WSS with integrated water quality data of water sampling point.

Materials and methods

The study area is Abbottabad in the Khyberpakhtun Khuwa (KPK) Province, which currently ranks second only to Baluchistan as the province with the poorest drinking water supply in the country in terms of quality and quantity. Abbottabad ranks third in KPK in terms of water supplied through pipelines, with the district Bannu first and Haripur second (Munabi *et al*., 2009).

The selective sub-sites for this study are the prominent urban settlements of Abbottabad such as Habibullah Colony (HC), Jinnahabad Colony (JC) and Kaghan Colony (KC). The research methodology comprised five steps *i.e.* 1) Meeting with TMA and Cantonment Board Authority (CBA) Abbottabad to get information regarding prevailing WSS in the area of study, 2) acquisition of maps for strategic water sampling and digitization. 3) water sampling by using Global Positioning System (GPS) device (for positioning the water sampling points). 4) Laboratory tests for drinking water quality parameters. 5) Mapping the water supply system and integration of drinking water quality data of targeted sites to visualize the whole scenario regarding water quality through WSS infrastructure at community level.

The focused areas were located in the cantonment limits of Abbottabad and hence the WSS of these target colonies was controlled by the CBA, Abbottabad. Overall 53 drinking water samples (DWS) were collected from important water supply components which are tube wells (TW), storage tanks (ST), filtration plants (FP) and consumer end in the study area. The water samples

were then subjected to physiochemical estimation *viz.*, pH, total dissolved solids (TDS), turbidity, electrical conductivity, total hardness, chloride, Ca^{2+}, Pb^{+2}, Cd^{+2} and Ni^{+2}; and bacteriological analysis *viz.*, total coliforms and fecal coliforms through membrane filtration method. The target points of WSS for water sampling were positioned by using GPS device. The important components of WSS and the water sampling points in the study area were mapped and digitized by using ArcView 3.1 software, a tool of Geographic Information System (GIS). Later on the water quality data of each sampling point was integrated with the digitized maps using this software.

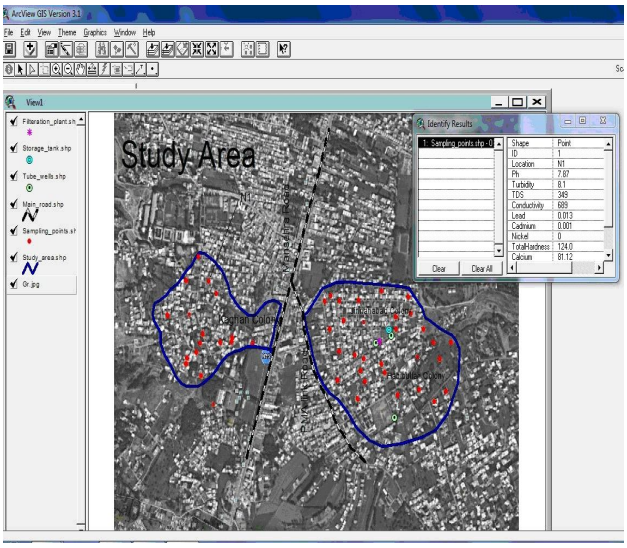

Fig. 1. Visualization of WQ Attribute Data of sampling points of WSS of Habibullah Colony, Jinnahabad Colony and Kaghan Colony in Abbottabad, KPK.

Mapping by using ArcView 3.1 Software

GIS technology is applied in a variety of problems in water distribution networks. It gives a visual model of the field conditions regarding water quality and hence can be used with ease, even by professionals with very little experience (Schindler and Garrard, 1999; Swati and Kurian, 2008; Preston, 2002). Figure 1 shows the overall study area showing components of water supply system and drinking water sampling points. The GIS software helped in visualization of the water quality data of a specific location.

Results and discussion

The centralized WSS of the selective urban areas (HC, JC, KC) comprised three TWs, one ST and one FP, located in JC from where the drinking water was then supplied to the housing units of HC, JC and KC.

Table I.- Total and fecal coliforms (CFU in 100 ml) in tube wells (TW), storage tanks (ST) and filtering plants (FP) in the WSS of study area

Samples	Total coliform	Fecal coliform
TW1	6	0
TW2	0	0
TW3	14	0
ST	50	0
FP	128	0

Bacteriological analysis

Two TWs out of three and ST showed total coliform contamination. The most alarming was the condition of FP which showed high total coliform contamination revealing its non functionality. No fecal coliform contamination was seen in any sample of TWs, FP and ST (Table I). According to WHO (2006), there should be no total or fecal coliform per 100 ml of drinking water. The study showed that consumer end is at higher risk of gastrointestinal diseases in the study area. All household water samples of HC, JC and KC showed severe total and fecal coliform contamination (Table II) which is an indication of recent fecal contamination in the water distribution network. (Wellington *et al*., 2008). The soft pipeline deposits were the main reservoir for microbes in the drinking water distribution networks (Zacheus *et al*., 2000). Disturbances such as change in pressure in the pipeline may release these bacteria into water (Paul *et al*., 2008). The presence of coliforms in drinking water implies failure of the water treatment system, a break or leak in the water mains, or contamination of the water distribution system usually by backflow from households.

Physiochemical analysis

The mean pH values of drinking water samples collected from TW1, TW2, TW3, ST, and FP were found within the range of WHO proposed

standard *i.e.* 6.5 to 8.5. The highest pH value of tap water was found in sample of JC. Increase in mean pH values of tap water as compared to the tube well, storage tank and filtered water was observed (Fig.2A). Water in distribution system may get contaminated either at the source or during the transit through pipelines because of contaminant intrusion (Kalbermatten, 1989).

Table II.- Percentage of total and fecal coliform (CFU in 100 ml) in household drinking water samples of Habibullah Colony (HC), Jinnabad colony (JC) and Kaghan Colony (KC).

Household drinking water samples	Total coliform	Fecal coliform
HC	100%	56.25%
JC	86%	68.75%
KC	100%	68.75%

The turbidity values of the drinking water samples of TW1, TW2, TW3, FP, ST and households of HC, JC and KC cross the permissible limit (5 NTU) of turbidity in drinking water (Fig. 2A), which showed that water had particulate and organic matter. The highest turbidity value (8.5 NTU) was found in water sample collected directly from FP (Fig.2A). A gradual increase in turbidity was found as water moved from TW to consumer end that might be due to leaks and cracks in supply pipelines through which organic matter and other loose material got deposited in the pipes. Scratching by water flow in pipelines ultimately enhanced the turbidity (Goshko *et al.*, 1983). Turbidity made water aesthetically unacceptable (Pritchard *et al.*, 2007). The turbidity causing colloidal particles harbor pathogenic microorganisms thus making disinfection ineffective (Ridgway and Olson, 1982; Camper *et al.*, 1986; Herson *et al.*, 1987).

The variations in permissible limit of WHO while the electric conductivity in all TW, STS in JC and tap water of HC, JC and KC were above the acceptable limit (400µS/cm) of WHO (Fig. 2B) which indicate high concentration of salts in water (WHO, 2006; Pooya *et al.*, 2009). Potable water should be clear, non saline and free from compounds that can cause color, taste and odor (Pritchard *et al.*, 2007).

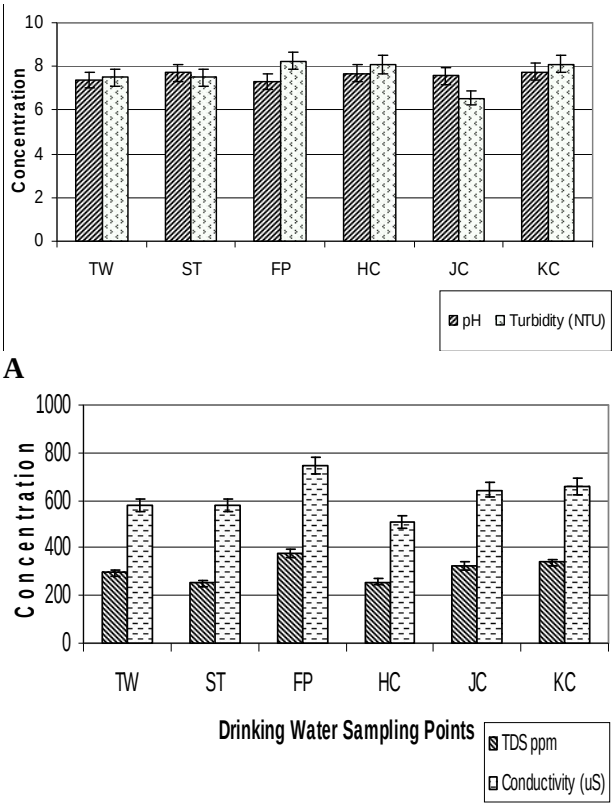

Fig. 2. Mean pH and turbidity (A) dissolved solids and conductivity (B) of drinking water samples of TWs, ST, FP and household points HC, JC, KC. For abbreviations seen Table I and II.

Total hardness Ca^{++} and chloride content of all water samples below the WHO permissible limit of 500 mg/l, 200 mg/l, and 250 mg/l (WHO, 2006; Pooya *et al.*, 2009). Pb^{+2}, Cd^{+2} and Ni^{+2} were not detectable in TWs, FP and ST samples while Pb^{+2} was found at higher concentration in water samples of HC, JC and KC, crossing permissible limit of <0.05 mg/l. Same is the case with Cd^{+2} crossing defined limit of WHO (0.003 mg/l), while no nickel was found in any of the samples.

Conclusions

It is concluded that, the consumers get contaminated water, as evidenced by the presence of coliform in the WSS. High turbidity and bacterial contamination in drinking water indicated fecal contamination in almost all household tap water samples. Detection of lead and cadmium predicts

the deterioration of the water supply pipelines due to overage and corrosiveness. The complicated water supply and sanitation networks, unplanned infrastructure development, lack of periodic water quantity and quality monitoring, poor management and water treatment have exacerbate the situation. The digitized maps developed by using GIS tool gave an opportunity to update the data in coming years on water quality issues.

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Penetrance of Novel Mutations of Endothelin-B receptor Gene in Pakistani Families with Waardenburg Syndrome

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Abstract.- Mutations in endothelin-ß-receptor gene (*EDNRB*) gene have been reported to cause Waardenburg syndrome (WS4) in humans. We investigated novel mutations in *EDNRB* gene and their association with WS4 in two Pakistani families named WSPK1 and WSPK2 using PCR and direct sequencing technique. A transition of T to C in codon (L361S) in exon 5 of EDNRB gene was found in family WSPK1. The mutation was found in the homozygote patients with WS4 and their asymptomatic heterozygote parents. In second family WSPK2, three mutations; a G to C transversion in codon 335 (C335S) in exon 5, a transition of T to C in codon (L361S) in exon 5 and a non coding transversion of T to A at –30 nucleotide position of exon 5 were identified in the homozyote patients and the heterozygote asymptomatic parents. The patients and asymptomatic parents carried the same mutations. In both families, the parents have consanguineous marriage. In this study, we have identified the penetrance of the novel mutations of EDNRB gene in two Pakistani families suffering with WS4. This is first report of WS4 and its correlation with the novel mutation described herein.

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Key words: Endothelin-ß-receptor gene, EDNRB, Waardenburg syndrome, penetrance.

Waardenburg syndrome (OMIM; 193500) is a rare disorder (1 in 40000 live births) characterized by sensorineural deafness along with defects of neural crest-derived tissues and pigmentary abnormalities (1). Waardenburg Syndrome is responsible for 1-3% of total congenital deafness cases (Read and Newton, 1997). Waardenburg-Shah syndrome (WS4; MIM277580) is one of the four types of Waardenburg Syndrome that is characterized by the association of pigmentation abnormalities, including depigmented patches of the skin and hair, vivid blue eyes or heterochromia irides, and sensorineural hearing loss along with dystopia canthorum, musculoskeletal abnormalities of the limbs, Hirschsprung disease, or neurological defects (Pingault *et al*., 2010). The syndrome has been associated with the mutations of Endothelin-B recpetor (EDNRB) gene (Puffenberger *et al*., 1994a), which is present on human chromosome 13 (Arai *et al*., 1993). Although genotype-phenotype association of EDNRB gene with WS4 has been established (McCallion and Chakravarti, 2001), but rarity of families suffering with WS intricate the investigation of novel mutations (unpublished) in EDNRB gene. The present study describes novel mutations of EDNRB gene and their penetrance in two Pakistani families suffering with WS.

Materials and methods

After obtaining a formal approval from the Institutional Review Board of Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences and written-informed consent from the families suffering with WS4, blood samples of two families (WSPK1 and WSPK2) were collected. Each of the families had two patients suffering with WS4. DNA extraction was performed from whole blood following standard phenol-chloroform extraction method (Sambrook and Russell, 2001) and was stored at –20°C. Seven pairs of primers were designed to amplify the exons and the flanking Intronic sequences of the EDNRB gene (Table I). Purified PCR products were sequenced with both forward and reverse primers using BigDye terminator cycle sequencing kit (Applied Biosystems, USA) on ABI 3130XL Genetic Analyzer. Sequence data were edited manually using Chromas Ver. 1.45, http://www.technelysium.com. au/chromas.html). Nucleotide sequences of all of the seven exons were used for multiple sequence alignments, which were performed with ClustalW freeware (http://www.ebi.ac.uk/Tools/clustalw2). The coding DNA sequences were conceptually translated to amino acid sequences using BioEdit software (http://www.mbio.ncsu.edu/BioEdit).

Results

A transition of T to C in codon (L361S) in exon 5 of EDNRB gene was found in the family WSPK1 (Fig. 1). The mutation was found in the homozygote patients with WS4 and their asymptomatic heterozygote parents. In second family WSPK2, three mutations; a G to C transversion in codon 335 (C335S) in exon 5, a transition of T to C in codon (L361S) in exon 5 and a non coding transversion of T to A at –30 nucleotide position of exon 5 were detected in homozyote patients and heterozygote asymptomatic parents (Fig. 1B). The patients and asymptomatic parents carried the same mutations. In both families, the parents have consanguineous marriage (Figs. 1A,B). All of the disease causing mutations was homozygous and novel.

Discussion

Endothelin B receptor protein is encoded by EDNRB gene which is present on the chromosome band 13q22 and consists of 7 exons with a total length of 2400 bases (Arai *et al*., 1993). A 442-residue protein of heptahelical receptors is encoded by the gene that is involved in the G-protein-mediated intracellular signaling pathway (McCallion and Chakravarti, 2001). EDNRB mutations are mainly inherited from unaffected parents (Zhang *et al*., 2007). The inheritance pattern of EDNRB mutations is complex, but it cannot be defied that homozygotes have a high probability of developing disease phenotype. The term “not fully recessive-not fully dominant” can be used for the transmission of the mutations (Pingault *et al*., 2010). Missense mutations are speckled along the EDNRB

Table I.- Primer used for amplifcation of *EDNRB* gene.

Exon	Forward Primer Sequence	Reverse Primer Sequence	Product size
Exon 1	TCCTGTCTTCCTTCCTCTGC	CTCAAGCCCACCATGATTTC	600 bp
Exon 2	ACCAGAGTTTATCCTACTCTGCAT	GCACAGTTTATTTTCTAAGTAACATGG	298 bp
Exon 3	CTGTGCAATTCAATAAAACTAAGG	GGGAACAGGGGAAAAATAGC	339 bp
Exon 4	GAAGATAATCATTCCCTGATGAA	CAAGAAAAAGGAAATATGCTCTGG	373 bp
Exon 5	AAATGTCGTTTTAGAAGATAGAATGC	AAGATCGATGGAAACACTTCTGA	277 bp
Exon 6	AAGCACAGAAGCTACAATGACTACA	GCAGTTTTGAAAGCTTATATTTGA	250 bp
Exon 7	AACCCTGGAGAGGAGGAAGA	TTTGTTTTGGCAAATGTTTCA	290 bp

bp: Base Pairs

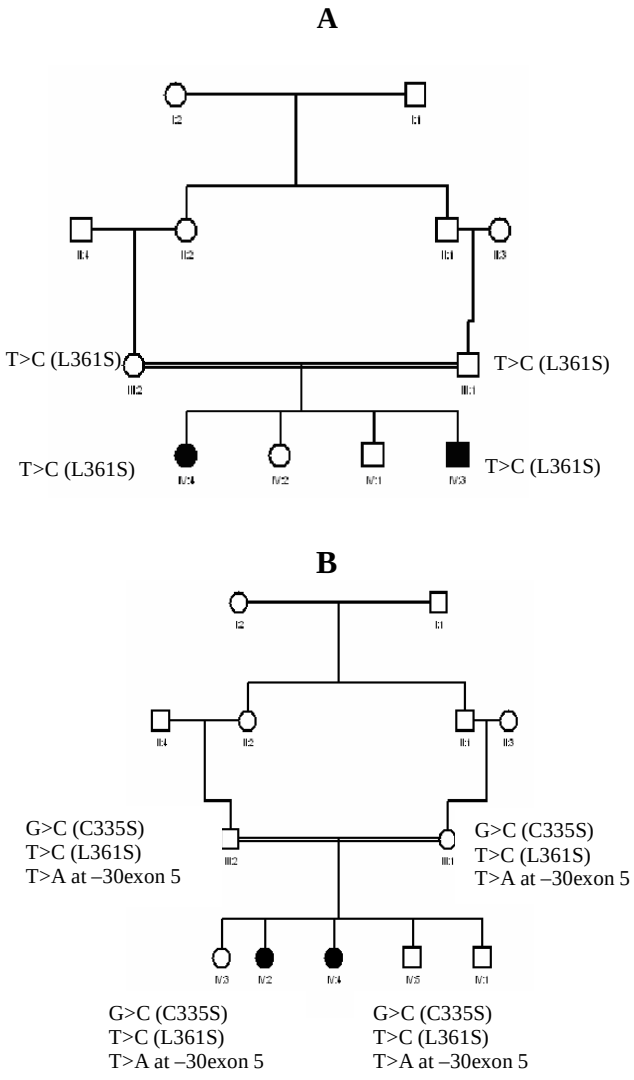

Fig. 1. Pedigree of Pakistani Family WSPK1 (A) and WSPK2 (B)

protein; in the extracellular, intracytoplasmic or the transmembrane domains. These can destabilize the protein and dwindle the number of receptors on the cell surface, impaired ligand binding, or alteration of the transduction signal (Pingault *et al*., 2010). Approximately 23 mutation have been reported in EDNRB gene so far (Lin *et al*., 2008). In this study, we have investigated two families with patients of WS4 for investigation of mutations in EDNRB gene. The study found a transition of T to C in codon (L361S) in exon 5 of EDNRB gene in the family WSPK1. The mutation was found in the homozygote patients with WS4 and their asymptomatic heterozygote parents. In second family WSPK2, three mutations; a G to C transversion in codon 335 (C335S) in exon 5, a transition of T to C in codon (L361S) in exon 5 and a non coding transversion of T to A at –30 nucleotide position of exon 5 were detected in homozyote patients and heterozygote asymptomatic parents. The association of EDNRB mutations with WS4 was also revealed in a study of a large Mennonite femaily (Puffenberger *et al*., 1994b). A WS4 associated missense mutation (Trp276Cys) in EDNRB gene was identified in the inbred population. The study demonstrated that the EDNRB mutations have pleiotropic effects. And non-enteric phenotypes in the population could only expressed by homozygotic mutations. The study also described the incomplete and dosage sensitive penetrance of the phenotype (Puffenberger *et al*., 1994a). Following the study, there have been at least three further reports of homozygous EDNRB mutation associated with WS4 (Sangkhathat *et al*., 2005). Considering the reports, our study persuaded that the EDNRB mutations follow the recessive

mode of inheritance to express the WS4 phenotype. Pingault *et al*. (2010) revealed their observations that among the EDNRB homozygous (or compound heterozygous, either proved or suspected) cases, about 70% seemed to segregate with a fully recessive transmission, whereas in the remaining families, some heterozygous relatives present with isolated HD, constipation or depigmentation features. The inconsistency in mode of penetrance could be explained as a upshot of mutation position or difference in genetic modifiers (Sangkhathat *et al*., 2005). Conclusively, the overall transmission of EDNRB mutations is complex, but it can be considered that homozygotes have a high probability of developing severe phenotypes (Pingault *et al*., 2010). In summary, we described the penetrance of the mutations of EDNRB gene in two Pakistani families having patients of WS4. This is first report of WS4 and its correlation with the novel mutation described herein.

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A Note on the Diet of Indian Wolf (*Canis lupus*) in Baltistan, Pakistan

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Abstract.-Ten scats of Indian wolf (*Canis lupus pallipes*) from Baltistan were analyzed on the basis of hair reference key of local wild and domestic mammals. Four species of domestic ungulates, one species of wild ungulate and one species of small mammals were found with a frequency of: domestic sheep, 6.25%, domestic goat, 25%; cow, 6.25%; yak, 12.5%; Himalayan ibex, 6.25% and marmot, 6.25%. Plant material was observed with a frequency of 12.5%. In terms of biomass, domestic livestock contributed 90.7%, while the rest 9.3% came from Himalayan ibex and marmot.

Keywords: Scats wolf, ungulates, Baltistan, biomass.

Wolf predation on wild and domestic animals disturbs the economy of rural areas and is the main cause of conflict between humans and wolves. The diet of wolves comes from diverse sources including small poultry to horses. The wild and domestic ungulates constitute the main prey of wolves. Depending on the local availability, wolves mainly prey on middle-sized wild ungulates (Jędrzejewski *et al*., 1992). In the absence of or near non-availability of wild ungulates, domestic livestock serve as the main diet. Diets of mammalian predators are usually assessed by two

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methods: analyzing scats and stomach contents (Kübarsepp and Valdmann, 2003). The knowledge about the diet composition of wolves is necessary for understanding and managing the predator prey relationship. This study provides some information on the diet composition of wolves through scat analysis in Baltistan region of Pakistan.

Materials and methods

The study was conducted in Tehsil Mushabrum (35° 27' 74" N, 76° 20' 92" E) District Ghanchey (35° 28' 72" N, 76° 21' 20" E) of Gilgit Baltistan province of Pakistan. Wolf scats (n=10) were opportunistically collected during the scat collection expedition of snow leopard (*Panthera uncia*) from November 2007 through March 2008. Scat samples were collected by following the trails of wolves between the elevation ranges 3,200 and 3,275 m (10,480-10,750 feet). The samples were stored in labeled polythene bags. The scats were confirmed/identified by using the genetic markers at Department of Veterinary Integrative Biosciences, Texas A&M University, College Station, Texas, USA. For this purpose field collected scats were stored in 15-mL centrifuge tubes and DNA was extracted by using Qiagen Stool kit. A small segment of cytochrome b was sequenced and compared to the reference sequences for genetical identification of scats (Janečka *et al.*, 2008).

For diet analysis, scats were washed and broken apart; undigested hairs were isolated for microscopic comparison with hair reference key (Oli, 1993; Anwar *et al*., 2011). A hair reference key of potential 4 domestic *viz.*, domestic goat (*Capra hircus*), sheep (*Ovis aries*), cow (*Bos taurus*) and yak (*Bos grunniens*); and 4 wild mammals *viz*., Himalayan ibex (*Capra ibex sibirica*), markhor (*Capra falconeri falconeri*), Kashmir marmot (*Marmota caudata*) and pika (*Pica royleana*), was made to identify the prey items. The key was developed on the basis of hair characteristics *viz.*, medullary pattern, hair width and medulla width. The diet composition and relative consumption of food items of wolf was determined by the use of percent frequency of occurrence (PFO). Biomass composition was estimated by averaging the two equations, $y=0.38+0.02x$ (Floyed *et al.*, 1978) and $y=0.135+0.0148x$ (Jethva and Jhala, 2004), where "*y*" is the estimated mass (kg) of prey consumed per scat and "*x*" is the assumed live adult mass of the prey species (Habib, 2008).

Results and discussion

Ten scats of Indian wolf were collected from 8 different localities (Fig. 1). The mean diameter of the scats was 2.4 cm (range=1.5-3.0), with an average total length of 16.2 cm (range=10-31). The average number of segments were 5.2 (range=2-9). Five scats were found with blunt ends and 5 with tapering ends. The mean weight of the scats was 38.59 g (range = 6.34-122.76).

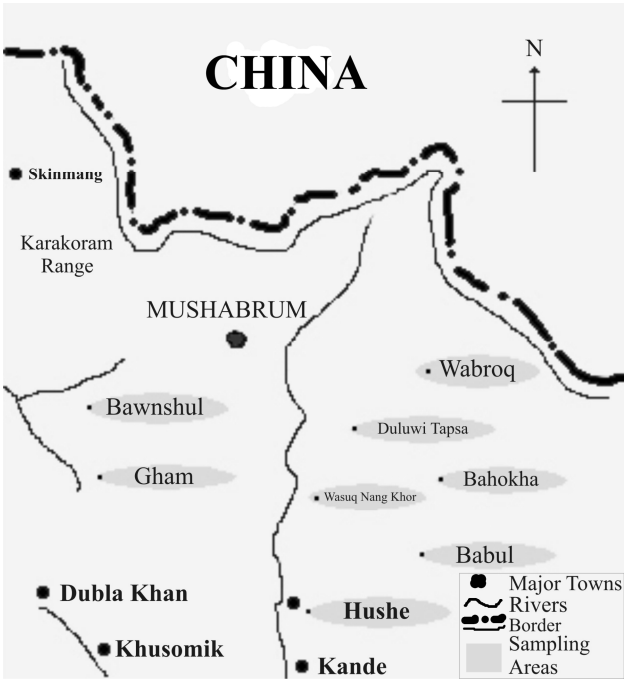

Fig. 1. Tehsil Mushabrum map showing sampling sites for scats of the Indian wolf.

The frequency of occurrence of six prey species of Indian wolf is shown in Table I. The domestic goat was the most frequently found (25%) prey species in the diet of Indian wolf. Yak was the second frequently occurring (12.5%) prey item. Other preys included domestic sheep, Himalayan ibex, Kashmir marmot and cattle, each with a frequency of 6.25%. Plant matter was observed with a frequency of 12.5%. Remaining unidentified

food items contributed about 25%. Roberts (1997) reported Himalayan ibex, marmot and domestic dogs in the diet of wolf from Gilgit and Baltistan region. Schaller (1976) analyzed 63 scats from Chitral and Khunjerab areas and found domestic stock (38%), Himalayan ibex (37%), marmots (17%) and 2% of each Shapu (*Ovis vignei vignei*) / Marcopolo sheep (*Ovis ammon polii*), cape hare (*Lepus capensis*) and grass in the diet of Indian wolf (Table III).

Table I.- Frequency of occurrence of the remnants of prey species in the scats (n=10) of the Indian wolf collected from Baltistan, Pakistan.

Prey items	No. of samples	Frequency of occurrence (%)
Domestic sheep	1	6.25
Domestic goat	4	25.00
Himalayan ibex	1	6.25
Marmot	1	6.25
Cattle	1	6.25
Yak	2	12.50
Plant matter	2	12.50
Unidentified remains	4	25.00

Floyed *et al.* (1978) conducted 9 feeding trials on different sized prey species and developed the linear relationship (y=0.38+0.02x) between ingested biomass and the live weight of the prey species. Jethva and Jhala (2004) also developed a linear relationship (y=0.135+0.0148x), their prey base was almost similar to that of the prey species present in Baltistan region. Habib (2008) studied the food habits of wolves in Afghanistan, and derived a new equation (y=0.217+0.0182x) by taking the average of equation derived by Floyed *et al.* (1978) and Jethva and Jhala (2004). Habib (2008) reported the similar type of prey fauna in the diet of wolves in Afghanistan and used averaged linear relationship in field conditions. In present study biomass consumption was estimated by using the same averaged linear relationship, y=0.217+0.0182x. Yak was found to be the most important prey item (59.2%) in terms of biomass. Domestic goat, cattle and ibex followed with 14.0%, 13.5% and 7.8% respectively. Domestic sheep contributed 3.98% biomass while the share of marmot was 1.56% (Table II).

The most preferred food item of Indian wolf in Baltistan areas was yak followed by domestic goat and cattle. The results of this study showed that 90.7% of the diet of Indian wolf in terms of biomass came from the domestic livestock. Roberts (1997) reported that wolves on getting opportunity feed on domestic goats and sheep and in retaliation hunted ruthlessly in all the northern mountainous region of Pakistan. Indian wolves together with snow leopards, another major carnivore species of the study area, set a pattern of livestock depredation. In future, the survival of this carnivore species can be threatened by the local herders.

IUCN Pakistan in a C.A.M.P. Workshop (2005) rated Indian wolf as endangered whereas the international status of Indian wolf according to IUCN Red Data Book (2011) is 'Least Concern with Stable Population Trend'.

The study rings an alarm bell. Heavy dependence on the domestic livestock shows that the prey base for wolf is extremely rare. This calls

Table II.- Calculation of the biomass (in kg) of various prey species consumed by Indian wolf (n=10).

Prey species	Assumed weight (kg) A	Biomass per scat (kg) B	Number of scats C	Biomass consumed (Relative %) D	Percentage consumption E
Domestic sheep	30	0.8	1	0.8	4.0
Domestic goat	25	0.7	4	2.7	14.0
Himalayan ibex	70	1.5	1	1.5	7.8
Marmot	4.5	0.3	1	0.3	1.6
Domestic cattle	130	2.6	1	2.6	13.5
Domestic yak	300	5.7	2	11.4	59.2

A, assumed weight (kg) of prey species; B, 0.217 + 0.0182 × A; C, scat number in which prey species was found; D, B × C; E, (B × C) / Σ (B × C) × 100.

Table III. Comparison of frequency of occurrence (%) of prey species in the diet of Indian wolf in Pakistan

Prey items	Schaller (1976) % Frequency	Present study % Frequency	Roberts (1997)*
Himalayan ibex	37	6.25	+
Marmot	17	6.25	+
Domestic sheep	-	6.25	+
Domestic goat	-	25.0	+
Cattle	-	6.25	-
Yak	-	12.5	-
Domestic dog	-	-	+
Domestic stock	38	-	-
Shapu	2	-	-
Marcopole sheep	2	-	-
Cape hare	2	-	-
Plant Matter	-	12.5	-
Unidentified	-	25.0	-

*Roberts (1997) reported the food item (+) in diet of wolf but % frequency is not given

for action by the concerned authorities to take measures to develop wild ungulate populations to minimize dependence of wolf on domestic livestock. In view of the local threatened status of wolf in Pakistan it gets all the more important to properly manage the wild ungulate and predator populations to help conserve the population of wild carnivores in general and that of Indian wolf in particular.

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Saprolegniasis in Two Commercially Important Carps

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Abstract.- Two species of carps, *Catla catla*, Hamilton and *Ctenopharyngodon idella*, Valenciennes, 1844, were examined for fungal infection from a private fish farm in Lahore. Both *C. catla* and *C. idella* were confirmed to have fungal infection, saprolegniasis, and concurrent infection of *Saprolegnia* sp. and crustacean copepod *Lernaea* sp. Two fungal genera *Saprolegnia* and *Achyla* and two *Lernaea* species, *L. cyprinacea* and *L. ctenopharngodonis* were identified from these fishes. The prevalence of saprolegniasis was 75% and 43.3% in *C. catla* and *C. idella* respectively. Concurrent infection of *Saprolegnia* sp. and *Lernaea* sp. was 25% in *C. catla* and 56.6% in *C. idella*. Occurrence of saprolegniasis is correlated to ecological and biological factors.

Keywords: Saprolegniasis, *Saprolegnia, Achyla, Lernaea cyprinacea, Lernaea ctenopharngodonis*

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The carp fish farming is practiced in many parts of Pakistan. The establishment of 7829 fish farms (area 45650 acres) in private sector is an indication of rapid growth of fisheries sector in the Province of the Punjab (DGOFP, 2010). The Chinese carp *Ctenopharyngodon idella* and major carp *Catla catla* are commercially very important especially in Asia. The fish fry are stocked in nursery ponds in autumn and kept under crowded condition prior to their stocking in rearing ponds in late winter (Jhingran and Pullin, 1985). During hot summer evaporation of water from fish ponds results in lowering of water level hence, cause reduction in living space for fishes in ponds. This situation makes fishes crowded in ponds and results in fish health problems (Sanathanam *et al.,* 1987*)*. The superficial and systemic infection in fishes eventually results in mortality (Bauer *et al.,* 1973).

Fungi are known to attack fish eggs, fry, fingerlings and adult fish (Roberts, 1989). The *Oomycetes* are economically significant group of mycotic agents which affect many freshwater fishes. Three genera of family saprolegniaceae, *Saprolegnia, Achlya* and *Aphanomyces* are pathogenic. Some species of these genera cause infection in carps (Srivastava, 2009), salmonids (Bruno and Wood, 1999), catfish (Durborow *et al.,* 2003; Loan *et al.,* 2006) and other fishes (Bucke *et al.,* 1979; Copeland and Willoughby, 1982; Xu and Rogers, 1991). Infection by these fungi sometimes results into epizootics among salmonids and other teleosts (Hatai and Hoshiai, 1992, 1993). High mortality in various species of carp cultured in ponds has been attributed to *Saprolegnia parasitica* (Krishana *et al.,* 1990) and in silver carp, *Hypophthalmichthys molitrix* cultured in cages (Jha *et al.,* 1984). Mortality in *Labeo rohita* has also been reported during winter when water had high organic load (Toor *et al.,* 1983). Significant suppression of growth in cultured carp was observed when *Saprolegnia* sp. was associated with *Argulus indicus* infection (Singhal *et al.,* 1990).

Fungal infection in fishes is considered difficult to prevent, especially in intensive freshwater system. Mayer (1991) reported fungal infection to be second to bacterial disease in economic terms in aquaculture. Outbreaks of saprolegniasis in cultured fish are usually restricted to chronic and steady loses. Whereas, in fish hatcheries the fish eggs mortality can increase quickly and cause significant number to die, this condition has big economic impact. When fungal infection in fishes and eggs is mild, it may be treated chemically with some success (Loan *et al.,* 2006). The aim of this study was to investigate fungal infection in commercial carps stocked in earthen nursery ponds and reared under semi intensive culture conditions.

Materials and methods

Disease fish samples of *C. catla* and *C. idella* were collected from a fish nursery pond (area 405m³, with stocking density of 2500) on a commercial fish farm in Lahore during December 2000. The recommended stocking density in nursery pond is 100-600 larvae/m³ (size of larvae 0.5-1.5cm; Horvath *et al.,* 1992). All the ten fishes in test sample appeared to have fungal and parasitic infection, which was visible to naked eye. Subsequently, 50 more fishes were collected. These fishes were wrapped in plastic bags laid in crushed ice and brought to Fish Pathology Laboratory, Fisheries Research and Training Institute, Lahore. The fishes were weighed, measured and examined thoroughly for parasitic and fungal infection on the body.

Sixty fishes were examined of which 54 were infected. The fungal infection appeared as cotton wool spread on different parts of the body of these fishes and was visible to naked eye. The material from the fungal growth was taken on a drop of water on a clean glass slide (wet mount preparation) and observed under a microscope (Olympus Model CH3ORF 200 Japan). Both *C. catla* and *C. idella* showed concurrent infection of crustacean parasite *Lernaea* sp. and *Saprolegnia* sp. *Lernaea* were removed from the fishes, observed under microscope and identified according to Bauer *et al.,* (1973) and Kabata (1985). The *Lernaea* parasites were preserved in 70% alcohol for subsequent study. The water quality parameters such as turbidity, water temperature, dissolved oxygen and pH of infected fish nursery pond was recorded at the site with the help of Secchi disk; DO meter YSI (Model 57) and digital pH meter. The photographs

of fungi were taken by camera fitted on microscope (Model SC 35.Type 12).

Results

The Secchi disk reading of infected nursery pond ranged from 8-10cm. The pond water was dark green in color with poor visibility. The mean water temperature was 13º C, pH 8.8 and dissolved oxygen was 3.8mg/L. Mean weight and mean length of *C. idella* was higher as compared to C. *catla.* However, the mean weight and mean length of 6 uninfected *C. catla* was higher than that of infected *C. catla* (Table I).

Table I.- *Ctenopharyngodon idella* and *Catla catla* infected with a *Saprolegnia* sp. and *Learnae* spp.

	C. idella	*C. catla*	
		Infected	Un-infected
No. of fish examined	30	30	6
No. of fish infected	30	24	-
Prevalence (%)	100	80.0	-
Mean total length (cm)	11.86±2.45	7.04±1.49	12.40±1.80
Mean weight (g)	119.07±26.47	70.05±17.05	125.0±20.g
Saprolegniasis	13 (43.3%)	18 (75%)	-
Concurrent infection	17 (56.5%)	6 (25%)	-

**C. catla* 6 uninfected fishes; (Concurrent infection means infection of fish with *Saprolegnia* sp. and *Lernaea* sp. at the same time)

Two types of infections were observed in these fishes; saprolegniasis and concurrent infection of *Saprolegnia* sp. and *Lernaea* sp. The occurrence of saprolegniasis was higher in *C. catla* (75%) compared to *C. idella* (43.33%). Concurrent infection was higher in *C. idella* (56.6%) than in *C. catla* (25%) (Table I). The clinical picture of fishes with saprolegniasis, revealed typical cotton wool like fluffy appearance on the body and fins. The infection was superficial and chronic on fins, skin, lateral side and abdomen of the fish. In mild cases of saprolegniasis, fishes had patchy lesions (2-4 mm in diameter) on the body. Infected skin was thin and de-pigmented, the tips of fins were damaged. The lesions on the skin were ulcerative and penetrating into muscles. Organs like eyes were infected in serious cases. Infected eyes showed endopthalmia and extensive growth of fungal hyphae causing complete blindness of the fish (Fig. 1.). Infection starts from the tip of the fin and proceeds towards base, and this result in complete erosion of fin as seen in caudal fin (Fig. 1). Erosion of fins leads to the destruction of epidermis and exposure of caudal peduncle. Two genera of fungi *Saprolegnia* sp. and *Achyla* sp. were identified by their characteristic hyphae and sporangium. *Saprolegnia* sp. was isolated from bodies of both *C. catla and C. idella* (Fig. 2) and *Achyla* sp. from eyes of *C. idella*. Both *C. catla* and *C. idella* seems very susceptible to pathogenic *Saprolegnia* sp. and *Achyla* sp.

L. cyprinacea and *L. Ctenopharyngodonis* were attached on ventral side of the body of the fish, at the base of pectoral fin, pelvic fin, caudal fin and near the orbit of the eye in *C. catla* and *C. idella* respectively. The mean intensity of *L. cyprinacea* was 4.5 parasite/fish (range 4-6) and that of *L. ctenopharyngodonis* was 3.0 parasite/fish(range 1-5). The microscopic examination showed that fungal hyphae covered and penetrated the body and egg sack of *Lernaea* (Fig. 3).

Discussion

The higher mean weight and mean length of *C. idella* as compared to *C. catla* may be attributed to their age which is normally three to four month older than C. *catla.* 100 % prevalence in *C. idella* may be due to their longer stay in the pond. Fungal infection observed in this study in *C. catla* and *C. idella* is suggested to be associated with factors such as, injuries caused by high stocking density; unsuitable water quality and excess of organic manure added in the pond. Mechanical damage to the skin of farmed brown trout *Salmo trutta* due to high stocking density was considered to be responsible for an increased *Saprolegnia* infection (Richards and Pickering, 1978). Environmental stress such as poor water quality, handling and overcrowding (Bailey, 1984), high organic load in water (Toor *et al.,* 1983) can all result in increased occurrence of fungal infection. This means that the fungi act as secondary pathogens. However, some fungi like *Saprolegnia parasitic* act as primary pathogen too (Durborow *et al.,* 2003). Fungal attack on fins leads to erosion of tips and complete damage of the fin, as seen in this study. Willoughby (1994) reported that a fish, with one fourth of posterior part of the body eroded due to heavy fungal infection

Fig. 1. *Saprolegniasis* in *C. idella,* with infected eyes and eroded caudal fin.

Fig. 2. *Saprolegnia* hyphae and sporangium (S) from *C. idella*

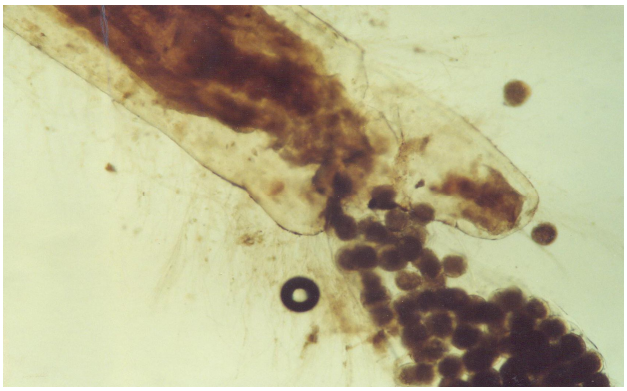

Fig. 3. *Saprolegnia* hyphae penetrating *Lernaea* and its egg sack.

still survived. In severe cases, 80% of the body of fish may be covered with fungal hyphae (Richards and Pickering, 1978).

The initial infection site may not be confined to gills, skin or fins but it may be any other site such as eye, as seen in this study. Eye infection damages cornea due to tight adherence of fungal hyphae. From eyes the fungus penetrates into the brain and in such condition treatment is impossible and eventually fish die (Srivastava, 2009). The cause of pathogenis of this serious disease is not clear. However, the risk factor may be the inability of fish to adjust to the low temperature during winter. As, in such condition the immune system is impaired, which cause loss of mucus from the skin and even suppress mucus production by goblet cells in the dermal layer of skin (Durborow *et al.,* 2003). The skin of the fish without mucus is unprotected and fungal spores start developing on it and result in growth of hyphae which may even extend into muscle tissues in chronic cases. The specific number of pathogenic zoospores in water may be regarded as risk factor. The initial *Lernaea* infection causes skin lesions on fish. These lesions become the sites of attack by secondary invader the zoospores of *Saprolegnia* sp., this results in concurrent infection. *Saprolegnia* sp. has also been observed in wild *Salmo salar* infected with *Gyrodactylus salaris* (Johnsen, 1978) and *Gyrodactylus* sp. which damaged the skin of the host (Heggberget and Johnsen, 1982). Concurrent infection of *A. indicus* and *Saprolegnia* significantly suppressed the growth of *C. carpio*, which shows that comparative growth rate can be used as indictor of parasite stress to fish (Singhal *et al.,* 1990). This means that concurrent infection can cause reduction in growth rate of fishes. The concurrent infection observed in present study initiated by primary pathogens such as, *L. cyprinacea* and *L. ctenopharyngodonis* in *C. catla* and *C. idella* which later on facilitated secondary *Saprolegnia* infection in these fishes.

The water in the infected pond was unsuitable for fish culture due to poor quality (high pH and low dissolved oxygen) according to Boyd and Tucker (2003). High turbidity and poor visibility of pond water due to organic load make the ecological conditions stressful for the fish. Moreover, overcrowding of pond with fishes leads to close contact between fishes, this condition facilitate infection by the pathogens such as *Lernaea.* In the present study the stocking density in nursery pond was high with regard to the weight and length of the

fishes (Table I). This infection cause superficial injuries on skin of the fish. These injuries become the footholds for fungus to attack. In these stressful ecological conditions the chances of fungal infection increases. Hence, it is suggested that fish farmer must minimize the chances of occurrence of such injuries to the fishes. Treatment of fish prior to stocking in ponds is recommended. *Achlya* sp. which dominates saprolegniasis has successfully been treated with gentian violet (1.5ppm) and hydrogen peroxide (Loan *et al.*, 2006). Nursery ponds must be stocked with healthy fish seed and appropriate stocking density may be maintained for short time.

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Lymphoid Leucosis in Chicken – Histopathology of Spleen

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Abstract.- Lymphoid leucosis has been reported in both captive and free living wild birds. It is caused by retrovirus. This disease causes severe loss due to mortality of poultry birds and depressed performance. The avian leucosis virus (ALV's) is prevalent throughout the world, new strains from one location may spread across borders, thereby undermining the disease control measures. Four commercial egg type white dead birds (33 weeks old) were received from a commercial layer poultry farm of Karachi. These birds had no previous sign of ill health. On post mortem the spleen was found diffusely enlarged and white in colour. A portion of the infected spleen tissue was preserved in 10% formalin and processed for 10 µm thick histological sections. The section of spleen showed diffused heavy infiltration of lymphoid cells. High magnification showed cells consisting of small and large lymphocytes. Cortex and medulla alongwith infiltration of lymphoid cells was prominent.

Key words: Lymphoid leucosis, chicken, spleen, lymphocytes, Pakistan.

Among important diseases causing mortality in poultry is avian lymphoid leucosis. The retrovirus which causes avian lymphoid leucosis are oncogenic and has been categorized into six groups namely A, B, C, D, E and J (Roberts *et al.*, 2007). Lymphoid leucosis causes severe loss due to mortality of birds and depressed performance. The avian leucosis virus (ALV) is prevalent throughout the world, new strains from one location may spread across borders, thereby undermining the disease control measures. Clinically the avian lymphoid leucosis is usually seen in chicken above 12 weeks of age. The most prominent symptoms are diarrhoea, inappetence, weakness, emaciation, dehydration, and depression before death (Mathew *et al.*, 2010). ALV can be transmitted vertically from parent to offspring or horizontally from bird to bird. Merck (1998) reported tumorous swelling in visceral organs in the absence of peripheral nerve enlargement as a gross pathological feature of diagnostic significance. In the present study histology of chicken spleen is being reported for the first time from Pakistan based on clinical and pathological features characteristic of avian leucosis.

Materials and methods

Four commercial egg laying white dead birds (33 weeks old) were received from a commercial layer poultry farm of Karachi. The birds had no previous symptoms of ill health. On postmortem examination, the spleen was diffusely enlarged and white in colour. A portion of infected spleen was preserved in 10 percent formalin and processed for histological studies. The tissue was dehydrated in different concentrations of ethanol, and embedded in paraffin wax at 52°C for 10 days. 10 µm thick sections stained with haematoxylin and eosin.

Results and discussion

In the present study no neural enlargement was observed but spleen revealed architectural distortion (Fig. 1A). The differential diagnosis of lymphoid leucosis and Marek's disease in chicken is usually made on a number of factors which include age, incidence, clinical signs, macroscopic observation and histology (Purchase and Burmester, 1978; Wadsworth *et al.*, 1981). Main histo-pathological change was diffuse heavy infiltration of lymphoid cells (Fig. 1B). High magnification showed cells consisting of small and large lymphocytes. The large lymphocytes were poorly stained whereas the smaller were deeply stained (Fig. 1C). Cortex and medulla alongwith infiltration of lymphoid cells was obvious (Fig. 1D). The avian leucosis virus induces an obvious lymphoma in chicken called lymphoid leucosis (Pizer and

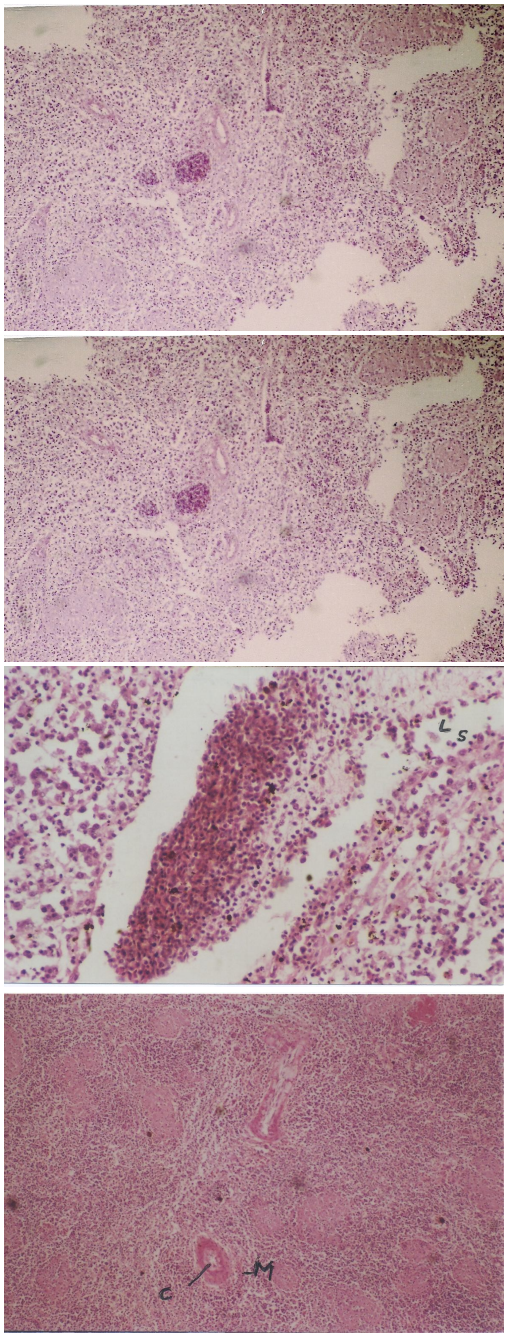

Fig. 1. A, Histology of spleen revealed distortion of organ rehitecture. X25; B, Main histopathological change was diffuse heavy infiltration of lymphoid cells (←) X25; C, High magnification showed cells consisting of small (S) and large (L) lymphocytes. The large lymphocytes are poorly stained while the smaller one are deeply stained. X50; D, Section showing cortex (C) and medulla (M) alongwith infiltration of lymphoid cells. X25.

Humphries, 1989). In studies conducted in India Balachandran *et al.* (2009) reported that lesion suggestive of lymphoid leucosis was observed in 15 tissues (2.16%) of layer birds, their study revealed that the lymphoid leucosis involved liver (26.67%) and spleen (73.33%) tissues. However, lymphoid leucosis was not detected in any of the tissue from broiler birds. The involvement of spleen in lymphoid leucosis is of considerable diagnostic importance (Wadsworth *et al.*, 1981). No neural involvement was present, while in Marek's disease neural involvement is a conspicuous feature (Halliwell, 1971). The age of birds in the present study with lymphoid leucosis was in agreement with earlier reports (Darcell, 1994); Balachandran *et al.*, 2009).

The rarity of lymphoid leucosis reports in commercial and free ranging chicken in Pakistan could possibly be due to under-reporting. For accurate definitive diagnosis of lymphoid leucosis can be made by the identification of the circulating viruses (Wang and Juan, 2002) will depend on identification of lymphoid leucosis.

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Table II.- Sensitivity of isolated microorganisms from the biodeteriorated paints against different biocides at different concentrations.

Strains	$HgCl_2$ (cm)			$CdCl_2$ (cm)			$Cu(CH_3COO)_2$ (cm)			$(NH_2)_2CO$ (cm)		
	100 ($\mu g/ml^{-1}$)	200 ($\mu g/ml^{-1}$)	300 ($\mu g/ml^{-1}$)	100 ($\mu g/ml^{-1}$)	200 ($\mu g/ml^{-1}$)	300 ($\mu g/ml^{-1}$)	100 ($\mu g/ml^{-1}$)	200 ($\mu g/ml^{-1}$)	300 ($\mu g/ml^{-1}$)	100 ($\mu g/ml^{-1}$)	200 ($\mu g/ml^{-1}$)	300 ($\mu g/ml^{-1}$)
MSF1	1.1±0.05	1.3±0.03	1.8±0.07	0.5±0.05	0.7±0.04	1±0.07	0.1±0.002	0.5±0.009	0.7±0.03	0.09±0.017	0.1±0.009	0.7±0.03
MSF2	1.5±0.03	1.6±0.09	1.9±0.05	1.9±0.05	0.2±0.001	0.6±0.009	0.18±0.01	0.25±0.07	0.4±0.02	0.2±0.05	0.5±0.03	0.8±0.03
MSB1	1.0±0.04	1.1±0.06	1.4±0.08	1.4±0.08	0.6±0.02	0.9±0.017	0.1±0.03	0.4±0.007	0.7±0.01	0.09±0.01	0.2±0.011	0.6±0.013
MSB2	0.8±0.025	1.2±0.05	1.5±0.03	1.5±0.03	0.3±0.017	0.5±0.03	0.09±0.007	0.12±0.001	0.5±0.003	0.2±0.011	0.5±0.04	0.7±0.05