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## CONTENTS




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# DEVELOPMENT OF DIPSTICKS FOR EASY AND QUICK DETECTION OF *BACILLUS THURINGIENSIS* (BT) CRYSTAL (CRY) PROTEINS AND BT-RECEPTORS

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**Abstract:** A new method, for quick and easy detection was established, which was based on sandwich ELISA technique. By applying that method, the types of Crystal proteins in Bt isolates and Bt Cry1A receptor varieties in crude BBMVs extracts of target pests can be detected within one hour, before going into the tedious work of protein purification and western blotting techniques.

**Key words:** Dipstick, Detection, *Bacillus thuringiensis*, receptor.

## INTRODUCTION

*Bacillus thuringiensis* (Bt) is a Gram-positive, aerobic, spore forming, and soil dwelling bacterium, which produces an enormous variety of intracellular proteinaceous crystalline inclusions known as δ-endotoxins, after the completion of exponential growth. The proteins comprising the crystal are vanguard of active ingredients for biological control of commercially important insect pests, household pests, and vectors of animal and human diseases (Goldberg, and Margalit, 1977; Macintosh, *et al.*, 1990; Feitelson, *et al*, 1992). Bt is highly specific, with toxicity limited to only some species of major groups of insects typically lepidoptera (butterflies/ moths), diptera (flies/ mosquitos), and coleoptera (beetles). New Bt proteins are being discovered which are active against other orders of insects and pests, such as mites, flatworms and nematodes. Key agricultural pests currently targeted with Bt insecticides include bollworms, stem borers, budworms, leafworms and beetles. Bt δ-endotoxin has been used successfully as a natural pesticide in agriculture, forestry, and health for several decades due to the adverse effects of agrochemicals including lack of selectivity toward beneficial insects, environmental hazards, and health concerns as well as evolution of resistance. Resistance to chemical pesticides in more than 500 species of insects has been reported (Georghiou, and Lagunes-Tejeda, 1993).

A new method was developed for easy and quick detection of Bt crystal proteins for screening of Bt isolates and Bt receptors in crude extract of solubilized BBMVs of target pests. Antibody-based Enzyme-Linked Immuno-Sorbent Assay (ELISA) method was used, by a different format for displaying immune response called a "sandwich" assay.

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## MATERIAL AND METHOD

All the reagents used were prepared from Sigma Biochemicals, USA or Merck Biochemicals, Germany. *Helicoverpa armigera* and *Earias vitella* larvae were obtained from the insectory of the Institute.

*Preparation of ICPs (Cry IAa, Cry IAb, CryIAc, Cry2A)*

Insecticidal Crystal proteins (ICPs) were prepared by the procedure described by Lee, *et al.,* (1992). *Bacillus thuringiensis* cultures were grown on T3 agar plates until complete sporulation. The spores were scraped off the plates and re suspended in sterile water. The suspensions were centrifuged, pellets resuspended in sterile distilled water with a ratio of about 1ml per culture, and stored at –20°C. To extract the Bt. delta-endotoxin for bioassay the suspension was centrifuged, supernatant discarded and pellet was resuspended in an equal volume of alkalic buffer (50mM Sodium Carbonate, 10mM dithiothreitol pH 10) and incubated for 3 hours to overnight at 37°C. The samples were then centrifuged and the supernatants taken for further use.

*Preparation of brush border membrane vesicles*

Brush border membrane vesicles were prepared from isolated midguts of the 4$^{th}$ instar target larvae (*Helicoverpa armigera*, *Earias vitella* and *Tribolium castaneum*). The larvae were placed on ice for 15 minutes; the head and abdomen were grasped with fine tweezers. Careful pulling was done to separate the gut from the cuticle. The isolated midguts were placed on ice chilled Buffer A solution (300 mM Mannitol, 5 mM EGTA, 17 mM Tris-HCl, pH 7.5). BBMV will be prepared by the differential Magnesium precipitation method (Wolfersberger, *et al.*, 1987). Chilled midguts were weighed and immersed in nine times their weight, of ice-cold Buffer A. Prepared a uniform homogenate of the midguts using a Dounce Homogenizer. After about ten excursions of the motor-driven pestle, saved a small aliquot of the homogenate and diluted the remainder in equal volume of ice-cold Magnesium Chloride solution (24 mM $MgCl_2$). Placed the mixture on ice for 15 min and centrifuged at 4,500 rpm for 15 min (4°C). Decanted the supernatant in a clean tube and centrifuged again at 16,000 rpm for 30 min (4°C). Resuspended the pellet in one half, original homogenate, volume of Buffer A and prepared a uniform suspension using the Homogenizer. Diluted with equal volume of 24 mM $MgCl_2$ and placed in ice for 15 min. Centrifuged the mixture at 4500 rpm for 15 min, saved pellet and centrifuged the supernatant again at 16,000 rpm for 30 min (4°C). The pellet from this centrifugation contained the Brush Border Membrane vesicles, & it was stored at –70°C.

The BBMV's were solubilized in solubilization Buffer containing 20 mM Tris-HCl (pH: 7.4), 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulphonate (CHAPS) at 25°C for 30 min. Removed the debris by centrifugation at 14,000 rpm for 15 min (4°C) and estimated the concentration using the Bio-Rad protein assay reagent.

*Production of antibodies*

One milligram of purified receptor protein was mixed with an equal volume of Freund`s Adjuvant Complete (Sigma F-5881) and used to inject rabbits subcutaneously. Sample of 5ml blood was taken from rabbit just before the first injection to prepare the preimmune serum. A second injection was given three weeks later using the same method except that Freund`s Complete Adjuvant was replaced by Freund`s incomplete Adjuvant (Sigma F-5506). About 1ml serum was recovered from 5ml blood samples taken from rabbit two days after the second injection. Rabbit was bled by heart puncture to collect maximum blood two days after the third injection. Antiserum separated and stored at -20°C. Antibodies were further purified from one aliquot of antiserum by affinity purification.

To affinity purify the antibodies by Immunoabsorbant technique as described in Rybicki, *et al.,* (1990), with slight modification.

After the binding of receptor protein to nitrocellulose membrane, washed the membrane 3 times, 5 minutes each wash with TBST (10mM Tris-HCL, pH8.0, 150mM NaCl, 0.05% Tween 20). The protein containing membrane was blocked for o/n at 4°C in blocking solution (TBST containing 5% skimmed milk and 0.05% Sodium Azide). Three washings of 5 minutes each, with TBST followed blocking, followed by incubation in antiserum for two hours at room temperature. After incubation with the antiserum, 3 washes, 5 minutes each wash were given to the membrane. Bound antibodies were then eluted by 0.1M glycine pH 2.9. The pH of the eluant was immediately adjusted to 7.00 with 0.1N NaOH solution (addition of 85ul of 0.1N NaOH per 500ul of 0.1M glycine pH 2.9 gave the required pH 7.0). Eluted antibodies were stored at -20C till further use.

To utilize this assay, one antibody (the "capture" antibody) was purified and bound to a solid phase. Antigen was then added and allowed to complex with the bound antibody. Unbound products were then removed with a wash, and a labeled second antibody (the "detection" antibody) was allowed to bind to the antigen, thus completing the "sandwich". The assay was then quantitated by measuring the amount of labeled second antibody bound to the matrix, through the use of a colorimetric substrate. A major advantage of this technique was that the antigen did not need to be purified prior to use, also that these assays are very specific.

*Dipstick assays*

Coated the antibodies (specific to antigens) on a nitrocellulose membrane strips, allowed to dry the strips then placed in blocking solution (5% skimmed milk in 1x PBST. After washing in 1xPBST,dip the strips in the sample to react prespotted antibody dots to the target analyte. After washing, passed the strips through secondary antibody and color solutions. The whole process was completed within 60 minutes. Visual interpretation of the test results was done by dot`s color intensity.

## RESULTS AND DISCUSSION

Purified antibodies of Bt Cry proteins and Bt-receptor were spotted on nitrocellulose membrane strips, passed the strips through sample and secondary antibody solutions. Positive results are indicated by the presence of colored reactive dots on the membrane as indicated in Fig I (2).

Crude samples of Bt isolates were spotted on nitrocellulose membrane strips, passed the strips through anti-Cry1Ac antibody and secondary antibody solutions. Positive results are indicated by the presence of colored reactive dots on the membrane as indicated in Fig I (4).

Bt isolates and Bt receptors were checked by a new immunoassay. In that assay, dipsticks were developed for quick and easy detection of types and novelty of Bt crystal proteins in screening of Bt isolates and Bt-receptors in target pests. By applying that method, kinds of Bt Cry1A receptor can be detected in crude BBMVs extracts of target pests before going into the tedious work of receptor purification. Dipstick method, which is very economic and specific, can be used in the detection of Cry protein types in Bt isolates. Other methods of detection such as gel electrophoresis and insect bioassay are labor-intensive and time-consuming.

To our knowledge, so far no one study has used dipstick test to detect Cry proteins in Bt samples and Bt receptor in insect pests. The test was shown to have 95% specificity and sensitivity, in the present study. This method has many useful applications. It will be helpful in screening of Bt and pest control programmes.

The main threat to the long-term success of Bt transgenic crop approach is evolution of resistance by pests. So far, evolution of insect resistance to Bt crops in the field has not been reported (Tabashnik, *et al.*, 2003). Nonetheless strains of more than a dozen species of insects have evolved resistance to *B. thuringiensis* toxins in laboratory selection experiments. Moreover, many cases of field-evolved resistance to *B. thuringiensis* sprays have been reported for the diamondback moth, *Plutella xylostella*, a pest of crucifer crops. The most common mechanism of resistance is altered binding of *B. thuringiensis* toxins to target sites in the brush border membrane of the larval midgut (Ferre, and Van Rie, 2002).

So the Bt receptor detection and identification will help in detailed understanding of resistance mechanisms. This understanding together with increasing knowledge of pest biology and plant molecular biology, and the possibility to experimentally evaluate resistance-management methods on a small scale should allow, for fine-tuning of existing management tactics and design alternative options. The careful implementation of such tactics should safeguard the value of Bt for insect control.

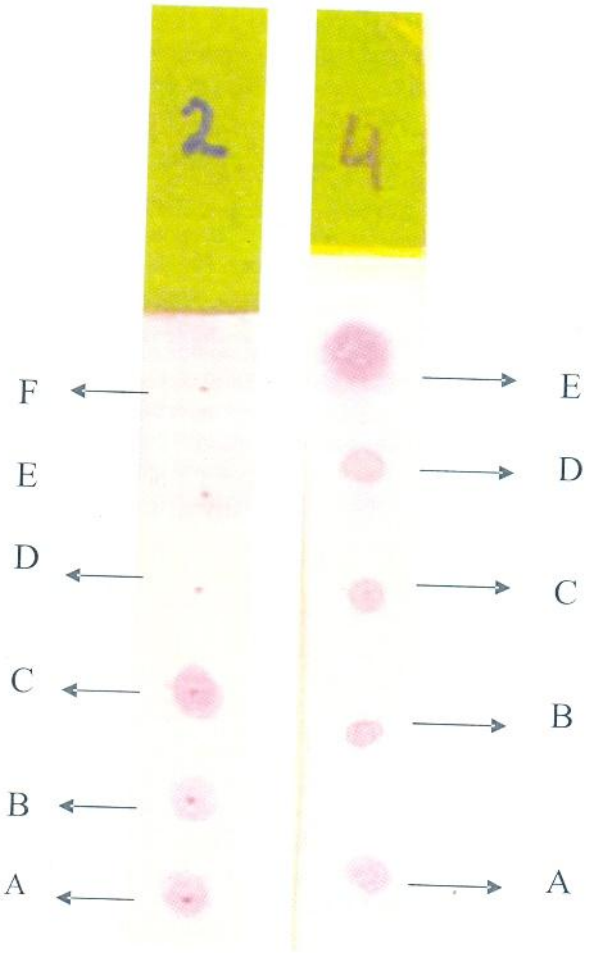


**Fig 1. Development of Dipsticks for easy and quick detection of Bt crystal proteins and Bt-receptors. Dipstick 2; (A) *H.armigera*`s BBMVs (B) *H.armigera*`s receptor, (C) *E.vitella*`s BBMVs, (D) *T. casteneum*`s BBMVs (E) Aphid`s BBMVs, (F) no antigen .Dipstick 4; (A)(B)(C)(D) Cry1Ac positive Bt isolates (E) anti-Cry1Ac & Cry1Ac as positive control.**

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# EFFECTS OF TENEKIL PLUS, AN ORGANOCHLORINE, ON DEVELOPMENT OF CHICK

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**Abstract:** Tenekil Plus, an organochlorine insecticide, was tested for embryotoxic and teratogenic effects in chick. Different concentrations of the insecticide (2.37, 4.75, 9.5 and 19 µg/egg were injected into the yolksac of eggs, at zero day of incubation. Recoveries were made at day 7 and 15 of incubation. Morphological studies revealed concentration dependent adverse' effects of the insecticide. The body weight and CR length decreased significantly ($P<0.001$) in each dose group. The developmental abnormalities observed were anencephaly, microcephaly, microphthalmia, anophthalmia, agenesis of beak, micromelia, ectopia cardis and phocomelia. The present study indicates that Tenekil Plus is potentially dangerous to developing chicks, which may be equally harmful to mammals especially human development too.

**Key words:** Avian development, Chick, Tenekil Plus, an organochlorine, Developmental anomalies.

## INTRODUCTION

Pesticides have a variety of effects on reproduction. In exposed people, pesticides cause birth defects, miscarriages, low birth weights and decreased fertility (Schwartz and LoGerfo, 1988; Garry, 1996; Savitz, 1997).

Organochlorine chemicals such as polychlorinated biphenyls (PCBs), Chlordane, Dichlorodiphenyltrichloroethane (DDT), Dichlorodiphenyldichloroethylene (DDE), and Lindane were greatly used worldwide during several decades for agricultural and industrial purposes. (Swain, 1991; Daston, *et al.,* 1997; Golub, *et al.,* 1991; Fisher, 1998; Battershill, 1994). Most chlorinated hydrocarbons pesticides are persistent, have propensity to bioaccumulate, biomagnify in the food chain and are toxic to non-target species (Eisler and Jacknow, 1985).

Organochlorines have been widely used during previous years (Lodha and Sexena, 1991). Extensive use and limited biodegradation are the two major factors involved in their worldwide contamination and biomagnification (Hargrave *et al.,* 1992; Fossi *et al.,* 1995; Nichols *et al.,* 1995). Several chlorinated hydrocarbons that exist in the natural systems are toxic to humans (Gribble, 1994).

Many organochlorines are endocrine disrupters or carcinogens. Of particular concern, is the finding of neonatal hypotonia or hyperflexia in relation to PCB exposure. Many chlorinated hydrocarbons are known as hepatic tumor promoters and stimulate the protein kinase C activity *in vitro* (Longnecker *et al.,* 1997).

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Organs that appear to be at particular risk for developmental abnormalities in offspring because of maternal exposure are those with receptors for gonadal hormones: in female fetuses this includes the mammary glands, fallopian tubes, uterus, cervix. and vagina, and in male fetuses it includes the prostate, seminal vesicles, epididymes, and testes. In both sexes the external genetalia, brain, skeleton, thyroid, liver, kidney and immune system are also targets for steroid hormone action and are thus potential targets for endocrine-disrupting chemicals although these chemicals have multiple mode of action, in addition to acting as hormone agonists and antagonists, in different target tissues (Colby, 1980; McEwen, 1981; Leatherland, 1982; Grossman, 1984).

Studies have found organochlorine chemicals to be the cause of widespread catastrophic effects on wildlife including: eggshell thinning, deformities and high mortality in birds and eagles, abnormal thyroid function in fish and birds, abnormal hormone levels in birds, alligators, and mammals, decreased fertility in birds, fish, shellfish, otters, and minks, emasculation and feminization of male fish, birds, turtles, alligators, otters, minks, beluga whales, polar bears, and panthers, defeminization and masculation of female fish, gastropods, turtles, birds, and mammlas, alteration of immune function in birds and mammals, birth defects and high infant mortality in mammals, behavioral changes in birds, abnormal sex organs and intersexed birds, turtles, alligators, sturgeon, etc., low testosterone levels and undescended testes in alligators and panthers, strongly significant dose related relationship to endometriosis in monkeys, production of vitelogin, a female protein, by male fish living near sewer outfalls, doubled rate of testicular cancer and reproductive defects in military dogs used in Vietnam and their offsprings in the Great Lakes area and Florida (Windham, 2001; Raloff, 1994; Seal Rehabilitation and Research center and Netherlands National Institute of Health, 1994; Kuehl, and Haebler, 1995; Gross, *et al.,* 1994; Guillete, *et al.,* 1994; Human and Ecological Risk Assessment; Guillete, 1994; Monks, 1994; Blus, 1984; Hickey, and Anderson, 1968; Colbom, *et al.,* 1996; Colbom, and Clement, 1992; Colbom, *et al.,* 1993; Colbom, *et al.,* 1995; U.S.EPA, 1994; U.S.EPA, 2002; Bimbaum, 1994; Endometriosis Association, 1995; Canadian Department of Health and Welfare, 1985; Jobling, *et al.,* 1995; Menzer, 1995; Davis and Bradow, 1995; Meersman, 1999).

Exposure to endocrine-disrupting chemicals in the environment has been. . associated with abnormal thyroid function in birds (Moccia, *et al.,* 1986) and fish ',. , (Moccia, *et al.,* 1981) decreased fertility in birds (Shugart, 1980), fish (Leatherland. 1992), shellfish (Gibbs, *et al.,* 1988), and mammals (Reijnders, 1986); decreased hatching success in fish (Mac, *et al.,* 1988), birds (Kubiak, *et al.,* 1989) and turtles (Bishop, *et al.,* 1991); demasculinization and feminization of male fish (Munkittrick, *et al.,* 1991), birds (Fry and Toone, 1981) and mammals (Beland, 1989); defeminization and masculinization of female fish (Davis and Bortone, 1992), gastropods (Ellis and Pattisina. 1990), and birds (Fry and Toone, 1981 ); and alteration of immune function in birds (Erdman, 1988) and mammals (Martineua *et al.,* 1988).

One type of deformity commonly caused among bird populations and in millions of commercially raised chickens exposed to low levels of dioxin or other dioxin-like chemicals is chick-edema disease, which causes twisted beaks, crooked legs, deformed

claws and feathers, and other abnormalities (Monks, 1994; Blus, 1984; Hickey and Anderson, 1968).

In the 1960's the organochlorine pesticide DDT was found to cause eagle eggs to have thin shells and thus crack easily. A recent report by Dr. Theo Colborn, of the World Wildlife Fund, and her colleagues, described the inability of bald eagles in Washington to reproduce after feeding on local fish. The eagles contained up to 10 times as much of the organochlorines DDT, PCBs and Chlordane as would allow them to reproduce successfully (RHWN No.365, 1993).

Male herring gulls on Lake Ontario, exposed to DDT and other organochlorines, were found with female sex organs. When gulls eggs were injected with DDT in the laboratory, the male offspring had female sex organs, strengthening the causal link with organochlorines (RHWN No.264, 1991).

In 1984, a Florida biologist found that in Lake Apopka, 90 % of the alligator eggs suddenly failed to hatch. The few males that did hatch had phalluses so tiny that they were useless for reproduction (Foreman and Globe, 1994).

Organochlorines may also be a factor in decreased sperm count in humans. In 1977, male workers at an Occidental Chemicals factory making the organochlorine pesticide dibromochloropropane (DBCP) noticed that none of their wives had had any children since the men had begun working with the chemical. Tests revealed that the majority of the men had zero or severely reduced sperm counts, but no other noticeable health effects (Sexton, 1993). Men with low sperm counts have been found to have significantly higher concentrations ora number of organochlorines in their semen. There is also evidence linking organochlorines with health effects in humans, including testicular cancer, lowered sperm count and birth defects (Thomton, 1993).

Evidence clearly exists that a number of organochlorine chemicals (such as Dioxin, PCBs, and DDT) have reached in aquatic food sources that can lead to substantial functional deficits in animals that consume this food. Male rats fed Lake Ontario fish showed hyperactivity to stress, and offspring of females fed Lake Ontario fish during pregnancy also expressed the same hyperreactive condition, although the offspring were never fed fish (Daly, 1992). In addition, offspring of women who ate two to three Lake Michigan fish a month for at least 6 years preceding their pregnancies were slightly preterm, had lower birth weight, smaller skull circumference, and cognitive, motor (hypotonicity and hypoflexivify), and behavioral deficits at birth compared with offspring whose mothers did not eat fish. The defects were associated with the mother's lifetime experience of eating fish, not just what they ate during pregnancy. These findings emphasize the importance of exposure of females to contaminants before pregnancy in terms of effects on their offsprings (Fein *et al.,* 1984).

Tenekil Plus is an organochlorine pesticide (C8-C20) prepared by chlorinating certain petroleum and is admixed with a pyrethroid to enhance its efficacy against termites. Tenekil Plus has been developed as a result of extensive research by PCSIR, Pakistan and has been thoroughly field tested (PCSIR, 1996).

All the known insecticides may cause damage to the living organisms in one way -or another. Above-mentioned studies have indicated that organochlorine insecticides are

toxic for non-target organisms and are also embrotoxic and feto-toxic. Thus the purpose of present study was to evaluate the teratogenic effect of Tenekil Plus in developing chicks.

## MATERIALS AND METHODS

Fertilized eggs of *Gallus domesticus* were used. The eggs were purchased from Veterinary Research Institute, Lahore. The eggs were selected randomly and divided into five different groups.

For dose administration, eggshells were cleaned with the help of alcohol, holes were made without rupturing the shell membranes. Then 0.1ml of each concentration (0.00, 2.37, 4.75, 9.50 and 19.0 µg) was injected into yolk sac of eggs of respective group, with microapplicator. All these treatments were applied in sterilized conditions. Following injection, the hole in the egg shell was sealed with liquid paraffin wax.

Eggs were incubated at 37.5±0.5°C in the presence of continuous water supply. Recoveries were made on day 7 and 15 of incubation. The embryos were fixed in Bouin's fixative for 48 hours. Then washed in 70% alcohol and finally preserved in 80% alcohol for morphological studies.

Morphological observations involved measurements of crown-rump length as well as gross anatomical observations. The developmental conditions of brain, eyes, ear, limbs, beak etc. were studied with the help of magnifying lens and with naked eye depending upon the size of the embryo. The data were analyzed using student, t test. The selected embryos were macrophotographed.

## RESULTS AND DISCUSSION

The purpose of present study was to evaluate the developmental toxicity of Tenekil Plus in avian system.

The main observations made during the present investigation were significant reduction ($P<0.01$) in CR length and body weight (Fig.1, 2; Table 1, 2) in embryos of all dose groups. Some major developmental anomalies such as ectopia cardis, microphthalmia, micromelia, amelia, phocomelia, agenesis of beak and twisted spinal cord were also noted in all treated groups (Table I and 2).

The results of present study are similar to the earlier results that organochlorines are toxic to embryonic and fetal organs and can induce teratogenicity in chicks. It was found that tenekil injected in the eggs before incubation even at very low concentrations produced embryotoxicity and teratogenecity.

A study, carried out to determine the effects of DDT, Endrin and various PCBs in cockerels after chronic oral administration (50-200 ppm) of various PCBs included depressed body weight and feed intake; general edema and hydropericardium; increased liver weight and decreased heart; spleen, and testes weight; depression of sexual characteristics; and some mortality (Iturri, 1974).

**Table 1:** **Embryotoxic effects and developmental anomalies induced by different concentrations of Tenekil Plus in 7-days developing chicks.**

| Dose (µg/egg) | Resorbed embryos (%) | C.R. Length (mm S.D) | Weight (mg S.D) | Head (%) | Beak (%) | Eyes (%) | Limbs (%) | Cardiac Position (%) | Neck (%) | Spinal cord (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| 0.00 | 0.00 n = 10 | 13.4 1.26*** | 1795.3 159.4*** | Well developed | Well dev. | Well dev. | Well dev. | Well dev. | Well dev. | Well dev. |
| 2.37 | 10 n = 9 | 6.77 1.64*** | 557.6 134.5*** | Microcephaly (55.55) | Short beak (66.66) Agenesis of beak (33.33) | Anophthalmia (33.33) Microphthalmia (44.44) | Phocomelia (22.22) Micromelia (55.55) | Ectopia cardis (25) | Thick and small (100) | Twisted (100) |
| 4.75 | 30 n = 7 | 6.42 1.61*** | 499.2 124.8*** | Microcephaly (85.7) Anencephaly (12.11) | Short beak (12.11) Agenesis of beak (85.7) | Anophthalmia (42.85) Microphthalmia (28.57) | Amelia (14.28) Phocomelia (28.57) | Ectopia cardis (42.85) | Small and thick (100) | Twisted (10) |
| 9.50 | 20 n = 8 | 7.37 1.06*** | 579.7 130.1*** | Microcephaly (37.5) Anencephaly (37.5) | Short beak (10) Agenesis of beak (90) | Anophthalmia (50) Microphthalmia (50) | Amelia (25) Phocomelia (12.5) Micromelia (62.5) | Ectopia cardis (37.5) | Small (100) | Twisted (12.5) |
| 19.00 | 40 n = 6 | 6.00 0.632*** | 441.0 0.632 | Microcephaly (33.33) Anencephaly (50) | Agenesis of beak (100) | Anophthalmia (66.66) Microphthalmia (33.33) | Amelai (16.66) Micromelia (33.33) | Ectopia cardis (50) | Small (100) | Curved and Twisted (33.33) |

*** = Significantly different against the controls (P<0.001)

**Table 2: Developmental anomalies induced by different concentrations of Tenekil Plus in 15-days developing chicks.**

| Dose (µg/egg) | Resorbed Embryos (%) | C.R. Length (mm S.D) | Weight (mg S.D) | Head (%) | Beak (%) | Eyes (%) | Limbs (%) | Cardiac position (%) | Neck (%) | Spinal cord (%) | Plumage (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.00 | 0.00 | 28.1 3.84*** | 4772.6 1276.6*** | Well dev. | Well dev. | Well dev. | Well dev. | Well dev. | Well dev. | Well dev. | Well dev. |
| 2.37 | 20 | 19.0 2.00*** | 2723.3 520.5*** | Microcephaly (62.5) | Short beak (75) Agenesis of beak (25) | Anophthalmia (25) Microphthalmia (50) | Phocomelia (37.5) Micromelia (50) | Ectopia cardis (25) | Small (100) | Twisted (12.5) | Not developed (100) |
| 4.75 | 10 | 14.8 1.69*** | 1798.4 313.4*** | Microcephaly (55.55) Anencephaly (11.11) | Short beak (22.22) Agenesis of beak (77.77) | Anophthalmia (33.33) Microphthalmia (44.44) | Amelia (11.11) Phocomelia (22.22) Micromelia (33.33) | Ectopia cardis (50) | Small and thick (100) | Twisted (0) | Not developed (100) |
| 9.50 | 30 | 10.0 1.63*** | 1076.1 346.02*** | Microcephaly (42.8) Anencephaly (28.57) | Short beak (14.28) Agenesis of beak (85.71) | Anophthalmia (71.42) Microphthalmia (28.57) | Amelia (14.2) Phocomelia (28.5) Micromelia (57.14) | Ectopia cardis (57.14) | Small (100) | Twisted (0) | Not developed (100) |
| 19.00 | 30 | 7.28 0.755*** | 526.0 144.3*** | Microcephaly (42.8) Anencephaly (57.10) | Agenesis of beak (100) | Anophthalmia (85.7) Microphthalmia (14.28) | Amelia (71.42) Micromelia (28.5) | Ectopia cardis (71.42) | Thin and small (14.28) | Twisted (14.28) | Not developed (100) |

*** = significant difference against controls (P<0.001)

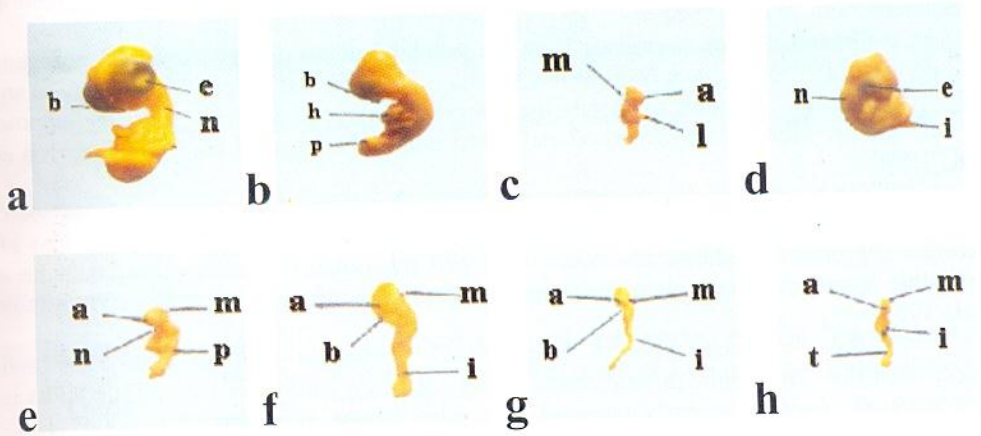


Fig. 1: Macrophotograph of chick embryos recorded on day 7 of incubation from eggs treated with different concentrations of Tenekil Plus. a) control embryo with normal organogenesis; b) embryo from 2.37µg/egg dose group: c,d) embryos from 4.75 µg/egg dose group; e,f) embryos from 9.5µg/egg dose group g,h) embryos from 19µg/egg dose group respectfully, showing different developmental anomalies. *Note:* microcephaly (m), anophthalmia (a), microphthalmia (e), phocomelia (p), micromelia (l), amelia (i), ectopia cardis (h), agenesis of beak (b), twisted spinal cord (t), and small neck (n),

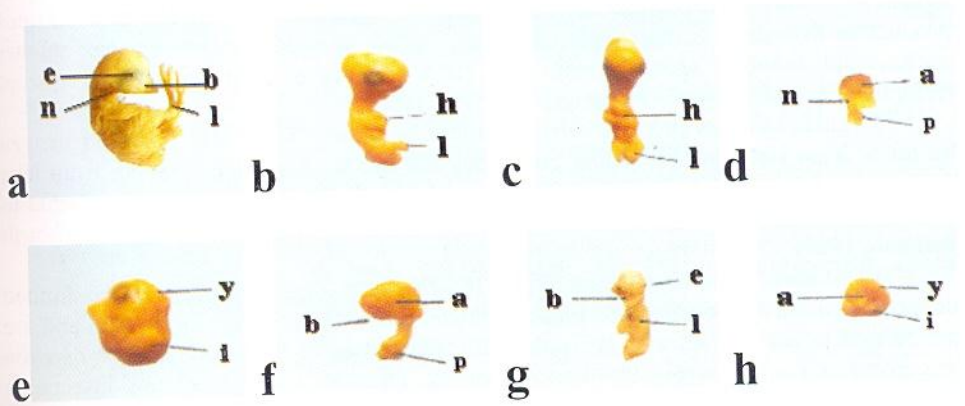


Fig. 2: Macrophotograph of chick embryos recorded on day 15, from eggs treated with different concentrations of Tenekil Plus. a) control embryo with normal organogenesis; b,c) 2.37 µg/egg (lateral and front view); d,e) embryos from 4.75 µg/egg dose group; f,g) embryos from 9.5µg/egg dose group; h) embryos from 19µg/egg dose group respectfully. *Note:* anencephaly (y); anophthalmia (a), microphthalmia (e), phocomelia (p), micromelia (l), amelia (i), ectopia cardis (h), agenesis of beak (b) and small neck (n),

Some of the organochlorines are able to alter the endocrine and reproductive systems by either mimicking or antagonizing endogenous hormone action, modulating the synthesis and metabolism of endogenous hormones, or altering hormone receptor expression (Sonnenschein, *et al.,* 1998).

Organochlorine chemicals have the potential to act as environmental estrogens (Soto *et al.,* 1995) and have been shown to adversely affect wild life. Organochlorines are lipid soluble and resistant to metabolism, these compounds accumulate in the adipose tissue and are found in human blood and breast milk (Wiemeyar *et al.,* 1984; Bergeron *et al.,* 1994).

The insecticide Aldrin, Dieldrin, and Toxaphene, known to have pleiotropic toxic effects in animals, were shown to inhibit gap junctional communication. Interpretation of results suggests that chemical inhibition of gap junctional communication could be a possible mechanism to explain their tumor promoting and neurotoxic effects (Trosko *et al.,* 1987).

Mirex exposed embryos demonstrated increased malformation rates and decreased total embryonic protein contents and decreased somite nwnbers. Thus Mirex is embryotoxic *in vitro* to early organigenesis stage mouse embryos (EI-Bayomy *et al.,* 2002).

Endosulfan caused hypertension, pupillary dilation and an increase in cardiac output and peripheral resistance. In conclusion, endosulfan acts within the brain to produce both autonomic and somatic toxicity (Anand *et al.,* 1981).

According to a review draft of EPA's current dioxin health assessment, reproductive and developmental effects may be occurring at levels lower than originally thought to have an adverse effect on animals (USEPA, 1994). For example, a single, tiny oral dose (0.064 micrograms per kilogram of body weight) of dioxin on day 15 of pregnancy in rats has no effect on the mother, but increases the likelihood of various reproductive disorders in their male offspring, including undescended testicles, smaller testicles, and reduced sperm count. The EPA also notes that dioxin can disrupt development at a large nwnber of stages (RHWN, 1993).

Organochlorines produce developmental problems. The study followed women who for at least six years preceding pregnancy ate two to three fish a month from lake Michigan (which is contaminated with organochlorines). Their offsprings were found to have lower birth weight, smaller skull, cognitive, motor and behavioral deficits at birth (Weltman, 1993).

All studies including present one, indicate that organochlorines have limited biodegradation, have propensity to bioaccwnulate, biomagnify in the food chain and are toxic to non-target species and are potentially dangerous to developing embryos even when given at comparatively low concentrations. Thus, it is suggested this insecticide should be used very carefully and under extreme necessities.

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# EPIDEMIOLOGICAL STUDY OF FASCIOLOSIS IN BUFFALOES AT DIFFERENT SITES OF PUNJAB PROVINCE

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**Abstract:** In present study certain epidemiological aspects of fasciolosis in buffaloes were investigated by fecal examination in six different areas of Punjab *i.e.*., Sheikhupura, Gujranwala, Kamoki, Muridke, Shahdara and Kasur. The epidemiological data showed overall infection of 15.36%. Highest infection was observed in Kamoki (20.17%) followed by Shahdara (19.67%), Kasur (18.5%), Muridke (17.0%), Sheikhupura (9.0%) and lowest in Gujranwala (7.8%). Month-wise overall prevalence was found highest in September (32.67%) and lowest in June (4.0%). Season-wise data revealed highest infection in autumn (28.33%) and lowest in spring (8.17%). Sex-wise prevalence did not show any significant difference between males and females.

**Key words:** Epidemiology, fasciolosis, buffaloes.

## INTRODUCTION

Fasciolosis or liver rot caused by *Fasciola* spp. (mainly *Fasciola gigantica* and *F. hepatica*) is cosmopolitan in distribution. It is normally a disease of bile duct of domestic herbivorous animals such as sheep, cattle and goats, which are the normal hosts (Mulcahy and Dalton, 2001; Pfukenyi *et al.*, 2005). It contributes to great economic and health losses in cattle industry in many countries of the world (Kofta *et al.*, 2000; Moll *et al.*, 2000; Ortiz *et al.*, 2000; Attallah *et al.*, 2002; Phiri *et al.*, 2005). On livestock it has both direct effects, actual liver condemnation at slaughter and indirect effects such as decrease in feed efficiency, weight gains, milk production and reproductive performance (Aal *et al.*, 1999; Shaikh *et al.*, 2004).

The appearance of symptoms of fasciolosis as well as the severity of the disease depends upon the intensity of infection. Symptoms may appear a few days after ingestion of metacercaria, when the immature worms reach the abdominal cavity and begin migrating across or in the liver. Animals fasciolosis is characterized by sudden death with blood stained froth at the natural orifices in acute cases while diarrhoea, jaundice and

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*Part of Ph.D. research of first author*

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bottle jaw are predominant features in chronic cases. The disease entity causes loss in term of morbidity and mortality in fluky areas and is of special interest in the waterlogged areas because of increased incidence in human infestation (Farid *et al.*, 1988; Hassan *et al.*, 1995).

Kendell (1954, 1965) was who first reported that fascioliasis caused by *Fasciola gigantica* and *F. hepatica* was one of the major problems in cattle raising area of Punjab, Pakistan. Later on epidemiology and chemotherapy of fasciolosis was studied in buffaloes and cattle in some areas of Punjab (Malik, 1984; Sheikh, 1984; Maqbool *et al.*, 1994, 2002).

The present study was aimed at investigating certain epidemiological aspects of fasciolosis by fecal examination including occurrence of disease and its relationship with meteorological factors. The epidemiological data thus obtained will facilitate in developing strategy for the prevention and control of fasciolosis in buffaloes in Pakistan.

## MATERIALS AND METHODS

*Area of the study*

Study was conducted for epidemiology of fasciolosis in buffaloes in 6 different sites of Punjab Province *i.e.*, in Shahdara, Muridke, Kamoki, Gujranwala, Kasur and Sheikhupura.

*Prevalence*

To record the prevalence of fasciolosis in humans and buffaloes above mentioned sites were visited every month. On each visit a total of 50 faecal samples of buffaloes were collected. These samples were examined by direct microscopic examination (Urquhart *et al.*, 2001). Prevalence of infection was recorded month-wise and season-wise. The age, sex and area of buffaloes were also furnished.

*Meteorological data*

Meteorological data including maximum and minimum temperature (°C), relative humidity (%), rainfall (mm) and pan evaporation (mm) were obtained from Meteorological Station, Lahore and their correlation with the occurrence of the disease was worked out.

*Statistical analysis*

Data was analyzed statistically by using computer software (Microsoft SPSS 6.0).

## RESULTS

*Epidemiology*

In present study overall infection of fasciolosis in six different areas of Punjab, from April 2003 to March 2004, was found 15.36% (Table 1).

*Area-wise prevalence*

Area-wise prevalence showed highest infection rate in Kamoki (20.17%) followed by Shahdara (19.67%), Kasur (18.5%), Muridke (17.0%), Sheikhupura (9.0%) and lowest in Gujranwala (7.8%) as shown in Table 1.

*Month-wise prevalence*

In month-wise, overall prevalence was highest in September (32.67%) and lowest in June (4.0%). All areas followed the same pattern except Muridke, which shows lowest infection in May (2.0%) (Table 2).

*Season-wise prevalence*

In season-wise data highest infection was observed in autumn (28.33%) followed by winter (17.08%), summer (10.75%) and lowest in spring season (8.17%) (Table 2).

*Age-wise prevalence*

It was also observed that adult animals were more infected (15.77%) than youngsters (6.0%) (Table 1).

*Sex-wise prevalence*

Overall sex-wise prevalence showed non-significant difference. In males 15.14% and in females 15.76% infection rate was observed (Table 1).

## DISCUSSION

Overall infection of fasciolosis in buffaloes was noted as 15.36% in Punjab province. In a similar study, conducted in some other areas of Punjab, Maqbool *et al.* (2002) noted 14.7% infection rate, which is very close to the results of present study. Area-wise results show highest infection in Kamoki (20.17%) followed by Shahdara (19.67%), Kasur (18.5%), Muridke (17.0%), Sheikhupura (9.0%) and lowest in Gujranwala (7.8%). Statistical analysis by Students t-test, showed non-significant difference of fasciolosis infection between Gujranwala and Sheikhupura, but other areas has significantly higher infection than Gujranwala, as well as from Sheikhupura ($P \leq 0.001$) as shown in Table 2. This may be due to fact that high level of infection was thought to be associated with the extension of the canal system providing additional areas of swamp and

marsh where the buffaloes were exposed to infective larvae of helminthes as was also noted by Chaudhri *et al.* (1993) and Misra *et al.* (1997).

**Table 1: Overall prevalence of Fasciolosis in buffaloes of Punjab (April 2003-March 2004).**

| Factors | | Total No. of samples observed | Total No. of samples infected | Infection (%) |
|---|---|---|---|---|
| Areas | Gujranwala | 600 | 47 | 7.83 |
| | Sheikhupura | 600 | 54 | 9.0 |
| | Muridke | 600 | 102 | 17.0 |
| | Kasur | 600 | 111 | 18.5 |
| | Shahdara | 600 | 118 | 19.67 |
| | Kamoki | 600 | 121 | 20.17 |
| Season | Spring | 600 | 49 | 8.17 |
| | Summer | 1200 | 129 | 10.75 |
| | Winter | 1200 | 205 | 17.08 |
| | Autumn | 600 | 170 | 28.33 |
| Sex | Male | 2325 | 374 | 16.09 |
| | Female | 1275 | 179 | 14.04 |
| Age | 0-2 years | 150 | 9 | 6.0 |
| | >2 years | 3450 | 544 | 15.77 |
| Months | April | 300 | 33 | 7.33 |
| | May | 300 | 14 | 4.67 |
| | June | 300 | 12 | 4.0 |
| | July | 300 | 41 | 13.67 |
| | August | 300 | 62 | 20.67 |
| | September | 300 | 98 | 32.67 |
| | October | 300 | 72 | 24.0 |
| | November | 300 | 61 | 20.33 |
| | December | 300 | 54 | 18.0 |
| | January | 300 | 51 | 17.0 |
| | February | 300 | 39 | 13.0 |
| | March | 300 | 27 | 9.0 |
| **Total** | | **3600** | **553** | **15.36** |

In month-wise, overall prevalence highest infection was noted in September (29.0%) and lowest in June (3.0%). All sites individually followed the same pattern except Muridke, which showed lowest infection in May instead of June, while Shahdara showed lowest infection in June as well as in March. Statistical analysis showed non-significant difference between infection rate of May and June, June and March and April and June,

while in all other months infection is significantly higher than in June *i.e.*, P<0.001 (Table 2). Study by Maqbool *et al.* (2002) favours our results.

**Table 2: Overall prevalence (%) of Fasciolosis in buffaloes of Punjab in study period (April 2003-March 2004).**

| Factors | | Sheikhu-pura | Gujran-wala | Kamoki | Muridke | Shahdara | Kasur | Mean ±S.E. |
|---|---|---|---|---|---|---|---|---|
| Season | Spring | 4.0 | 4.0 | 12.0 | 7.0 | 7.0 | 15.0 | 8.17±1.82 |
| | Summer | 4.0 | 6.0 | 13.5 | 13.5 | 13.5 | 14.0 | 10.75±1.84 |
| | Autumn | 22.0 | 13.0 | 35.0 | 29.0 | 40.0 | 31.0 | 28.33±3.93*** |
| | Winter | 10.0 | 9.0 | 23.5 | 19.5 | 22.0 | 18.5 | 17.08±2.51** |
| Sex | Male | 9.6 | 9.6 | 18.74 | 17.71 | 21.0 | 19.55 | 14.98±2.18 |
| | Female | 6.0 | 6.6 | 25.6 | 16.0 | 18.33 | 15.33 | 16.78±3.86 |
| Age | 0-2 Yrs | - | 7.0 | - | - | 4.0 | - | 5.5±0.63 |
| | >2 Yrs | 9.0 | 8.0 | 20.17 | 17.0 | 21.1 | 18.5 | 15.63±0.60*** |
| Months | April | 6.0 | 4.0 | 6.0 | 4.0 | 10.0 | 14.0 | 7.33±1.6 |
| | May | 2.0 | 4.0 | 4.0 | 2.0 | 6.0 | 10.0 | 4.67±1.24 |
| | June | 0.0 | 2.0 | 2.0 | 10.0 | 4.0 | 6.0 | 4.0±1.46 |
| | July | 4.0 | 8.0 | 20.0 | 18.0 | 18.0 | 14.0 | 13.67±2.6** |
| | August | 10.0 | 10.0 | 28.0 | 24.0 | 26.0 | 26.0 | 20.67±3.4*** |
| | September | 24.0 | 16.0 | 48.0 | 32.0 | 42.0 | 34.0 | 32.67±4.76*** |
| | October | 20.0 | 10.0 | 22.0 | 26.0 | 38.0 | 28.0 | 24.0±3.8*** |
| | November | 18.0 | 8.0 | 28.0 | 14.0 | 34.0 | 20.0 | 20.33±3.84** |
| | December | 10.0 | 14.0 | 20.0 | 26.0 | 16.0 | 14.0 | 18.0±2.28*** |
| | January | 8.0 | 8.0 | 24.0 | 24.0 | 24.0 | 22.0 | 17.0±3.28*** |
| | February | 4.0 | 6.0 | 22.0 | 14.0 | 14.0 | 18.0 | 13.0±2.82** |
| | March | 2.0 | 4.0 | 18.0 | 10.0 | 4.0 | 16.0 | 9.0±27.76 |
| **Mean ± S.E.** | | **9.0 ± 2.24** | **7.83 ± 1.2** | **20.17 ± 3.6*** ** | **17.0 ± 2.74*** ** | **19.67 ± 3.81*** ** | **18.5 ± 2.28*** ** | **15.36 ± 2.82** |

* P≤0.05; ** P≤0.01; *** P≤0.001

Season-wise data revealed highest infection in autumn (26.5%), followed by winter (17.08%), summer (10.75%) and lowest in spring (7.5%). Season-wise statistical analysis showed that spring (March-April) and summer (May-August) seasons have non-significant difference in prevalence of fasciolosis, while winter (November-February) and autumn (September-October) season showed a significantly higher infection (Table 2). Study of meteorological factors showed that after maximum rainfall in July and August (252.3 mm and 108.5 mm) infection increases to maximum in September (32.67%) (Tables 1, 3). These results are in line with those of Hayat *et al.* (1986) and Maqbool *et al.* (1994, 2002) who reported that in Pakistan, rainy season starts in the month of July and changes the environmental temperature and humidity so as to favor the emergence of

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# A NOTE ON THE BIOSTRATIGRAPHIC EVENTS OF THE SIWALIKS

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**Abstract:** The Mio-Pliocene Siwalik Group was deposited in the Himalayan foreland basin in response to uplift and erosion in the Himalayan fold-thrust belt. This 7-km thick succession of fluviatile deposit is rich in vertebrate fauna. The Siwalik fauna has been regarded as one of the most important Cenozoic fossils anywhere in the world and has been studied intensely for more than a century. The British and American workers did the pioneering work on the Siwalik stratigraphy and palaeontolgy in the nineteenth and early part of twentieth centuries. Several American and Pakistani scientists did post-independence geological work in the Siwaliks of Pakistan. As a result, a fine-scale biostratigraphic work has emerged, supplemented by vertebrate faunal zone.

**Key words:** Siwaliks, biostratigraphy, stratigraphy.

## INTRODUCTION

Terrestrial Neogene sediments, all of which can be referred loosely as "Siwaliks" are present through out India, Nepal, and Pakistan, where they are associated with young, active orogenic belts resulting from the collision of India and Asia. The important sedimentologic and taphonomic features of the rocks have been extensively discussed by Behrensmeyer (1987), Behrensmeyer et al. (1995), Willis and Behrensmeyer (1995), and Willis (1993a, 1993b). The formations are fluvial in origin and comprise alternating sandstones and fine-grained sediments, with occasional conglomerates, especially in the upper parts of the section. Typical depositional environments include channels, crevasse splays, fills, and floodplain soils. Individual formations are somewhat arbitrarily distinguished on the basis of ratios of sand to clay and silt, and because local depositional conditions vary greatly, the lithostratigraphy of the formations is complex. Although, some lithostratigraphic units (e.g., the "Chinji" or "Nagri" Formations) are recognized over a broad region, most units are very restricted in their geographic extent. This aspect, which has only recently been recognized, means that the lithostratigraphic correlations used in earlier syntheses of the Siwalik stratigraphy (e.g., Pilgrim, 1910) are of limited use, if not actually misleading. Understanding of Siwalik geology, stratigraphy, sedimentation, and chronology has greatly improved in the past two decades. The classic formulation of the Siwalik stratigraphy was developed by G. E. Pilgrim in a series of papers on the occurrence of fossils and sediments throughout the Indian subcontinent (Pilgrim, 1908, 1910, 1912, 1913, 1914, 1917, 1926, 1932, 1937, 1939). He recognized

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seven successive "faunal zones" for the Early Miocene through Early Pleistocene: Gaj, Kamlial, Chinji, Nagri, Dhok Pathan, Tatrot, and Pinjor (Fig. 1). These terms were at first used only as faunal units, but they subsequently became confused with lithostratigraphic units and are now used mainly with reference to some of the lithological formations recognized throughout Pakistan and India (Colbert, 1935; Shah, 1977). Age estimates by Pilgrim and others for the formations and their contained fauna were based on imprecise faunal correlations to European and even North American Neogene sequences (Pilgrim, 1926; Matthew, 1929; Colbert, 1935). Consequently, since 1973 much effort has been directed at refining Pilgrim's biostratigraphic sequence and placing the faunas within a secure chronostratigraphic framework.

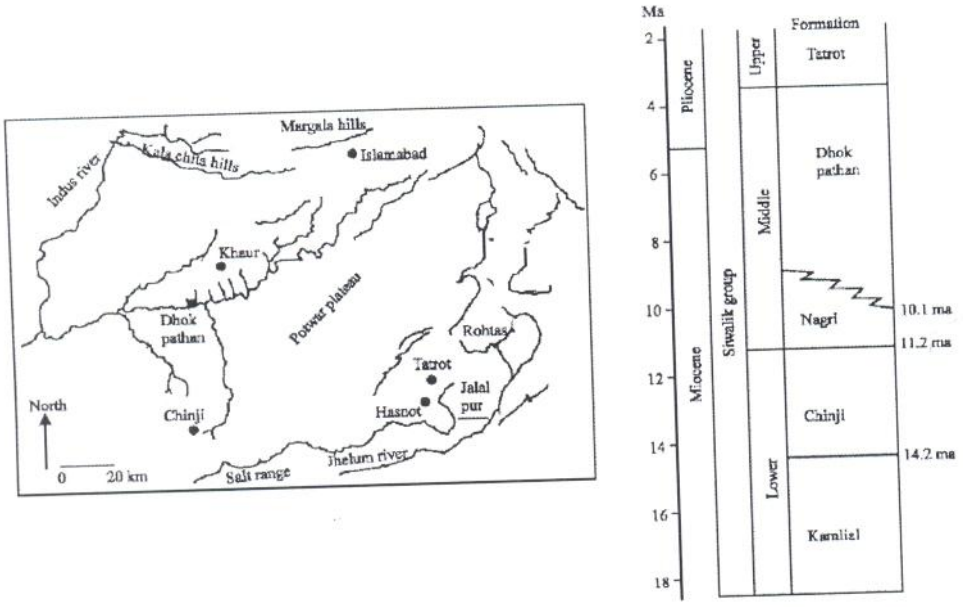


**Fig.1: Stratigraphic sections of the Siwaliks**

## BIOSTRATIGRAPHY

The Siwalik sediments are well suited for magnetostratigraphic studies; it has been possible to establish a regional chronostratigraphic framework that now spans the Early Miocene through the Pleistocene (Opdyke *et al.*, 1979; Johnson, G. D., *et al.*, 1982, Tauxe and Opdyke, 1982; Johnson, N. M., *et al.*, 1982, 1985; Kappelman, 1986; Friedman *et al.*, 1992; Downing *et al.*, 1993). This framework also allows Siwalik faunal assemblages to be more precisely correlated to well dated sequences in Africa, Europe,

and elsewhere (Flynn *et al.*, 1990). The Potwar Plateau sequence is probably the most complete terrestrial Neogene sequence and is currently the best documented and most intensely collected of the widespread Siwalik deposits. Johnson, N., *et al.* (1982, 1985) have shown that the Potwar Siwalik sediments range between 18.3 and 0.6 ma. During this period only one major depositional hiatus has been identified (Opdyke *et al.,* 1979). With reference to the Geomagnetic Reversal Time Scale of Berggern *et al.* (1985) the ages of the Miocene Potwar formations (Kamlial, Chinji, Nagri, and Dhok Pathan Formations) and the Late Pliocene through Pleistocene formations are now fairly well understood.

Older, fossiliferous formations are also known in Pakistan outside the Potwar Plateau but in most cases are not well dated. These include parts of the Murree Formation near Banda Daud Shah, the Chitarwata and lower Vihowa Formations in the Zinda Pir Dome near Dera Ghazi Khan (Friedman *et al.*, 1992; Downing *et al.*, 1993) and correlative rocks at Dera Bugti and the upper Gaj and Manchar Formation in Sindh (Raza *et al.*, 1984; de Bruijn and Hussain, 1984). The Zinda Pir Dome sequence is now the best dated, with a series of superposed large and small mammal localities ranging between 22 and 15 ma and extending the mammal sequence of southern Asian for another 4 million years.

Changes in the rates of sediment accumulation and an apparent widespread depositional hiatus have made the stratigraphic relations and ages of the Early Pliocene formations of the Potwar less certain. These include the Tatrot Formation and its equivalents, which contain an important fauna with recorded occurrences of *Elephas, Hippohyus, Sivachoerus*, and *Sus* (Barry *et al.*, 1982; Hussain *et al.*, 1992) and some older sediments near Rhotas and Jalalpur. Previously, the Tatrot was interpreted as being in the upper part of the Gauss Chron (Barry *et al.*, 1982), while critical parts of the Rhotas and Jalalpur sequences have been interpreted as being in the Gilbert Chron (Opdyke *et al.*, 1979; Johnson *et al.*, 1982). The upper part of the Gauss is between 2.5 and 2.9 ma on the Berggren *et al.* (1985) time scale, whereas the Gilbert spans the considerably longer interval between 3.4 and 5.35 ma. Hussain *et al.* (1992) suggested that the Tatrot Formation might be older than previously thought and could be in the lower part of the Gauss, between 3.2 and 3.4 ma and Barry *et al.* (2002) suggested an age for the Tatrot Formation between 3.5 to 3.3 ma (Table 1). Support for this older age also comes from new, unpublished collections of the Harvard-Geological Survey of Pakistan group. Similarly, unpublished work by Kappelman and Stubblefield suggests problems in the dating of the Rhotas section, and this leaves only the Jalalpur section as spanning from 5.4 to 3.4 ma. The new correlation to the geomagnetic time scale that makes the Pliocene fossiliferous horizons between 0.5 and 1.5 million years are older than previously thought (Barry *et al.*, 1982). The stratigraphic relationships between the younger Potwar formations (referred to under various formational names, including the Samwal, Kakra, and Mirpur Formations of Hussein *et al.,* (1992) and the Pliocene and Pleistocene formations of northwestern India and Kashmir are still unresolved. The latter includes the important Pinjor and all the younger faunas, which are thought to span between 2.5 and 0.9 ma (Azzaroli and Napoleone, 1982; Tandon *et al.*, 1984; Barry, 1987). The well-dated fossiliferous Potwar formations, as exposed at Mirpur and in the Pabbi Hills, as well as

elsewhere throughout the Potwar, span to a slightly longer interval between 3.4-0.6 ma (Opdyke *et al.*, 1979; West, 1981; Johnson, G. D., *et al.*, 1982; Johnson, N. M., *et al.*, 1982), but it is not yet clear where within this interval the Indian sediments and fossils lie. Because Pinjor and the other younger formations contain both cervids and *Equus*, which are known to appear in the Potwar sections in the upper Gauss and near the Gauss – Matuyama boundary, respectively (Opdyke *et al.*, 1979; Hussain *et al.*, 1992), a likely age for the oldest appearance of the Pinjor fauna is 2.4-2.5 ma. Some progress has been made in developing a comprehensive Siwalik biostratigraphy that will be separate from the current lithostratigraphic usage. Barry *et al.*, (1982) proposed a series of biostratigraphic interval zones that begin with the first appearance of equids and end with the local extinction of three-toed hipparionine equids. These zones were defined in two designated reference sections on the Potwar Plateau, spanning approximately between 10.0 to 1.5 ma. Hussain *et al.* (1992) have modified the scheme by dividing the youngest zone into two range zones and showing that its lower boundary was older than previously thought.

**Table 1: Siwalik formations of the Potwar Plateau. * In the southern Potwar, the Kamlial and Murree Formations are not differentiated. In the north, the Murree is considerably older than 18 ma. (Source, Barry *et al.*, 2002).**

| Formations | Age Range (ma) |
|---|---|
| Tatrot | 3.5-3.3 |
| Dhok Pathan | 10.1-ca 3.5 |
| Nagri | 11.2-10.1 |
| Chinji | 14.2-11.2 |
| Mamlial/Murree* | 18.3-14.2 |
| Murree | ?-18.3 |

## CONCLUSION

It is often difficult to delineate the boundaries between the formations, however, from the geological or the sedimentological perspective it is best to view the Siwalik sequence as a single genetic unit (Table 2). Nevertheless, the Siwalik formations have always been cryptic chronostratigraphic units and from the paleontological point of view recognition of the formations and their boundaries has been a crucial step in dating the fossils (e.g. Colbert, 1935). This practice has in the past produced much confusion and sterile debate, but it is now possible to assume that with the contribution of magnetostratigraphy as a means of dating the rocks, this era of confusion is past. Murphy (1977) has noted the distinctions between the operations of definition, characterization, and identification in the practice of stratigraphy. The biostratigraphic interval-zones of Barry *et al.* (1982) were defined and characterized in reference sections and criteria were stated for identifying or recognizing them in other sections. Because they were related directly to stratigraphic sections, the interval-zones and their boundaries can, like stages,

**Table 2: Stratigraphic sections of the Siwalik group showing Formations and Zones. (Boundary dates are set from Barry *et al.*, 2002).**

| Ma | Siwalik Formations | Siwalik Subgroups | Siwalik Zones |
|---|---|---|---|
| 0 -- | | | |
| -- | | | |
| 2 -- | Soan | Upper Siwalik Subgroup | Boulder Conglomerate Zone<br>Tatrol Zone Pinjor Zone |
| -- | | | |
| 4 -- | Dhok Pathan | Middle Siwalik Subgroup | Dhok Pathan Zone |
| -- | | | |
| 6 -- | | | |
| -- | | | |
| 8 -- | | | |
| -- | | | |
| 10 -- | Nagri | | Nagri Zone |
| 11 -- | | | |
| 12 -- | Chinji | Lower Siwalik Subgroup | Chinji Zone |
| -- | | | |
| 14 -- | | | |
| -- | | | |
| 16 -- | Kamlial | | Kamlial Zone |
| -- | | | |
| 18 -- | | | |
| -- | | | |
| 20 -- | | | |
| -- | | | |
| 22 -- | | | |

be correlated to other geological phenomena, such as sedimentological or geochemical events, magnetopolarity zones, or to geologic time. However, the Siwalik interval-zones should not be confused with stages, which are chronostratigraphic units. Each interval-zone's lower boundary is defined by a biological event, not a stratigraphic level with a specific age. At the time the defining taxa were selected, the stratigraphic levels and the ages of their first appearances were thought to be accurately known.

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# INFECTION OF *PROTEOCEPHALUS FILICOLLIS* RUDOLPHI (CESTODE) IN *GASTEROSTEUS ACULEATUS* L., THREE-SPINED STICKLEBACK, IN RELATION TO SEX AND LENGTH OF THE HOST

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**Abstract:** A population of the cestode *Proteocephalus filicollis* from *G. aculeatus*, three-spined stickleback was sampled over a two years period from Airthrey loch, Scotland. Infection of fish showed that there is no significant difference in prevalence and abundance in both sexes. In general, female fish dominated the male in this wild stickleback population. Almost all the length classes of fish were infected throughout the year. Yet, there was no statistical difference in prevalence and abundance in all four length classes of fish. The parasites were over-dispersed in both sexes, in all length classs of fish and in all seasons. The infection of fish in relation to sex and size of the host is discussed.

**Key words:** *Proteocephalus filicollis;* prevalence; abundance; *Gasterosteus aculeatus.*

## INTRODUCTION

The trends of temporal variation in the infection and maturation of temperate freshwater fish parasitic helminths are diverse. Some species exhibit a definite cycle of occurrence or maturation, whilst other shows a variable pattern. Some species show inconsistently variable behaviour along the years and follow changes in the environment (Kennedy, 1975). Seasonal cycles of maturation, growth and recruitment have commonly been observed in species of *Proteocephalus* (Kennedy, 1977). In temperate climate water temperature is a factor causing temporal changes in maturation and infestation level of helminth population in fish (Kennedy, 1975; Chubb, 1979). There are many studies on the seasonal cycle of *Proteocephalus* sp. including *P. filicollis* (Hopkins, 1959); *P. stizostethi* Hunter & Bangham, 1933 (Connor, 1953); *P. torulosus* Batsch, 1786 (Kennedy and Hine, 1969); *P. percae* (Wootten, 1973); *P. ambloplitis* Leidy, 1887 (Fischer and Freeman, 1968; Eure, 1976); *P. macrocephalus* Creplin (Nie and Kennedy, 1991). The basic seasonal pattern shows recruitment in summer and autumn with eggs production from spring to summer.

*Proteocephalus filicollis* is host specific of its final host, three-spined stickleback. (Freze, 1969; Willemse, 1968). The first intermediate hosts of *P. filicollis* are Cyclops copepods. According to Iqbal (1998) *Proteocephalus filicollis* stay in the intestine of the

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fish for almost a year and *P. filicollis* show migration in the intestine of the fish, but do not have serious pathological effects on the site of attachment in the intestine of the fish. Hopkins (1959) observed a clear seasonal cycle of infection in this parasite in Scotland. However, Chappel (1969) in his study on *P. filicollis* from England emphasized on duration of low winter temperature and the geographical location of the host as an important factors in determining the seasonal cycle in the fish parasites.

The aim of this study was to investigate in detail the features of the population biology of *P. filicollis* from a wild population of three-spined stickleback from Airthrey Loch with emphasis on sex and size of the host and to compare this with previous studies done in Britain.

## MATERIALS AND METHODS

A total of 1351 three-spined stickleback were collected from the littoral zone of Airthrey Loch from April 1993 to July 1995 on monthly basis. Fish were caught with a hand net (mesh size 3.0 x 3.5 mm). Sample size ranged from 20 to 85 per month. Fish were most plentiful in late summer and autumn. In winter the Loch was often at least partly frozen, which made fishing difficult. Fish were also not abundant in spring and early summer.

The fish samples were examined fresh as soon as possible. Total length, weight and sex were recorded. Fish samples were divided into four arbitrary length classes as under:

< 2.0 cm = LC-1; 2.1-3.0 cm = LC-2; 3.1-4.0 = LC-3; 4.1cm > = LC-4

The gut from the stomach to rectum was removed intact and placed in a Petri dish containing tap water. The gut was opened from posterior to anterior and then examined under the dissecting microscope using transmitted light at x 10 and x 40 magnification. Numbers of parasites were counted, removed and were preserved in 70-80 % alcohol for further examination.

Observed parasite distribution was fitted to the theoretical negative binomial (Elliot, 1983) and variance to mean ratio was calculated (Anderson and Gordon, 1982). Significance level was determined by the Chi-square test (Elliot, 1983). A probability of $P< 0.05$ was considered significant. The prevalence rate between two sexes was compared by 2 x 2 contingency table. The relationship of the abundance to the size of the fish and difference in abundance between sexes of the fish were tested by using Non-parametric Kruskal and Wallis test (Sokal and Rohlf, 1987). All tests were performed by using computer package (Minitable and Quatro-pro Version 1.00). Prevalence, mean intensity and abundance are defined as in Margolis *et al.* (1982). Infrapopulation size: All individuals of a species of parasite occurring in an individual host.

## RESULTS

*Infection with sex of the host*

A total of 1351 fish were examined of which 516 fish were infected, making an overall prevalence rate of 38.1 % and abundance was 1.4 (Table 1). *Proteocephalus filicollis* were found in the intestine and the rectum of the fish. Of the total infected fish 119 were male, 280 were female and in 117 fish sex could not be determined. The prevalence of infection in male was 37.9% and in female it was 42% and in fish of undetermined sex was 31.5%. There was no significant difference in prevalence between male and female fish ($P = 0.75 = 1.323$ (I.d.f): $P > 0.75$ N.S.D). Abundance in male was 1.1 in female 1.7 and in fish of undetermined sex was 1.3. There was no significant difference in the abundance in both sexes ($P = 0.75 = 0.575$, $P > 0.90$).

In male fish prevalence was high (55% & 50%) in spring and summer 1993, but it declined slightly from autumn 1994 (33.9% to 27.2%). During 1994-95 male fish showed a higher prevalence than in the previous year. Prevalence was 56.6% and 64.3% in autumn and winter respectively, which declined from spring to summer 1995 (30.9% to 26.8%). Abundance increased from spring to summer 1993 but then declined and was much lower from autumn 1993 to summer 1994. Abundance was higher in autumn 1994 and winter 1994 and declined in spring and summer 1995.

In female fish prevalence was high in spring 1993 (41.3%) but it was lower in the following summer and autumn. Prevalence was highest in winter (47.6%) after which there was a gradual decline from spring to summer 1994 (38% to 28.5%). Prevalence was generally higher in 1994-95 in all seasons than in the previous year. Prevalence was 60% in autumn and than rose again in winter and spring before decline in summer 1995. Abundance dropped from 2.2 in spring 1993 to 0.92 in summer. From autumn 1993 to winter 1993 it increased from 0.75 to 1.55 and then declined in spring and summer 1994. In autumn 1994 abundance was high (2.86) and it then rose again to reach a still higher level of 3.72 in spring, which declined to 1.03 in summer 1995. In general abundance was high in spring and winter.

In general female fish dominated the stickleback population of Airthrey loch (Table 2).

*Infection with length of host*

All length classes of fish were observed to be infected in almost every month except LC-1, which was only available from mid summer to early autumn. The prevalence rate remained between 8.0 to 52.0 in LC-2, LC-3 and LC-4 during the year 1994 whereas it was higher and ranged from 11.1% to 100% in LC-2 to LC-4 during 1995. In the same way abundance ranged from 0.2 to 2.1 in LC-2 to LC-4 during the year 1994 whereas it ranged from 0.1 to 7.0 in LC-2 to LC-4 during 1995.

The overall prevalence of *P. filicollis* in each length class of fish is sown in Table 3. Prevalence was highest (41.55%) in LC-3 and the lowest (30.76%) in the LC-1. In all four length classes of fish, prevalence fluctuated between 30-40%. A rising trend in

prevalence from LC-1 to LC-3 was observed. There was no statistical difference in prevalence between all four length classes of fish (Chi-square, P = 0.50 = 2.366, P> 0.50).

**Table 1: Prevalence and abundance of *P. filicollis* in male, female and undetermined sex *G. aculeatus* in Airthrey Loch.**

| Sex of fish | Total fish examined | No. of fish infected | Total No. of worms | Prevalence (%) | Abundance |
|---|---|---|---|---|---|
| Male | 314 | 119 | 352 | 37.9 | 1.1 |
| Female | 666 | 280 | 1138 | 42.0 | 1.7 |
| Sex? | 371 | 117 | 486 | 31.5 | 1.3 |
| Total | 1351 | 516 | 1976 | 38.1 | 1.4 |

**Table 2: Seasonal prevalence in male, female fish and male to female ratio of *G. aculeatus* population in Airthrey Loch**

| Season | Sex | Fish examined | Fish infected | Prevalence (%) | M:F ratio (infected) |
|---|---|---|---|---|---|
| Summer | M | 47 | 15 | 31.9 | 1: 3.12 |
| | F | 165 | 48 | 29.1 | |
| Autumn | M | 79 | 32 | 40.5 | 1: 1.59 |
| | F | 148 | 51 | 34.4 | |
| Winter | M | 55 | 22 | 40.0 | 1: 2.9 |
| | F | 112 | 64 | 57.1 | |
| Spring | M | 133 | 50 | 37.6 | 1: 2.34 |
| | F | 241 | 117 | 48.5 | |

**Table 3: Prevalence, abundance and variance to mean ratio of *P. filicollis* in different length classes of *G. aculeatus* population in Airthrey Loch.**

| Length class | No. of fish Exam | Infect | Prevalence, Abundance Prev. | Abun. | Total No. of worms | No. of worms Maxi. | Mean | Variance | Variance/mean ratio |
|---|---|---|---|---|---|---|---|---|---|
| LC-1 | 104 | 32 | 30.7 | 1.3 | 144 | 46 | 1.38 | 24.04 | 17.36 |
| LC-2 | 483 | 174 | 36.0 | 1.3 | 649 | 20 | 1.34 | 07.77 | 5.57 |
| LC-3 | 462 | 192 | 41.5 | 1.8 | 859 | 35 | 1.85 | 17.10 | 9.19 |
| LC-4 | 302 | 118 | 39.0 | 1.0 | 324 | 23 | 1.07 | 5.59 | 5.21 |
| Total | 1351 | 516 | 38.1 | 1.4 | 1976 | 46 | | | |

The highest abundance was found in LC-3 and lowest in LC-4. In each year class of the fish examined, there was no significant difference in prevalence and intensity

between different length classes but there was a general tendency for the parasite population to fall in the largest fish. The mean intensity was also not statistically different between length classes (Chi-square, P = 0.90 = 0.584, P > 0.90).

The distribution of *P. filicollis* in different length classes of fish fitted the negative binomial distribution. The variance to mean ratio was always greater than unity, which indicated an over-dispersion of the parasite in the host population. Over-dispersion peaked in LC-1 and declined in the larger hosts (Table 3).

## DISCUSSION

More female than male fish were caught from Airthrey Loch throughout the year. No significant difference in overall prevalence rate between male and female fish was observed or in any particular season. Similarly, the abundance in male and female sticklebacks was not significantly different. No sexual preference by *P. filicollis* was recorded in previous studies from sticklebacks (Hopkins, 1959; Chappell, 1969a; Dartnall, 1972). Similarly, Fischer and Freeman (1969) reported no significant difference in the prevalence and mean intensity of penetrating plerocercoids of *P. ambloplitis* in male and female smallmouth bass from a single sample of 24 fish. Althoguh no significant difference in the prevalence in male and female stickleback is observed in the present study, yet the number of infected male remained lower and their mean length was always more than female fish especially in spring and early summer, which may be associated with peculiar biology of male in spawning period, being engaged in nest building and neither do they eat, which may result in decreasing possibilities of infection by intermediate host as suggested by Banina and Isakov (1974). The difference in prevalence rate and abundance in male and female may be result of difference in habitat of two sexes during breeding season, as female may be leaving the littoral zone after breeding and moving to deeper part of the loch. However, normally male and female stickleback shoals together.

*Infection and length of the host*

A rising trend in prevalence of *P. filicollis* with increasing host length was observed, but this did not show significant variation in all four length classes of fish. Abundance was high in the lowest length class and lowest in the highest length class. The drop in mean number of parasite in larger fish may reflect the dietary and behavioural change in older fish. The result of this study resembles those of earlier studies on this parasite. Two to three cm ($0^+$ year class) fish were heavily infected than $1^+$ year class and mean intensity varied from 1.3 to 1.6 but even raised to 2.6 and 2.9 (Hopkins, 1959). No significant variation in prevalence of *P. filicollis* with increasing fish size was observed, whereas mean intensity showed rising trend in the beginning and then dropping in larger fish (Chappell, 1969a). A significant decrease in prevalence with fish size, and reduction in intensity was observed in *P. filicollis* from Chew River in England (Dartanall, 1972). Whereas, low prevalence (8.1%) of *P. filicollis* was recorded in 5.1 to 6.0 cm fish from a

anadromus population of stickleback in Norway (Rodland, 1979). Similarly the degree of infection increased with size of fish for *P. torulosus* in dace (Kennedy and Hine, 1969) and mean intensity of infection of *P. perace* generally rose with the increase in length of roach (Wootten, 1972). Prevalence and mean intensity of *P. neglectus* increased with increasing length of chub (Scholz, 1991) and these were maximum 56% and 6.7% in the largest fish. Whereas no significant effect of length of European eel on the abundance of *B. claviceps* was observed by Nie and Kennedy (1992).

*Distribution of parasite in the host population*

The distribution of *P. filicollis* in fish population from Airthrey Loch was over-dispersed in both sexes, in all sizes of fish and in all seasons throughout the study period. An important factor, which increases the degree of over-dispersion is repeated exposure of the host to the infective stage of the parasite (Crofton, 1971; Anderson, 1974). The negative binomial distribution may be generated by the exposure of the host to many waves of infection, by the spatial clumping of the infective stages of the parasites or by changes in probability of infection caused by previous infection (Crofton, 1971a). As suggested by Anderson and Gordon (1982) the over-dispersed pattern of parasite number per host in natural habitat act to increase density dependent regulation of both host and parasite abundance. According to them, the mechanism of "environmental stochasticity" implies the rate processes, which govern the population growth of parasites species. These rates processes are birth, death, immigration and emigration, which are not constant for a given species and depend on environmental factors like climate, host susceptibility and host behaviour. They even suggested that for parasites in which a host is the main environment for population growth, difference in host behaviour or host susceptibility are the major factors generating over-dispersion in the distribution of the parasite within the host population.

As parasites were continuously acquired by the fish hence are accounted for higher variance to mean ratio. Abundance value fluctuated, which corresponded too with few heavily infected fish in the sample, or the regular expulsion of the worm population or the parasite-induced host mortality. Host mortality results in eliminating heavily infected fish thus decreasing mean parasite burden. Over-dispersion and parasite induced host mortality need further experimental investigation. Over-dispersion of *Proteocephalus* species has been reported by other authors (Kennedy and Hine, 1969; Wootten, 1974; Hanzelove *et al.,* 1990) and in *B. claviceps* (Nie and Kennedy, 1992).

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# EPIDEMIOLOGY AND CHEMOTHERAPY OF SARCOPTIC MANGE IN GOATS

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**Abstract:** The epidemiology and chemotherapy of sarcoptic mange in goat was studied at Civil Veterinary Hospital, Faisalabad, Pakistan. Out of 782 goats accessions to Civil Veterinary Hospital over a period of one year (January to December 1996), sarcoptic mange was recorded in 71(9.07%) animals. The prevalence was significantly higher (61.97%) among the animals below the age of one year than in animals aging more than one year (38.03%). Incidence is more (54.92%) in females than males (45.08%). Relative humidity showed a positive correlation with the occurrence of the disease. Fluctuations in the environmental temperature also affected the prevalence of the disease. The winter season was most strongly associated with the occurrence of the disease (20.87%), followed by spring (13.84%), autumn (4.76%) and summer (2.47%). Neem oil (*Azadarachta indica*) 14 applications showed a 100 percent clinical and parasitological cure with 28 days.

**Key words:** Epidemiology, chemotherapy, mange, goats, Pakistan.

## INTRODUCTION

Sarcoptic mange caused by *Sarcoptes scabiei* var. *Ovis* is a very common condition among goats in Pakistan with the symptoms of intensive irritation and dermatitis (Kambarage, 1992). Although the disease may occasionally terminate fatally (Kulkarni *et al.,* 1980; Satiji *et al.,* 1981), the economic importance of sarcoptic mange stems mainly from its high prevalence associated with unthriftiness, reduced body growth and productivity. Scabies in human resulting from contact with goats has also been reported (Chakrbarti *et al.,* 1981).

The present study was designed to determine the prevalence of sarcoptic mange in goats as well as to investigate the effects of host (age, sex) and meterological (relative humidity, environmental temperature) factor on the occurrence of the disease. An additional objective was to determine the therapeutic effect of Neem oil in the treatment of sarcoptic mange in sheep. As herbal preparations are gaining popularity in the hands of

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modern veterinary practioners owing to their low cost, no considerable side effects and quick adaptability by rural farmers.

## MATERIALS AND METHODS

*Study Animals and Epidemiological Parameters*

The study was conducted at the Civil Veterinary Hospital, Faisalabad. During the study period (January to December, 1996), a total of 782 sheep (of various age groups) were brought to the hospital for the treatment of different ailments. Of these 71 (9.075) were found positive for sarcoptic mange.

The month and season-wise prevalence of sarcoptic mange was recorded. For this purpose, the year was apportioned into 4 seasons with the following break up; winter (November thru Febrauary), spring (March, April), Summer (May thru August), and autumn (September, October). The prevalence of sarcoptic mange in relation to temperature, humidity age and sex was also calculated, the meteorological data for the relevant period were obtained from Department of Agri Meteorology, University of Agriculture, Faisalabad.

*Collection and Examination of Skin Scrapings*

In suspected cases, the skin scrapings collected from the edges of the lesions (with a blunt scalpel from 1x1 cm area from deep layer of epidermis till blood and lymph oozes out from the affected parts as head and nose region) in petri plates were warmed at 38°C for approximately 2 minutes and then examined under a stereo microscope for the presence of various stages of life cycle of mites (Sloss, 1970; Soulsby, 1982). The scrapings, which were found negative were transferred to test tubes containing 10 ml of 10% KOH and heated for 5 minutes in a beaker containing water. Later the tubes were centrifuged for 3 minutes at 2000 rpm and supernatant fluid was discarded. About 5 ml of water was added to the sediment and tubes were again centrifuged. The supernatant fluid was again discarded and a drop of sediment was examined microscopically for presence of mites and their eggs (Soulsby, 1982).

*Therapeutic Trials*

Thirty naturally infested goats of various age groups and both sex having either localized or generalized mange lesions were selected for therapeutic trials. Mange lesions were present on the head, neck, eyelids, ears, choulders, brisket, chest, abdomen, thighs, perineum and legs. The infested goat had alopecia, intense itching, pruritis and shaking of head and ears with marked emaciation and weakness. These goats were randomly divided into two groups taking 20 animals in one group (A) and 10 animals as untreated control in another (B). Animals in group A were treated with Neem oil (*Azadarachta indica*). Neem oil was smeared daily for 14 days on the effected parts by using cotton swabs so that the Neem oil may penetrate well in the skin. The efficacy of Neem oil was accessed on the day 0, 7, 14, 21, 28 post treatment and compared with infected control. Negative skin

scrapings, gradual disappearance of gross lesions, stoppage of itching, smoothing of skin surface and re-growth of normal hair were taking as the criteria to access the efficacy of Neem oil.

## RESULTS

During the one ear study period i.e., from January to December, 1996, a total of 782 goats of various ages were brought to the Civil Veterinary Hospital, Faisalabad. Of these, 71 were found positive for sarcoptic manage. The prevalence of sarcoptic mange was thus 9.07%.

*Month-wise prevalence*

The month-wise prevalence of sarcoptic mange is shown in Fig. 1. The highest prevalence of mange was recorded in the month of February with the infestation rate of 34.28%. The lowest incidence was recorded during the month of August being zero percent.

*Season-wise prevalence*

The highest prevalence was recorded in winter season i.e., 20.87%, while the lowest in summer i.e., 2.47% (Fig. 2). The data obtained were subjected to statistical analysis. The correlation between percentage of infestation, temperature (minimum and maximum) and relative humidity is presented in Fig. 1. The correlation co-efficient for ambient temperature ($r = -0.947$) was found to be highly significant ($P < 0.001$). Similarly, correlation ($r = 0.811$) between relative humidity and infestation rate was also highly significant ($p < 0.01$). As such the data revealed that fluctuation of temperature and humidity had a definite effect on the incidence of disease.

*Effect of host sex and age on the prevalence*

Analysis of prevalence data in relation to the patient's sex indicated that his variable did not affect the prevalence as well as severity of infestation. The prevalence of infestation was higher in animals under one year (61.97%) than in those aging over one (38.03%). In females, infection was 54.4 percent where as 45.6 percent was recorded in males.

*Effects of chemotherapy with Neem oil*

The results of therapeutic trials with Neem oil showed that after the treatment with *Neem oil*, clinical lesions started disappearing and there was growth of smooth shining hair over the affected parts. All the treated animals showed complete (100%) clinical and parasitological recovery within 28 days and no recurrence of lesions was reported by the owners.

## DISCUSSION

The overall prevalence of sarcoptic mange was 9.07 percent. The highest prevalence (34.28%) was observed in the month of February followed by January (21.87%), and March (17.77%). No case of the disease was recorded in the month of July. These findings are consistent with those of Blood and Radostits (1989). As regards seasonal impact on the disease frequency, the highest prevalence was recorded during the winter season (20.87%), while the lowest during summer season (2.47%). It was reported that during winter and autumn there was increased humidity and lowered temperature, which favor the multiplication of mites resulting the increased incidence of clinical cases of goats scab. During summer mites usually remain dormant in cryptic site, including the inguinal regions, infra orbital and inter digital fossae and the external auditory meatus. The results of the present study with respect to seasonal predisposition to sarcoptic mange are in accord with those of Guillot (1981), Sargison *et al.* (1995), Neog *et al.* (1992) and Roberts *et al.* (1971). In face Basu *et al.* (1952) reported the manage is definitely seasonal and is almost restricted to a few months of the year viz. January to April. Sarcoptic mites have been found to survive better at 20-27°C than at 31-39°C, which may explain in part at least for the much higher prevalence in winter as compared to other seasons.

The prevalence of sarcoptic mange in relation to sex indicated that this variable did not affect the prevalence as well as severity of infestation since animals of either sex were nearly equally affected. Animals under one year of age were significantly more frequently affected (61.975%) than over one year (38.03%). These finding are in congruence with those of Chakrabarti and Pradhan (1985). Since the disease spread by direct contact, the higher prevalence in animals under one year of age could be due to their tender skin and huddling tendency.

The therapeutic efficacy of Neem oil (*Azadarachta indica*) in the present studies was 100%. Nearly similar results with this preparation had been reported by previous workers Hirudkar *et al.* (1997). They observed this preparation cured all cases of mange tithing 30 days of treatment and no side effects were observed.

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# THE GROUND ACTIVITY OF SPIDERS COMMUNITY INHABITING VINEYARD IN FAISALABAD, PAKISTAN

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**Abstract:** To estimate the effect of residential spider populations on insect pests, spider species composition, relative abundance and seasonal occurrence were determined. The ground surface of a vineyard was sampled for spiders using pitfall traps from March 1992 through February 1994. Thirty-three species of spiders were recorded, representing eight families. The most common species were *Pardosa oakleyi, Lycosa vulgaris, Pardosa birmanica,* and *Marpissa tenebrosa.* The remaining 27 species contributed less than 4 percent of the total capture. The number of spiders in the samples peaked during late spring and summer months. Richness and diversity index values were high and the evenness index values were low during these months.

**Key words:** Cursorial spiders, species diversity, vineyard, Pakistan.

## INTRODUCTION

Increasing dependence on chemical insecticides for inhabiting populations of pest insects in the cultivation is becoming a serious threat to the ecological balance of the agroecosystem. Loading of the environment with toxic chemicals is detrimental to non-target organisms including those inhabiting the sub-soil fauna. Insecticides may even enter into the bodies of man and wildlife through food chains and cause health problems.

Spiders are common both in natural and agricultural ecosystems. These polyphagous insectivores comprise a significant portion of the beneficial natural enemy complex in agroecosystem (Luczak, 1979; Mansour *et al.*, 1980; Nyffeler and Benz, 1980; Dean *et al.*, 1982; Wise, 1993 and Nyffeler *et. al.* 1994a, b). In vineyards, spiders are abundant predators on grapevines, *Vitis vinifera* L. (Cate, 1975), and they may contribute to the natural control of some insect pests (Wilson *et al.* 1992; Costello and Daane, 1997 and 1998). For effective pest management decisions, the knowledge of

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ecological processes that govern the predator and pest population dynamics is an essential prerequisite. The present study documents temporal changes in the biological diversity of the spiders inhabiting the ground surface of a vineyard at Faisalabad.

## MATERIALS AND METHODS

This study was conducted in the vineyard of the Experimental Fruit Orchards of the University of Agriculture, Faisalabad (Punjab), from March 1992 through February 1994. In the field, grapevines were planted in rows each of which ran through the full length of the yard. The vines were spread over wires about 2m above ground supported by metallic poles. Thus, the ground floor remained shadowed by the vines from April through September. Outside this period the floor was exposed to almost full sunlight. The vineyard was surrounded by date palms and citrus groves from its western and southern sides.

The spider populations inhabiting the ground surface of the vineyard were sampled each month. The sampling were done using the pitfall traps which comprised of 10 cm long and 6 cm wide glass jars containing 150 ml of 70% ethyl alcohol and a little quantity of kerosene oil. The traps were sunk in the ground so that their mouths were at level with the ground surface. The traps were set in four rows, each row comprised of six traps. The distance between the traps within a row was 10 m. All the traps were operated for four consecutive days each month through out the study period. A fresh trap replaced each of the traps after two days. The spiders captured in the traps were taken to the laboratory, cleaned of debris, placed in 70% ethanol solution and sorted to species level.

For analysis, spider samples of all the traps operated during a trapping session were pooled. For the density analysis, abundances of spiders in the month were ranked and two way ANOVA was performed on ranks. The species diversity was calculated using Hill's diversity numbers, $N_1$ and $N_2$. $N_1 = e^{H'}$ ,where H' is shanon's index. $N\ 2 = 1/\lambda$, where $\lambda$ is Simpson's index. Evenness was calculated using modified Hill's ratio, $E = N_2 - 1 / N_1 - 1$ (Ludwig and Reynolds, 1988; Magurran, 1988). As the sampling effort was the same for the each of the trapping session, species richness per month was measured by counting the number of species present in that monthly samples. The diversity of yearly samples was compared by using Wilcoxon Signed – rank test.

## RESULTS

During the present study 1298 spiders were collected from the field, out of which 33 species were recorded representing eight families (Table 1). The first year (March, 1992 through February, 1993) of the study yielded 68% of the spiders, with a mean number of spiders per trap-night of 18.5. This was more than the second year (March, 1993 through February, 1994) which had a mean of 8.5 spiders per trap night. Except for the September samples, each monthly sample of 1992-93 had more spiders than did the similar trap per month in 1993-94 (Fig. 1a and b).

Table 1. Spiders collected from vineyard during March, 1992 through February, 1994

| Species | No. of specimens (%) | | |
|---|---|---|---|
| | 1992-93 | 1993-94 | Total |
| **Lycosidae** | **64.7** | **67.6** | **65.6** |
| *Pardosa oakleyi* | 17.1 | 27.0 | 20.2 |
| *Pardosa birmanica* | 10.3 | 9.8 | 10.2 |
| *Hippasa dignus* | 2.7 | 7.4 | 4.2 |
| *Lycosa vulgaris* | 25.2 | 5.5 | 19.0 |
| *Lycosa terrestris* | 2.2 | 2.9 | 2.5 |
| *Lycosa remota* | 1.4 | 2.9 | 1.9 |
| *Lycosa nigricans* | 4.7 | 12.3 | 7.1 |
| *Lycosa erronis* | 0.2 | 0.0 | 0.2 |
| *Lycosa macculata* | 0.9 | 0.0 | 0.6 |
| **Gnaphosidae** | **12.6** | **8.8** | **11.4** |
| *Gnaphosa elegantis* | 0.9 | 1.0 | 0.9 |
| *Gnaphosa subpoonaensis* | 0.7 | 1.0 | 0.8 |
| *Zelotus illustris* | 6.1 | 3.4 | 5.2 |
| *Zelotus faisalabadensis* | 1.1 | 0.5 | 0.9 |
| *Zelotus Pakistaniensis* | 0.5 | 0.5 | 0.5 |
| *Zelotus Pulchellus* | 0.7 | 0.0 | 0.5 |
| *Drassodus comatus* | 0.9 | 1.0 | 0.9 |
| *Sosticus mirus* | 0.0 | 0.5 | 0.2 |
| *Talinites extranea* | 0.2 | 0.0 | 0.2 |
| *Gnaphosidae 1* | 1.6 | 1.0 | 1.4 |
| **Salticidae** | **11.5** | **12.3** | **11.7** |
| *Marpissa carinata* | 1.1 | 0.5 | 0.9 |
| *Marpissa tenebrosa* | 9.9 | 9.8 | 9.9 |
| *Marpissa mirabilis* | 0.2 | 0.0 | 0.2 |
| *Plexippus primaries* | 0.0 | 0.5 | 0.2 |
| *Phidippus notabilius* | 0.2 | 1.5 | 0.6 |
| **Thomisidae** | **0.9** | **2.0** | **1.2** |
| *Oxyptila nemestrina* | 0.2 | 0.5 | 0.3 |
| *Misumens laudata* | 0.0 | 0.5 | 0.2 |
| *Philodromus amoenus* | 0.5 | 1.0 | 0.6 |
| *Thanatus promptus* | 0.2 | 0.0 | 0.2 |
| **Erigonidae** | **7.9** | **6.9** | **7.6** |
| *Erigone dentate* | 7.4 | 5.9 | 6.9 |
| *Erigonidae 1* | 0.5 | 1.0 | 0.6 |
| **Clubionidae** | | | |
| *Castianeira amiantis* | 1.6 | 1.0 | 1.4 |
| **Oxyopidae** | | | |
| *Oxyopes azhari* | 0.5 | 1.5 | 0.8 |
| **Sparassidae** | | | |
| *Eusparassus sp.* | 0.5 | 0.0 | 0.3 |

Numerically, the Lycosidae were the most dominant spiders (65.6%) in the total samples. They were represented by three genera and nine species. On the whole, *Lycosa vulgaris, Pardosa oakleyi*, and *Pardosa birmanica* were three most dominant species and constituted 49.4% of the total specimens. Family Ganaphosidae was represented by ten species and Salticidae by five species and each contributed >11% of the total collected spiders. All the other remaining species contributed 11.3% of the total specimens. The taxonomic composition and the order of dominance of the common species were different in the two yearly samples. During 1992-93, *Lycosa vulgaris* and in 1992-93 *Pardosa oakleyi* were the dominant species. Although in both years family Lycosidae remained dominant and contributed more than 65% of yearly collected specimens (Table 1).

In the present study, four species (*Lycosa vulgaris, Pardosa oakleyi, Pardosa birmanica* and *Marpissa tenebrosa*) constituted >59% of all spiders collected. In two years combined data, *Lycosa vulgaris* and *Pardosa oakleyi* co-shared the dominance by contributing 20% and 19% of all specimens. They were followed by *Pardosa birmanica* (10.2%) and *Marpissa tenebrosa* (9.9%). The relative abundance of these two species was similar in both the years but *Lycosa vulgaris* was most dominant in 1992-93 and *Pardosa oakleyi* in 1993-94 samples. However, statistically the difference was not significant.

The abundance of all the species in the monthly samples revealed that the number of specimens started to increase from March through May and June, and then declined continuously till December (Fig. 1a and b). From December through February, the number of spiders in the samples remained low. ANOVA showed that the size of the monthly ($F= 10.3$; d.f. $=11$; $P<0.01$) as well as yearly ($F= 11.9$; d.f. $=1$; $P<0.01$) samples was different during the two years.

**Table 2: Species diversity indices for the spiders captured from the vineyard during the study period**

| Month | 1992-93 | | | | 1993-94 | | | |
|---|---|---|---|---|---|---|---|---|
| | N0 | N1 | N2 | E | N0 | N1 | N2 | E |
| March | 7 | 4.8 | 4.1 | 0.8 | 7 | 5.4 | 5.4 | 1.0 |
| April | 11 | 5.4 | 3.4 | 0.6 | 8 | 5.2 | 4.4 | 0.8 |
| May | 19 | 11.5 | 8.0 | 0.8 | 11 | 7.3 | 6.1 | 0.8 |
| June | 13 | 4.8 | 3.0 | 0.5 | 9 | 6.9 | 6.3 | 0.9 |
| July | 14 | 8.0 | 6.5 | 0.8 | 12 | 9.8 | 11.3 | 1.2 |
| August | 11 | 7.5 | 6.6 | 0.9 | 7 | 6.0 | 8.3 | 1.5 |
| September | 6 | 5.7 | 21.0 | 4.2 | 8 | 4.0 | 2.8 | 0.6 |
| October | 10 | 9.2 | 17.1 | 2.0 | 2 | 2.0 | 1.7 | 1.7 |
| November | 4 | 3.8 | 10.0 | 3.2 | 4 | 4.0 | 1.7 | 5.7 |
| December | 1 | 1.0 | 0.0 | 1.0 | 3 | 1.1 | 3.0 | 8.5 |
| January | 3 | 3.0 | 0.0 | 1.0 | 2 | 2.0 | 1.7 | 1.7 |
| February | 7 | 5.8 | 6.4 | 1.1 | 2 | 1.8 | 2.0 | 1.3 |

No = the total number of species; $N1= e^{H}$, where H' is shanon's index; $N2 = 1/\lambda$, where $\lambda$ is Simpson's index; $E = N_2 - 1 / N_1 - 1$ (evenness index).

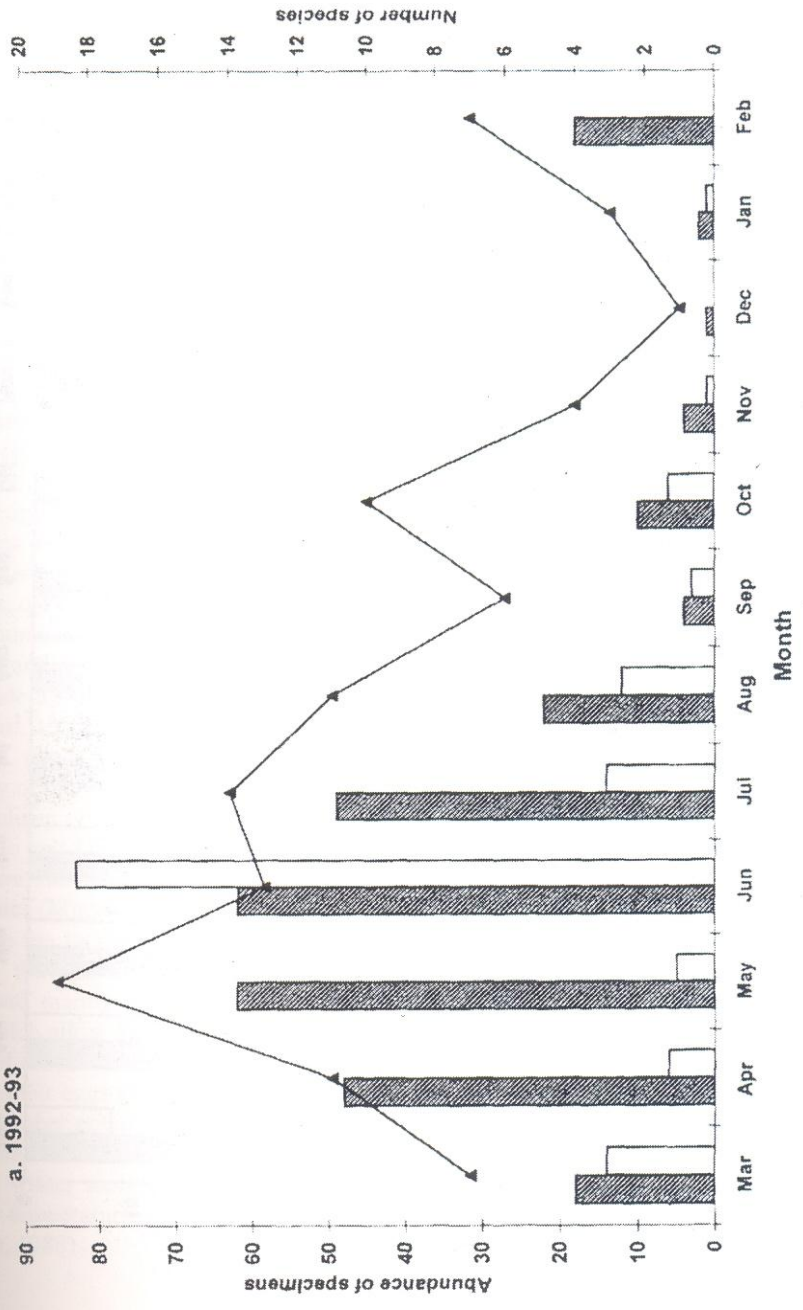
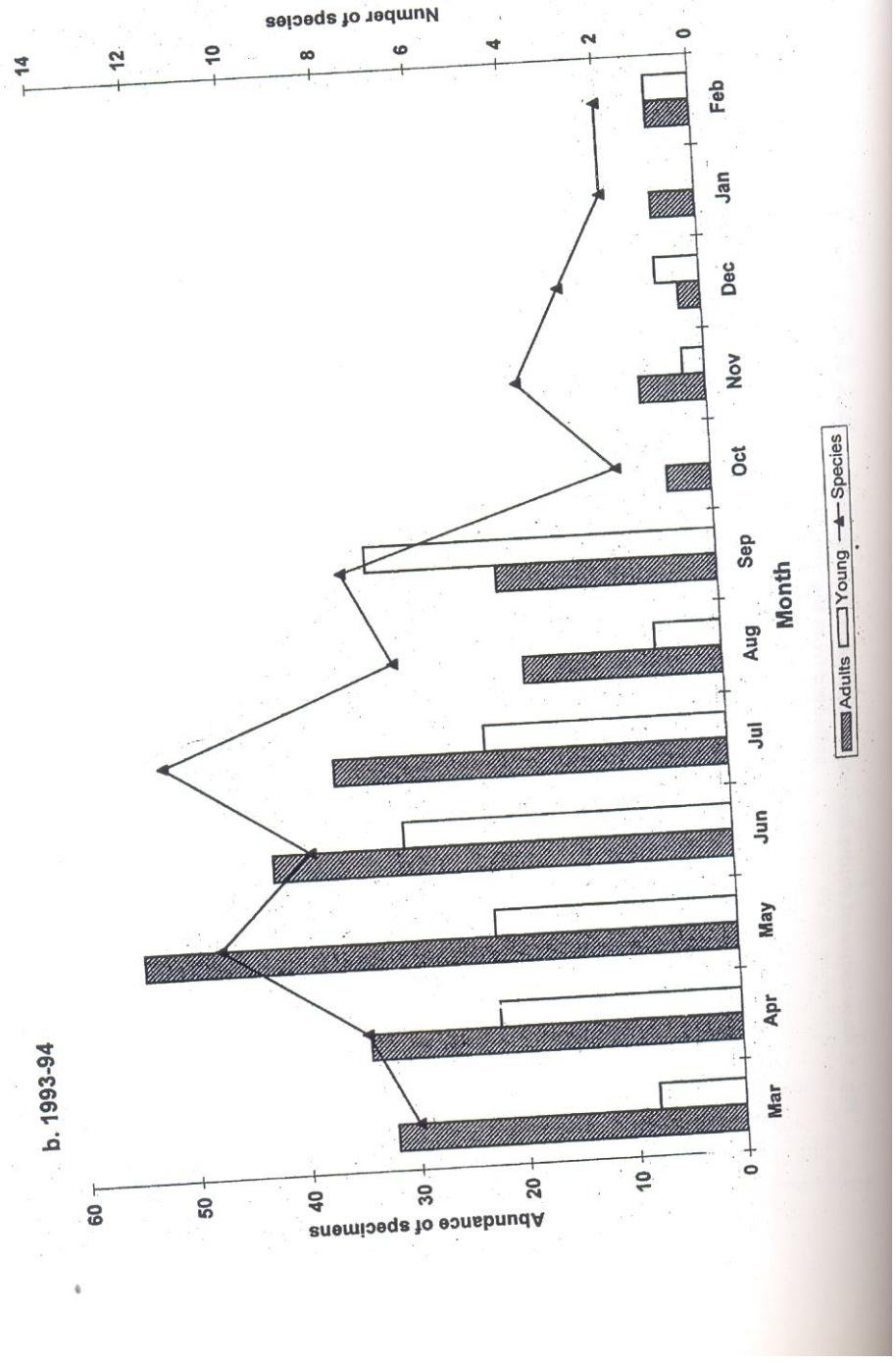



Fig. 1. Abundance of young and adult specimens and number of species in the monthly samples collected from the vineyard


b. 1993-94
Abundance of specimens
0
10
20
30
40
50
60
Number of species
0
2
4
6
8
10
12
14
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Month
Adults
Young
Species

Species richness in the monthly samples varied considerably. High numbers of species were generally recorded during late spring and the summer months. The values of diversity indices in the monthly samples of both years showed trend similar to that of the species richness. The highest richness and diversity was recorded in the months of May in 1992 and July in 1993. Species richness in the two years' monthly samples ($F=5.89$; $d.f.=11$; $p<0.01$), and the pooled yearly samples ($F=7.04$; $d.f.=1$; $p<0.05$) were significantly different. However, overall diversity values of the two yearly samples were not different from each other. Evenness also showed fluctuations and remained high from October to January. The evenness index value showed that no species gained a clear dominance in any of the monthly samples (Table 2).

## DISCUSSION

During the present study, Lycosidae, Gnaphosidae, Salticidae and Erigonidae were better represented in the samples. These four families comprised almost 96 percent of the total abundance. Out of these four families, Lycosidae was the most abundant group, as it constituted nearly 66 percent of the total abundance. Dominance of lycosids in the samples of cursorial spiders has also been reported by Oppenheimer and Tikader (1975), Patel *et al.* (1988), and Patel and Pillai (1988). Of the 33 species recorded during the two years, only four species were dominant. Dominance monopoly by a few species in spider communities of agroecosystem is a common phenomenon (Specht and Dondale, 1960; Leigh and Hunter, 1969, Costello and Daane, 1995). Three out of 41 species collected made up from 50 to 76% of the spider community in a Quebec apple orchard (Dondale *et al.* 1979) and 7 out of 97 species collected half of the spiders found in East Texas cotton (Dean *et al.* 1982). It is reported that spatial, temporal and physical heterogeneity of habitats usually lead to contradictory results in experiments performed at different localities or in different years in the same localities (Hairstone, 1981; MacNally, 1983; Castello and Daane, 1998). Variation in the annual population size may also relate to temperature and air moisture variations, paucity and abundance of food supply and natural enemies (Muma, 1973).

With the increase of temperature during late spring and summer, the biological diversity of the spiders increased and peaked during this period. The increase in abundance was mainly due to the lycosid spiders. The lycosid spiders have waterproof epicuticle which allow them to be more active in high temperature and low humidity than the spiders of other families (Oppenhaimer and Tikader, 1975). Furthermore, most of the lycosids sun their eggs (Norgaard, 1951; Vlijm *et al.*, 1963). During the summer months, the greater number potential prey is also available to spiders due to high activity of insect in these months. Following the spring upsurge, the spider population often returned to low level during winter months. This might have been brought about due to mortality as a result of low temperature and shortage of food or low activity of the overwintering spiders (Gunnarsson, 1988).

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# EFFECT OF INOCULATING *SALMONELLA GALLINARUM* INTO YOLK SAC ON HEALTH STATUS OF BROILER CHICKS

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**Abstract:** One hundred (day-old) broiler chicks were randomly divided into two groups, A and B comprising fifty chicks each. The chicks of group A (treatment) received confirmed pathogenic isolate of *Salmonella gallinarum* (*S. gallinarum*) into the yolk while those of group B (control) received no bacteria. The experimental parameters studied were body weight, yolk sac, weight, pathology of yolk, packed cell volume (PCV) and Haemagglutination inhibition (HI) antibody titre against Newcastle disease virus (NDV) in serum. The reduced body weight, enhanced yolk sac weight and abnormal yolks were noted in the treatment group as compared to control one. Also PCV and the geometric mean HI antibody titre values were found significantly lower in treatment group. The results showed that inoculation of *S. gallinarum* into yolk sac affected health status of chicks by reducing weight gain and maternal antibody absorption from unabsorbed yolks.
**Key words:** Salmonella, yolk sac, PCV, antibody titre, broiler chicks

## INTRODUCTION

When the chick emerges from shell, its main nutrient source is the portion of yolk not utilized during incubation (Jin *et al.,* 1998). It is generally contended that initiation of growth in newly hatched broiler chicks is correlated directly with the absorption of yolk sac (Chamblee *et al.,* 1992). The nutrient substances and immunoglobulins present in yolk sac are used by the chick via blood circulation. The residual yolk is absorbed during first week of life. Sometimes its retention occurs due to certain factors. Presence of fat and water in yolk favour the bacterial growth, which may lead to yolk sac infection (Anonymous, 2000). In other words, infection of yolk sac deprives the chick of essential nutrients and antibodies.

The infection of yolk sac occurs frequently in young chicks. Different types of bacterial agents are attributed for causation of yolk sac infection. Major bacteria involved in yolk sac infection include *Salmonella gallinarum, Salmonella typhimurium, Escherichia coli, Staphylococcus aureus, Pseudomonas, Bacillus cereus, Enterococci, Clostridium* spp. and *Proteus* (Anjum, 1997). Present project was taken in hands to study the effect of inoculating *S. gallinarum* into the yolk sac on health status of broiler chicks.

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## MATERIALS AND METHODS

*Preparation of Inoculum (S. gallinarum)*

*S. gallinarum* was isolated from the viscera of birds suspected for fowl typhoid infection. Identification and confirmation of isolation organisms was made on the basis of cultural, morphological, staining characters and biochemical tests as described by Buxton and Fraser (1977). Viable count of the isolated organisms was determined by Miles and Misra technique (Quinn *et al.*, 1994).

*Experimental Design*

One hundred (day-old broiler chicks were taken from local market and were randomly divided into two groups, A and B having 50 chicks each.

Group A: Chicks in this group were inoculated with *S. gallinarum* into yolk sac.
Group B: Chicks in this group received no bacteria and were kept as control.

*Sampling Days*

Ten birds from each group were slaughtered on 3rd, 5th, 7th and 9th day of the experiment. Before slaughtering blood (with and without anticoagulant) was collected from each chick, on all sampling days in sterilized disposable syringes. Serum was obtained from clotted blood samples. The anticoagulant added blood was used for PCV estimation.

*Experimental Parameters*

The following experimental parameters were studied:

1. Body weight: Each bird was weighed before slaughtering.
2. Yolk sac weight: The unabsorbed yolks were removed and weighed.
3. Pathology of yolk: The yolks were thoroughly examined to record any gross pathological changes at the time of slaughtering.
4. Packed cell volume: Packed cell volume was determined by microhaemetocrit method (Khan and Aslam, 2001).
5. Antibody titre against NDV: Antibody titre against NDV in serum was determined by HI test as described by Thayer and Beard (1998).

*Statistical Analysis*

Data collected was statistically analyzed by applying unpaired t-test (Steel and Torrie, 1982).

## RESULTS

The birds of group A, inoculated with *S. gallinarum* appeared weak, dehydrated, droopy, ruffled and pot bellied. They also showed somnolence, poor growth, pale heads

and white diarrhea that adhered to the vent. Out of 50 chicks in treatment group one died each on day-2 and day-5, two died each on day-4 and day-7, and three died on day-8 of the experiment. The chicks of control group maintained a normal healthy appearance with no mortality.

Results of different parameters are presented in Tables 1-5.

**Table 1: Mean body weight (g) of treatment (A) and control (B) groups**

| Groups | Sampling Days | | | |
|---|---|---|---|---|
| | Day 3 | Day 5 | Day 7 | Day 9 |
| A | 42.2* | 50.3* | 62.9* | 78.3* |
| B | 58.3* | 71.4* | 114.8* | 126.6* |

* Significant difference (P< 0.05) A = treatment B = control

**Table 2: Mean yolk sac weight (g) of treatment (A) and control (B) groups**

| Groups | Sampling Days | | | |
|---|---|---|---|---|
| | Day 3 | Day 5 | Day 7 | Day 9 |
| A | 2.68* | 2.41* | 2.93* | 3.18* |
| B | 1.26* | 1.08* | 0.66* | 0.42* |

* Significant difference (P< 0.05) A = treatment B = control

**Table 3: Gross pathological changes in yolk sac of treatment (A) and control (B) groups**

| Day | Group | Discolouration | Offensive Odour | Consistency | | |
|---|---|---|---|---|---|---|
| | | | | Watery | Gaseous | Hard |
| 3 | A | ++ | +++ | +++ | + | - |
| | B | - | + | ++ | - | + |
| 5 | A | +++ | +++ | +++ | ++ | - |
| | B | - | - | - | - | ++ |
| 7 | A | +++ | +++ | +++ | ++ | - |
| | B | + | - | - | - | ++ |
| 9 | A | ++++ | +++++ | ++++ | + | - |
| | B | - | - | - | - | +++ |

A = treatment B = control Number of + signs indicates intensity of a parameter

**Table 4: Mean PCV value (%) of treatment (A) and control (B) groups**

| Groups | Sampling Days | | | |
|---|---|---|---|---|
| | Day 3 | Day 5 | Day 7 | Day 9 |
| A | 20.21* | 21.27* | 20.81* | 22.73* |
| B | 32.00* | 33.40* | 32.98* | 36.15* |

* Significant difference (P< 0.05) A = treatment B = control

**Table 5: HI titer against NDV in serum samples of treatment (A) and control (B) groups**

| Groups | Sampling Days | | | |
|---|---|---|---|---|
| | Day 3 | Day 5 | Day 7 | Day 9 |
| **Treated (A)** | 134.7 | 108.9 | 63.1 | 32.6 |
| **Treated (B)** | 279.5 | 244.2 | 103.5 | 55.3 |

## DISCUSSION

The chicks of treatment group showed significantly lower body weight as compared to control group. Khan *et al.* (2002) also observed reduced weight gain in yolk sac infection. It is alluring to speculate that experimental inoculation of *S. gallinarum* into yolk sac adversely affects metabolic activities of the chick, which ultimately lead to malabsorption and decreased weight gain.

In this study weight of unabsorbed yolk sac was significantly higher in the experimental than control group. Similar observations were made by Deeming (1995) and Shah (2002). This finding can be attributed to the fact that bacterial contamination of the yolk reduced the absorption of yolk material thus rendering the yolk of treatment group heavier than control group.

The yolks from treatment group were discoloured, enlarged, putrefied and watery as compared to yolks of control group, which were normal in colour, odour and consistency. Skeeles (1991) and Sainsbury (1992) reported similar observations. The theory underlying this phenomenon can be explained by the fact that when bacteria utilize the yolk as a food source, they convert it into metabolic breakdown products, thus rendering yolk abnormal.

Packed cell volume (PCV) analysis showed significant difference between treatment and control chicks. PCV values of treatment group were significantly lower than that of the control. A lower value of PCV in *S. gallinarum* infection has also been reported by Pomeroy and Nagaraja (1991) and Mdegela *et al.* (2002).

Haemagglutination inhibition (HI) titres of serum against Newcastle Disease Virus (NDV) were significantly lower in treatment group. Similar findings have been reported by Sander *et al.* (1998) and Rai *et al.* (2003). Our observation may be justified with the fact that in infected chicks, maternal antibodies against NDV are not absorbed from infected yolks so the level of serum antibodies falls.

From the above discussion it can be inferred that inoculation of *S. gallinarum* into yolk sac of broiler chicks resulted in reduced body weight, increased yolk sac weight with marked pathological changes in yolk, decreased PCV and antibody titre values, which adversely affect the health status of broiler chicks.

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# COMPARISON OF THE BODY COMPOSITION PARAMETERS OF FISH OF VARIOUS SPECIES WITH RELATION TO POND DEPTH

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**Abstract:** 36 specimen of *Labeo rohita, Cirrhinus mrigala, Hypophthalmicthys molitrix* and *Catla catla* were sampled from three ponds of different depth (5, 4 and 2.5 feet, respectively) to compare the body composition parameter of these fish of various species in relation to pond depth. It was revealed that there was significant effect ($P < 0.001$) of pond depth on % water, % ash, % organic, % fat and % protein contents (all % wet and dry body weight). It was observed that pond depth has significant effect ($P < 0.01$) on condition factor in pond B (4 feet depth) and no effect in pond A and C. The present study suggested that maximum mean values of body composition parameters were observed in *Labeo rohita* in all three ponds. While values of the same body composition parameter varied in ponds of different depths for the same species.

**Key words:** Body composition, inter comparison, pond depth, carps.

## INTRODUCTION

The main body constituents of the fish include water, lipid, ash and protein. Carbohydrates and non-protein compounds are also important constituents but are present in small amounts and are usually ignored during analysis (Love, 1980 and Wootton, 1990)). The live weight of majority of fish usually consists roughly of water, 70-80%, protein, 20-30%, lipid, 2-12% (Ali, 1999). However, the values vary considerably within and between species, and also with size, sexual condition, feeding, time of the year and activity. The distribution of these substances among the various organs and tissues of the body may also show considerable differences (Weatherley and Gill, 1987).

The term growth will signify change in magnitude. The variable undergoing change may be the length or other physical dimensions, including volume, weight, or mass either of an organism's whole body or its various tissues or it may relate to the contents of protein, lipids or other chemical constituent of the body. Growth may also relate to the change in the number of animals in population (Jhingran and Pullin, 1985).

Proximate body composition is the analysis of water, fat, protein and ash contents of fish (Love, 1970). The percentage of water is good indicator of its relative contents of energy, proteins and lipids. The lower the percentage of water, greater the

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lipids and protein contents and higher the energy density of the fish (Dempson *et al.*, 2004). It means that from measuring the relative amount of water in the fish one can obtain relative good estimates of the energy, fat and lipid contents (Salam and Davies, 1994, Jonsson and Jonsson, 1998).

The Catla, Rohu, Mrigal are all fast growing and highly esteemed food fishes in India. Fish has assumed great importance as a result of anti-cancerous effects, minimized risk of heart ailments and consequently prolongs life expectancy (Kulikove, 1978; Jhingran and Pullin 1985). The fish can convert food in the body tissues more efficiently than any other form of animals. The reason for superior food conversion efficiency of fish is that it assimilates diet with higher %age of protein because of their lower dietary energy requirements. These requirements for metabolisms are less in fish, which evolve in a protein rich energy deficient environment (Rath, 1993).

The present study was designed to make comparison between the body compositions of fishes of same species cultured in ponds of different depth.

There is increasing problem of shortage of water in Pakistan under the current dry spell. It is very expensive to keep the level of water in aquaculture ponds. If some species are performing better in specific water table then farmers can be encouraged to culture those species in those ponds to get fish with desirable body contents.

## MATERIALS AND METHODS

Thirty six specimens of *Labeo rohita*, *Cirrhinus mrigala*, *Hypophthalmicthys molitrix* and *Catla catla* (nine of each) were sampled from Abdullah fish farm Muzaffar garh from three ponds of different depths. All these specimens were caught with the help of a cast net. These fishes were transported in plastic containers to the Fish Research Laboratory, Institute of Pure and Applied Biology, Bahauddin Zakariya University Multan, where they were removed and killed with a blow on the head. After the fish became motionless they were blotted dry with a paper towel. All specimens were weighed on an electronic digital balance (Chyo, Japan) to the nearest 0.01gram. Total body length were measured to the nearest of 0.01cm using a Perspex measuring tray fitted with a sheet of millimeter ruler. All measurements were made from the tip of the maxilla to the longest caudal fin ray. To estimate the water contents, each fish was placed as a whole in pre weighed aluminum foil tray for drying till constant weight in an electric oven (Gallen Kamp, England) at 65-80°C.

To calculate ash content in each individual fish, 500 mg of sample was taken in a pre weighed, heat resistant China clay crucible and ashed in a Muffle furnace (Sybron thermolyne 1300) for 7 hours at 500°C and reweighed after cooling.

The fat contents were estimated using dry tissue by dry extraction method in which a mixture of Chloroform and Methanol in a ratio of 1:2, following the method of Bligh and Dyer (1959), Cui and Wootton (1988) and Salam and Davies (1994), was taken and 3 mg sample of powdered dry tissue was mixed into 10 ml of this mixture and stirred with a glass rod. The resultant mixture was left over night and then centrifuged. After

centrifugation, the clear supernatant was removed carefully into washed, dried and pre weighed small bottles. These bottles were then put in an oven at 40 to 50°C to evaporate the solvent to dryness leaving the lipid fraction. Lipids were then weighed on an electronic balance to nearest 0.0001 grams.

Total protein present in dry mass can be calculated by the difference method from the mass of other main constituents i.e. Ash, Fat and Water (Caulton and Bursell, 1977; Dawson and Grimm, 1980; Salam and Janjua, 1992; Salam and Davies, 1994; Amanuallah, 2000; Iqbal, 2000; Dempson *et al.,* 2004). Carbohydrates do not form a major component of fish and thus are generally neglected due to their egligible amounts (Elliott, 1976; Caulton and Bursell, 1977; Salam, *et al.,* 1993 and Salam and Davies, 1994). Excel and Minitab were used for statistical analysis.

## RESULTS

*Response of various fish species in pond A (depth 5 feet)*

It was observed that depth of pond A (5 feet) had a highly significant ($P < 0.001$) effect on all the body composition parameters (both dry and wet body weight) in fish of all four species and no effect on condition factor (Table 2).

In pond A, minimum values of % water contents were observed in *Catla catla* and *Labeo rohita* indicating maximum gain in body composition parameters followed by *Cirrhinus mrigala* and *Hypophthalmicthys molitrix.* % Ash and % fat contents (dry and wet weight) were highest in *Cirrhinus mirigala* while maximum % protein contents (dry and wet weight) were observed in *Labeo rohita* (Table 1).

*Labeo rohita* and *Catla catla* showed overall better growth among the four species in 5 feet deep pond.

*Response of different fish species in pond B (4 feet)*

It was observed that depth of pond B (4 feet) had a highly significant ($P < 0.001$) effect on all the body composition parameters (both dry and wet body weight) and significant effect ($P < 0.01$) on condition factor in fish of all four species (Table 4).

In pond B, minimum values of % water contents were observed in *Labeo rohita* indicating maximum gain in body composition parameters followed by *Catla catla, Hypophthalmicthys molitrix* and *Cirrhinus mrigala* respectively. % Ash contents (dry and wet weight) were maximum in *Catla catla* and minimum in *Labeo rohita.* Highest values of % organic and % protein contents were observed in *Labeo rohita* while *Catla catla* showed minimum value of % organic and *Cirrhinus mirigala* showed the lowest value of % protein contents (dry and wet weight). *Labeo rohita* showed overall better growth among the four species in 4 feet deep pond (Table 3).

**Table 1: Body constituents of different fish species of pond A (5 feet). Standard deviations are given in Parentheses**

| Body constituents | *Labeo rohita* | *Cirrhinus mrigala* | *H. molitrix* | *Catla catla* |
|---|---|---|---|---|
| Percent water | 66.757(0.369) | 69.523(0.351) | 71.060(0.394) | 66.750(0.340) |
| Percent ash (dry) | 9.66390.583) | 18.193(0.495) | 16.667(0.577) | 16.667(1.000) |
| Percent ash (wet wt.) | 3.220(0.190) | 5.5833(0.175) | 4.8733(0.167) | 6.6467(0.335) |
| Percent organic contents (dry) | 90.337(0.583) | 81.507(0.495) | 83.333(0.577) | 80.007(1.000) |
| Percent organic contents (wet) | 30.130(0.191) | 24.670(0.150) | 24.379(0.172) | 26.580(0.310) |
| Percent fat (dry wt.) | 15.990(0.00) | 27.323(1.115) | 24.00(0.000) | 20.663(1.149) |
| Percent fat (wet wt.) | 5.330(0.00) | 8.2700(0.346) | 7.0200(0.000) | 6.860(0.381) |
| Percent protein (dry wt.) | 74.333(0.577) | 54.167(1.258) | 59.333(0.577) | 59.33(1.528) |
| Percent protein (wet wt.) | 25.127(0.508) | 16.393(0.383) | 17.360(0.173) | 19.723(0.150) |
| Condition factor | 1.3467(0.362) | 1.1400(0.145) | 1.1800(0.155) | 1.480(0.306) |

**Table 2: ANOVA table showing the comparison of body constituents of different fish species belonging to pond A (5 feet).**

| Body constituents | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Percent water | 3,8 | 41.101 | 13.700 | 103.35 | <0.001*** |
| Percent ash (dry wt.) | 3,8 | 87.783 | 62.594 | 130.51 | <0.001*** |
| Percent ash (wet wt.) | 3,8 | 18.63 | 6.210 | 119.77 | <0.001*** |
| Percent Organic contents (dry wt.) | 3,8 | 187.783 | 62.594 | 130.51 | <0.001*** |
| Percent Organic content (wet wt.) | 3,8 | 63.045 | 21.015 | 545.87 | <0.001*** |
| Percent fat (dry wt.) | 3,8 | 210.734 | 70.245 | 105.89 | <0.001*** |
| Percent fat (wet wt.) | 3,8 | 13.0626 | 4.3542 | 65.670 | <0.001*** |
| Percent protein (Dry wt.) | 3,8 | 682.56 | 227.52 | 198.56 | <0.001*** |
| Percent protein (Wet wt.) | 3,8 | 137.548 | 45.849 | 263.99 | <0.001*** |
| Condition factor | 3,8 | 0.2216 | 0.0739 | 1.0900 | 0.406n.s |

n.s = Non significant, P>0.05
*** = Highly significant, P<0.001

**Table 3: Body constituents of different fish species of pond B (4 feet)**

| Body constituents | *Labeo rohita* | *C. mrigala* | *H. molitrix* | *Catla catla* |
|---|---|---|---|---|
| Percent water | 63.553(0.075) | 73.150(0.080) | 69.190(0.240) | 65.637(0.200) |
| Percent Ash (dry) | 13.323(0.577) | 18.993(1.005) | 16.333(0.577) | 20.490(0.500) |
| Percent Ash (wet) | 4.5833(0.213) | 5.0967(0.265) | 5.033(0.179) | 7.0200(0.170) |
| Percent Organic contents (dry) | 86.667(0.577) | 81.007(1.005) | 83.667(0.577) | 79.510(0.500) |
| Percent Organic contents (wet) | 31.580(0.208) | 21.740(0.270) | 25.777(0.179) | 27.250(0.170) |
| Percent Fat (dry) | 18.663(2.315) | 24.657(1.155) | 29.333(2.309) | 26.657(3.055) |
| Percent Fat(wet) | 6.7967(0.837) | 6.6200(0.311) | 9.0400(0.710) | 8.4500(0.398) |
| Percent protein(wet) | 68.00(1.732) | 56.333(1.528) | 54.327(2.076) | 54.833(1.258) |
| Percent protein(dry) | 24.777(0.635) | 15.120(0.412) | 16.737(0.640) | 18.797(0.432) |
| Condition factor | 1.2267(0.160) | 0.9933(0.180) | 1.1967(0.150) | 2.2200(0.502) |

**Table 4: ANOVA table showing the comparison of body constituents of different fish species belonging to pond B (4 feet)**

| Body Constituents | DF | SS | MS | F | p |
|---|---|---|---|---|---|
| Percent water | 3,8 | 159.725 | 53.242 | 1940.17 | <0.001*** |
| Percent ash (dry) | 3,8 | 89.373 | 29.791 | 61.85 | <0.001*** |
| Percent ash (wet) | 3,8 | 9.3271 | 3.1090 | 70.34 | <0.001*** |
| Percent O.contents(dry) | 3,8 | 89.135 | 29.712 | 61.68 | <0.001*** |
| Percent O.contents (wet) | 3,8 | 148.56 | 49.519 | 1118.88 | <0.001*** |
| Percent fat (dry) | 3,8 | 185.02 | 61.67 | 11.55 | <0.001*** |
| Percent Fat (wet) | 3,8 | 13.013 | 4.338 | 11.88 | <0.001*** |
| Percent Protein (dry) | 3,8 | 377.22 | 125.74 | 44.80 | <0.001*** |
| Percent Protein (wet) | 3,8 | 160.521 | 53.507 | 182.98 | <0.001*** |
| Condition Factor | 3,8 | 2.7265 | 0.9088 | 10.90 | 0.003** |

*** = Highly significant, P<0.001
** = Significant P<0.01

*Response of different fish species in pond C (2.5 feet)*

It was observed that depth of pond C (2.5 feet) had a highly significant ($P < 0.001$) effect on all the body composition parameters (both dry and wet body weight) and no effect on condition factor in fish of all four species (Table 6).

In pond C, minimum values of % water contents were observed in *Labeo rohita* indicating maximum gain in body composition parameters followed by *Cirrhinus mrigala, Catla catla* and *Hypophthalmicthys molitrix* respectively. % Ash contents (dry and wet weight) were maximum in *Catla catla* and minimum in *Labeo rohita.* Highest values of % organic contents (dry and wet weight) were observed in *Labeo rohita* and minimum in *Catla catla.* Value of % organic and % protein contents were maximum in *Labeo rohita*

while minimum values were observed in *Catla catla* and *Hypophthalmicthys molitrix* respectively. *Hypophthalmicthys molitrix showed maximum values of* % fats contents (dry and wet weight) and minimum values were observed in *Labeo rohita.* It was found that *Labeo rohita* showed overall better growth among the four species in 4 feet deep pond (Table 5).

**Table 5: Body constituents of different fish species of pond C (2.5 feet)**

| Body Constituents | *Labeo rohita* | *Cirrhinus mrigala* | *H. molitrix* | *Catla catla* |
|---|---|---|---|---|
| Percent water | 66.873(0.091) | 70.750(0.230) | 72.960(0.160) | 72.137(0.065) |
| Percent ash (dry) | 12.657(0.577) | 17.990(0.100) | 15.990(0.090) | 19.663(0.583) |
| Percent ash (wet) | 4.1900(0.190) | 5.2600(0.009) | 4.3200(0.010) | 5.4767(0.161) |
| Percent Organic contents (dry) | 87.343(0.577) | 82.010(0.014) | 84.00(0.000) | 80.333(0.577) |
| Percent Organic contents (wet) | 28.940(0.191) | 23.980(0.001) | 22.700(0.001) | 22.373(0.162) |
| Percent fat (dry) | 17.327(2.315) | 23.990(0.001) | 26.657(2.309) | 19.993(4.005) |
| Percent Fat (wet) | 5.7400(0.762) | 7.0100(0.001) | 7.200(0.623) | 5.5667(1.115) |
| Percent Protein (dry) | 70.00(2.646) | 58.00(0.001) | 57.333(2.309) | 60.333(4.041) |
| Percent Protein (wet) | 23.193(0.877) | 16.960(0.001) | 15.493(0.629) | 16.803(1.122) |
| Condition Factor | 1.500(0.488) | 1.0600(0.135) | 1.300(0.175) | 1.7333(0.220) |

**Table 6: ANOVA table showing the comparison of body constituents of different fish species belonging to pond C (2.5 feet)**

| Body Constituents | DF | SS | MS | F | p |
|---|---|---|---|---|---|
| Percent water | 3,8 | 65.447 | 21.8159 | 959.29 | <0.001*** |
| Percent ash (dry) | 3,8 | 81.707 | 27236 | 161.79 | <0.001*** |
| Percent ash (wet) | 3,8 | 3.8143 | 1.2714 | 81.46 | <0.001*** |
| Percent Organic contents (dry) | 3,8 | 81.734 | 27.245 | 163.47 | <0.001*** |
| Percent Organic contents (wet) | 3,8 | 83.2401 | 27.7467 | 1777.68 | <0.001*** |
| Percent fat (dry) | 3,8 | 154.53 | 51.51 | 7.71 | <0.001*** |
| Percent Fat (wet) | 3,8 | 6.421 | 2.140 | 3.87 | <0.001*** |
| Percent Protein (dry) | 3,8 | 309.58 | 103.19 | 14.40 | <0.001*** |
| Percent Protein (wet) | 3,8 | 107.151 | 35.717 | 58.96 | <0.001*** |
| Condition Factor | 3,8 | 0.7401 | 0.2467 | 2.93 | 0.099n.s |

n.s = Non significant, P>0.05
*** = Highly significant, P<0.001

## DISCUSSION

Readily and easily measured growth is one of the more complex activities of organisms. It represents the net outcome of series of behavioral and physiological processes that begins with food intake (the consumption of an appetitive behavior) and terminates in deposition of animal substance (Bret *et al.*, 1969).

The analysis of four main tissue constituents that is Protein, water, lipids and ash contents is some times described " approximate analysis" (Love, 1970). Carbohydrates and non-protein compounds are also important constituents but are present in small amounts and are usually ignored during analysis (Love, 1970, 1980; Weatherly and Gill, 1987).

Lipids are regarded as one of the most important food reserve contributing to the condition and this has led to the use of fat indices as a measure of relationship b/w percent water and percent fat (Sinclair and Duncan, 1972). Such estimates are used simply because the measurement of water is easy and rapid. These relationships have been shown to exist in various fish species and have been extensively used for predictive estimates (Iles and Wood, 1965; Brett *et al.*, 1969; Salam *et al.*, 1993).

The informations obtained on fats, protein and minerals contents and how they vary in relation to size and condition factor are important for the fish used as food by the consumers. It also facilitates the selection of most appropriate species having higher protein contents and optimum size and condition for human consummation. This information can helpful to the overall techniques and knowledge of aquaculture in country (Dempson *et. al., 2004)*. The body composition parameters are used as indicator to assess the nutritional status and condition of fish. Water, fat, protein, organic and ash contents of culturable species analyzed have more or less similar ranges to the earlier reports published for the species.

In present study when body composition parameter analyzed in different ponds, it was found that *Labeo rohita* performed better in pond of all depths indicating that growth of *Labeo rohita* is independent of pond depth. When fish of different species were compared with respect to pond depth, significant differences among body composition parameters were observed. It was observed that body composition parameters like % ash, fat, organic and protein contents significantly vary in different fish species with respect to pond depth. It was also noted that fish of same species have different values of body composition parameters in ponds of different depths (Table 1,3 and 5). Generally fish is considered as a rich source of protein. Present studies demonstrates that fish kept in ponds of different depths have different values of protein which help guide the formers to select best pond depths to produce protein rich fish. Same is the case for other body composition parameters. Direct comparison of the present studies is not possible as there was no previous record of comparison of body composition parameters among various fish species with respect to pond depth.

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# EFFECTS OF HYPO-&-HYPERTHYROIDISM ON THREE BELLIES OF TENOTOMIZED GASTROCNEMIUS MUSCLE IN PIGEONS (*COLUMBA LEVIA*)

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**Abstract:** Experimentally induced tenotomy caused degenerative changes such as decrease in muscle weight (mg/g body weight), decrease in muscular length and atrophy of the overall muscle. Histologically the loss of muscular mass with its replacement by the connective tissue and certain focal degenerations were recorded. Average cross-sectional aria of muscle fibers was also found to be decreasing, in three bellies of Gastrocnemius muscle. These bellies present three different distinct types of skeletal muscles: namely Gastrocnemius Internus which mainly comprises on slow oxidative muscle fibers, Gastrocnemium medius containing mixed muscle fibers and Gastrocnemius externus having mostly the fast glycolytic fibers. These degenerative changes were seen much more pronounced in Gastrocnemius Internus, whereas least in Gastrocnemius externus. Progressive muscular regressions with enhanced development of connective tissue along with some focal degeneration were seen in such animals with thyroxin administration. On the other hand general necrosis and some central core lesions of muscle fibers were seen in experimental group with induced hypothyroidism. However, this experimental group showed very little development of connective tissue. Morphometric analysis shows that there is no significant difference in reduction in the average cross-section areas of the muscle fibers after simple tenotomy, and tenotomy with induced hypothroidism. Huge increase in muscle fiber cross sectional area followed by the secondary atrophy was noted in the tenotomized group with induced hyperthyroidism. Gastrocnemius Internus showed faster degenerative changes among the three experimental conditions.

**Key words:** Tenotomy, skeletal muscle, thyroxin.

## INTRODUCTION

It has been quite well known that many hormones such as androgens, growth hormone, thyroxin and insulin have anabolic effects on skeletal muscle (Kuhn, 1985). In mammals circulating levels of thyroxin have diverse biochemical actions on skeletal muscle. Hyperthyroidism increases the protein catabolism, blood flow, and basic metabolic rate (Carter *et al.,* 1982).

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Muscle growth stops in thyroidectomized animals. Physiologic doses of thyroxin in such animals increase both the protein synthesis and degradation. The large doses of thyroxin cause and catabolic effect on skeletal muscles, which may lead to muscle wasting and general atrophy. Hyperthyroidism on the other hand reduces the rate of protein synthesis along with an increase in gluconeogenesis and general regression in muscular tissue (Tischler, 1981).

Skeletal muscle is one of the major target organs for thyroid hormone. The muscles most commonly affected are those used during prolonged effort (slow-twitch muscles). One of the major clinical features is the shortening of the Achilles-tendon reflex time in hyperthyroidism and its prolongation in hypothroidism (Everts, 1996).

Thyroid hormones induce a well known decrease in muscle oxidative capacity (Zoll, 2001). It mobilizes muscle Nitrogen. Grofte *et al.,* 1997 indicated that Nitrogen contents in the soleus and extensor digitorum longus muscles, of chronically hyperthyroid rats decreased by 22% and 11% respectively. Furthermore, chronic hyperthyroidism reduces N-contents of muscles, urinary urea-N excretion and N-balance. *In vivo* and *in vitro* lipid peroxidation significantly increases in hyperthyroidism and does not change in the hypothyroid state (Gredilla, 2001).

Thyroid hormones have a profound effect on mitochondrial oxidative activity, synthesis and degradation of proteins and vitamin E, the sensitivity of the tissues to catecholamine, the differentiation of muscle fibers, and the levels of antioxidant enzymes (Asayama, 1990). Silva *et al.,* 2004 claim that Thyroid hormones stimulate Un-Coupling Protein-3 (UCP-3) in skeletal muscle independently of angiotensin II or the β-adrenergic system. This probably reflects a direct action of the hormone on UCP-3 gene expression.

Influences, which interfere in the normal functioning of a muscle such as immobilization, plaster-casting, tenotomy or denervation bring negative responses, (Booth, 1982). In such muscles there was observed an atrophy of the muscle as shows by a general loss in the muscle weight, a significant reduction in protein synthesis as well as increase in protein breakdown (Tucker *et al.,* 1981).

Tenotomy specifically produces many degenerative changes in skeletal muscle in addition to general loss of muscular weight. Most obvious histological and morphometric changes are central core lesions and reduction in the average fiber size (Mufti and Ahmad, 1988), development of connective tissue, decrease in sarcoma length, splitting and distension of muscle fibers (Kvist *et al.,* 1991).

Most of the studies pertaining to the effects of tenotomy on skeletal muscle are confined to mammalian system. A relatively lesser work has been done on the avian skeletal muscles; moreover any hormonal effects on different types of traumatic conditions in such cases are yet to be reported. Keeping all the above mentioned information in mind it was decided to investigate the effects of hypo- and hyperthyroidism on tenotomized avian skeletal muscles.

Gastrocnemius muscle, which has three bellies, was selected for tenotomy. Each one of the three bellies reflect distinct fiber type distribution. Gastrocnemius medius (GM) largely consists of twitch or glycolytic fibers. Gastrocnemius Internus (GI) mostly contains slow osidative fibers and Gastrocnemius Externus (GE) has mixed fibers (Mufti

and Ahmad, 1988). All three bellies of this muscle were used in the present study, to record their differences in response to the same set of experimental conditions.

## MATERIALS AND METHODS

Thirty six young male domestic pigeons, ranging in weight from 250-350 grams, were used. These were divided into four groups each comprising nine animals. Group 01 animals were sued as control. Group 02, 03 and 04 were tenotomized. Group 02 pigeons received distilled water on alternate days {the vehicle control (VC) group}; group 03 pigeons received an oral dose of 1 *pg/g* body weight thyroxin sodium B.P. {thyroxin treated (T4) group} on alternate days, similarly the group 04 pigeons received an oral dose of 10 *μg/g* body weight thiourea on alternate days {thiourea treated (TU) group}.

Both legs of the experimental animals were tenotomized by cutting the tendons of Gastrocnemius muscle. For this purpose the tendon of each Gastrocnemius muscle was first isolated and then snipped with sharp scissors. The cut end of tendons were then sutured near the proximal end of the muscle on the underside of the skin flap in such away that it faced away from its original point of attachment. This procedure minimized the possible chance of reattachment. At the end the skin was sutured back.

Three animals were recovered from each group, on $5^{th}$, $10^{th}$ and $15^{th}$ day. In the recovery procedure three bellies of each Gastrocnemius muscle were removed carefully form each animal, weighed and fixed in Bouin's fixative for 10-15 hours. These were then processed for Wax imbedding in a routine way; sectioned at 6 μm, stretched on microscopic slides and stained with Hematoxylin and Eosin for histological and morphometric observations.

For histological analysis, selective sections from experimental and control animals, for all three bellies were photographed using digital camera fitted stereoscopic research microscope.

For morphometric analysis, camera lucida drawings of the muscle fibers were used. For this study, five randomly selected areas from six different sections were drawn on plain paper from each such muscle at 400X magnification. The average Cross-sectional area of each muscle fiber was calculated by using polar planimeter (Kt-type). The planimeter readings were converted into measurements in "$\mu m^2$" by using the following formula.

$$\text{Area in } (\mu m^2) = \frac{\text{Planimeter reading X } (10)^6}{(\text{Magnification})^2}$$

Average muscle fiber size was calculated for control and each experimental stage and shown in tables.

## RESULTS

In comparison with control a general loss of muscle tissue as indicated by overall contracture in size in all three bellies, along with reduction in overall weight (mg/g. Body Weight) in all three experimental groups were seen.

Generally a decline in muscle weight was observed after tenotomy in all three experimental groups; however maximum muscle weight loss was seen in TU group and

minimum in T4 group after 5 days of tenotomy. Whereas after 15 days of tenotomy the greatest weight loss was noted in T4 group while minimum in VC group (Fig.13).

*Histological observations*

In control group, all three bellies of Gastrocnemius muscle contained compactly arranged muscle fibers with very little connective tissue in between the muscle fascicles (Figs. 01, 02 & 03). All three bellies appeared quite well vascularized and innervated.

Although a typical pattern of muscle regression and development of connective tissue was observed after simple tenotomy (VC Group), GE showed degenerative changes with least intensity as compared to the other two bellies (Fig.4). Many focal degenerations (obliteration of a complete fiber) and increased fibrosis were observed in GM belly (Fig.5). GI showed maximum fibrosis with fairly large number of focal degenerations (Fig.6).

A general increase in the development of connective tissue was seen in T4 group in all three bellies, never the less maximum fibrosis was seen in th eGI belly after 15 days of tenotomy. GE was least affected in this context also. Some focal degenerations were seen even after 5 days of tenotomy in GI; this effect increased in intensity with the increase in the duration of tenotomy. While GM belly showed some of them only after 10 days, no focal degenerations were seen in GE even after 15 days of tenotomy. Some central core lesions of the individual muscle fibers were seen in the GI in all three stages. These types of lesions were present in the medius belly only in the 10 and 15- days stages; whereas no such defects were recorded in the externus belly (Figs.7, 8 & 9).

Rapid loss of muscle proteins as indicated by poor staining properties, along with the dissolution of muscle fibers margins and development of a far little connective tissue were the most obvious common observations in TU group. Focal degenerations were seen in GI and GM bellies even at 5 day stage, however no such changes were observed in GE even after 15 days (Figs.10, 11 & 12).

*Morphometric observations*

A gradual regression in average size $(\mu m)^2$ of fiber cross-sectional area (CA) in comparison with control was noted in all three bellies in VC group (Tables 1, 2&3).

With a slight increase in CA after 5 days of tenotomy in TU group in GE (117.38%) & GI (114.05%) bellies, afterwards it decreased. GM belly remained more consistent for the decrease in CA for this group.

All three bellies responded through a huge increase in average C.A in T4 group after 5 days of tenotomy. A negative trend was observed at the later stages (10&15 days0 in this group. Even so the average G.A value remained higher than control even after 15 days of tenotomy in GE & GM bellies (Tables 1, 2&3).

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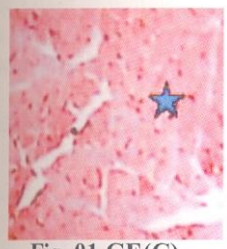

Fig. 01 GE(C)

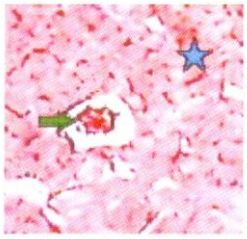

Fig. 02:GI (C)

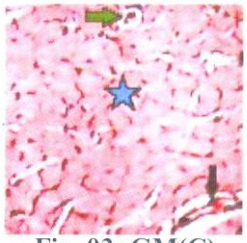

Fig. 03: GM(C)

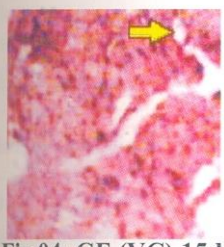

Fig.04: GE (VC) 15days

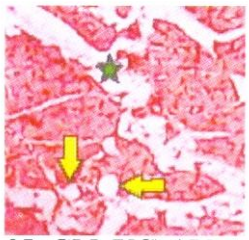

Fig.05: GM (VC) 15days

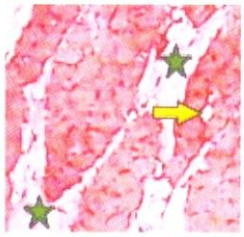

Fig.06: GI (VC)15days

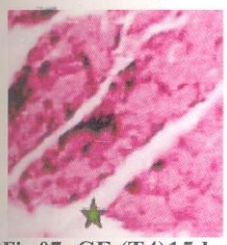

Fig.07: GE (T4)15days

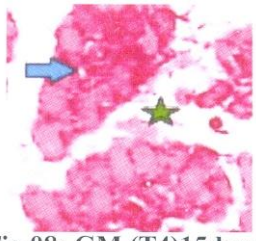

Fig.08: GM (T4)15days

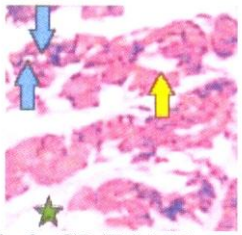

Fig.9: GI (T4)15days

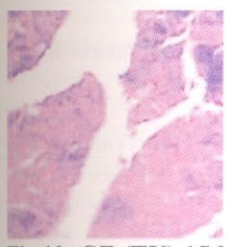

Fig.10: GE (TU) 15days

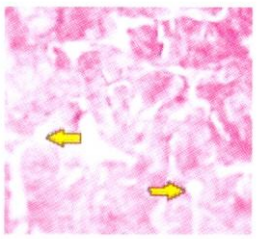

Fig.11: GM (TU) 15days

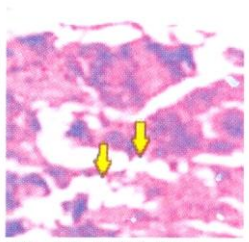

Fig.12: GI (TU) 15days

**Fig.1-12 (200x):** Selected photographs from control and various experimental conditions; **Blue star** indicates control muscle fascicle showing compact fiber arrangement; **Green arrows** indicate muscle spindle; **Gray arrow** indicates an artery; **Yellow arrows** show focal degenerations; **Blue arrows** indicate central core lesions; **Green stars** show fibrosis.

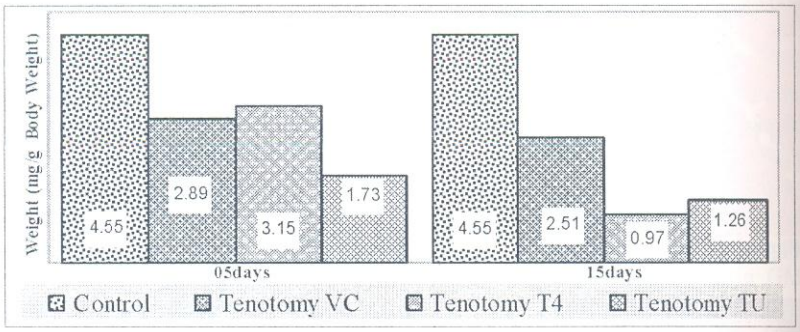


**Fig. 13: Weight comparison of gastrocnemius muscle in experimental animals with control.**

With a slight increase in Ca after 5 days of tenotomy in TU group in GE (117.38%) & GI (114.05%) bellies, afterwards it decreased. GM belly remained more consistent for the decrease in CA for this group.

**Table 1: Gastrocnemius Externus: Average cross-sectional area of muscle fibers in various experimental conditions in comparison with control.**

| | Control | Tenotomy VC | | Tenotomy T4 | | Tenotomy TU | |
|---|---|---|---|---|---|---|---|
| | Average size | Average size | % Control | Average size | % Control | Average size | % Control |
| 5 days | 2278 | 2219 | 97.41 | 4361 | 191.44 | 2674 | 117.38 |
| 10 days | 2278 | 2177 | 95.57 | 4381 | 192.32 | 2556 | 112.20 |
| 15 days | 2278 | 2131 | 93.55 | 3578 | 157.07 | 1996 | 87.62 |

**Table 2: Gastrocnemius Medius: Average cross-sectional area of muscle fibers in various experimental conditions in comparison with control.**

| | Control | Tenotomy VC | | Tenotomy T4 | | Tenotomy TU | |
|---|---|---|---|---|---|---|---|
| | Average size | Average size | % Control | Average size | % Control | Average size | % Control |
| 5 days | 2293 | 2115 | 92.24 | 3136 | 136.76 | 2193 | 95.64 |
| 10 days | 2293 | 1926 | 83.99 | 2858 | 124.64 | 2164 | 94.37 |
| 15 days | 2293 | 1898 | 82.77 | 2509 | 109.42 | 1857 | 80.98 |

All three bellies responded through a huge increase in average C.A in T4 group after 5 days of tenotomy. A negative trend was observed at the later states (10&15 days) in this group. Even so the average C.A value remained higher than control even after 15 days of tenotomy in GE & GM bellies (Tables 1, 2&3).

Table 3: **Gastrocnemius Internus: Average cross-sectional area of muscle fibers in various experimental conditions in comparison with control.**

| | Control | Tenotomy VC | | Tenotomy T4 | | Tenotomy TU | |
|---|---|---|---|---|---|---|---|
| | Average size | Average size | % Control | Average size | % Control | Average size | % Control |
| 5 days | 2356 | 2152 | 91.34 | 3179 | 134.93 | 2687 | 114.05 |
| 10 days | 2356 | 1750 | 74.28 | 3009 | 127.72 | 2048 | 86.93 |
| 15 days | 2356 | 1669 | 70.84 | 2181 | 92.57 | 1830 | 77.76 |

## DISCUSSION

Results obtained in the present study confirm that tenotomy causes degenerative changes in all types of skeletal muscles. These include a general loss of muscular weight, decrease in the muscular length, atrophy and degeneration of the muscle fiber, focal degeneration, and decrease in average cross-sectional area along with increase in fibrosis. These results are well in accordance with the similar studies in mammalian as well as avian systems (Hikida, 1972; Baker and Hall-Craggs, 1980 a & b; Dasse, 1981; Mufti and Ahmad, 1988; Kvist *et al.,* 1991). Similarly a direct correlation between thyroxin and metabolic rate is very well documented. Although results obtained in the present study cannot be compared with any of the existing literature, probably the initial massive weight loss in TU group and least in T4 group (after 5 days of tenotomy) indicate that thyroxin gives an anabolic cover to the tenotomized muscles, but prolonged exposure to hyperthyroidism in such muscles causes the catabolic changes to occur on a more faster rate than normal leading to a sharp decline in muscular weight (Fig. 13).

Development of fibrotic mass at the expense of muscular tissue, seen throughout the course of tenotomy in T4 group, is probably due to a mitogenic effect of thyroxin on cells of connective tissue. That is why; probably the slowest speed of muscular weight loss and least development of connective tissue were seen after prolonged (15 days) tenotomy in TU group animals.

Central core lesions, as has been seen earlier, are transitory in nature and occur at initial stages of tenotomy i.e., after 01 to 02 days (Baker & Hall-Craggs, 1980 a & b). The presence of central core lesions were restricted only to T4 group, which indicates that such changes may also be present in very early stages of tenotomy (probably after 1 or 2 days) in the VC group, where these might have been recovered rapidly. The same may not develop at all in TU group, primarily because of the slow metabolic rate stages of tenotomy. The anabolic cover given by the thyroxin hormone might have prevented the

development of central core lesions at the initial stages. Moreover the present of central core lesions only in GI and GM bellies indicate that the intermediate fiber containing muscles (such as GE) are least affected in this context.

Another consistent observation in TU group is poor staining properties with vague muscle fiber margins. This probably is due to a loss of functional proteins in muscle fibers i.e., actin and myosin. Such a loss can partly be due to immobilization caused by tenotomy and partly due to the sharp decline in anabolic rate of the experimental muscle caused by hypothyroidism.

Focal degenerations as reported to occur in all three bellies of Gastrocnemius muscle by Mufti & Ahmad, 1988, was confirmed in the present study. Focal degeneration was seen in GM and GI in all three experimental groups while in the case of GE only a little of such degenerations was seen in VC group at 15 days stage. Although no counting was made to show the intensity of such degenerations per muscle fascicle, in various experimental groups at various stages of duration of tenotomy, it seems that such number of degenerations was augmented with increase in the duration time of tenotomy. Moreover generally greater numbers of focal degenerations was seen in T4 group than VC group and minimum of such degeneration was recorded in TU group at any given stage. It was previously claimed that such obliteration of whole muscle fiber might be due to the development of fibrotic mass, which was supported to cause an interference in the blood supply to these fibers (Mufti & Ahmad, 1988) or due to the lack of innervations as the amount of fibrosis is inversely proportional to the degree of innervation (Mufti, 1977; Carlson *et al.,* 1979). Under present situation it seems that it is probably the rate of metabolism of such muscles, which can cause this type of changes to occur. So it is T4 group, which show much more focal degenerations; while TU group showed minimum fibrosis and least number of focal degenerations.

Decrease in the average cross-sectional area of the muscle fibers following tenotomy is also previously shown by Mufti & Ahmad, 1988. The same was also observed in the present study. In comparison with VC group hypo-thyroid condition seems not to severely alter the results of morphometric analysis; on the other hand interesting major changes were noted in the hyper-thyroid condition. Increase in average CA of muscle fibers after 5 days of tenotomy in T4 group indicate a general hypertrophy of the muscle fibers, which may probably be an anabolic effect of thyroxin. Prolonged exposures of tenotomized animals to thyroxin probably led to catabolic and degenerative activities and thus caused a significant decrease in the average CA value leading to a secondary atrophy in all three bellies of Gastrocnemius muscle.

All in all the degenerative changes shown by morphological, morphometric and histological parameters by the three bellies of the Gastrocnemius muscle were more pronounced in GI and GM; while GE was least affected. The differental response to same set of experimental conditions, such as tenotomy by various muscles of the same animal, is already well documented (Baker & Hall-Craggs, 1980 a.b; Mufti & Ahmad, 1988).

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# HEAT STRESS ON SERUM PROTEINS PROFILE IN STEEL FOUNDRY WORKERS

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**Abstract:** The study was based on serum protein profile of steel foundries workers following heat stress. Blood samples of the heat stressed subjects were collected from two steel foundries (Tayyab & Barkat Steel Mills, Lahore), Whereas, blood samples of control subjects were collected from Punjab University, Quaid-e-Azam Campus, Lahore. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was employed to study the low molecular weight serum protein fractions in the samples. The data of molecular weights and percent raw volumes, exhibited by each of the fractions was analyzed statistically using Student-t-test in finding the enhancement or reduction and appearance or disappearance of particular protein fractions for comparison between the heat stressed & the control subjects. The low molecular weight protein fractions ranged between 28-17 kDa in control and 28-14 kDa in heat stressed subjects. The protein fraction of 28 kDa remained unchanged in control as well as heat stressed subjects. Another protein fraction of 23 kDa indicated a non-significant elevation of 48% in heat stressed compared to control subject. Protein fraction of 20 kDa was encountered in all of the heat stressed and control subjects with non-significant reduction of 13% in heat stressed compared to control subjects. A 17 kDa protein fraction was appeared in all of heat stressed subjects and in all control subjects except one control subjects. This fraction exhibited no change in heat stressed as compared with control subjects. Protein fraction of 14 kDa was detected only in seventeen out of twenty four heat stressed subjects while totally absent in control subjects. The appearance of this fraction is, particularly, of significance concerning its probable role as a marker for the assessment of heat stress.

**Key words:** Heat stress, Protein, Electrophoresis.

## INTRODUCTION

Heat stress is the buildup of heat in the body to damaging levels, heat generated by the muscles during work and heat coming from the surrounding environment. Workers who become overheated get weaker and become tired sooner. They may be less alert (Rowe & Bellinger, 2002).

Larry Thurston (2004) defined heat stress as "the total heat imposed on the body by the combined effect of environment and metabolic heat factor". Excessive exposure to heat or to a hot work environment can bring about a variety of heat induced disorders: Heat cramps, Heat stroke, Heat exhaustion, Heat rash. Heat stress can also aggravate the effect of other toxins. Dehydration and loss of minerals through sweat decrease the body's ability to detoxify chemicals (U.S. Department of Health & Human Service, 1992).

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The effects of heat stress on cellular function include 1) inhibition of progression through the cell cycle; 2) inhibition of DNA synthesis, transcription, RNA processing and translation; 3) denaturation and misaggregation of proteins; 4) increased degradation of proteins through both proteasonal and lysosomal-pathways; 5) disruption of cytoskeleton components; 6) alterations in metabolism that lead to a net reduction in cellular ATP; and 7) changes in membrane permeability that lead to an increase in intracellular $Na^+$, $H^+$ and $Ca^{2+}$ (Kuhl and Rensing, 2000).

Heating causes a protein's conformationally sensitive properties, such as optical rotation, viscosity and UV absorption, to change abruptly over a narrow temperature range. Such sharp transition indicates that the entire polypeptide unfolds or "melts" cooperatively, that is, nearly simultaneously. Most proteins have melting points well below 100°C. Among the exceptions are the proteins of thermophilic bacteria, organisms that inhabit hot springs or submarine volcanic vents with temperature near 100°C. Proteins such as albumins, globulins, etc. are coagulated by heat (Voet *et al.,* 1999).

Serum albumin when heat treated goes through two structural stages. The first stage is reversible heating up to 65°C while the second stage above that is irreversible but does not necessarily results in a complete destruction of the ordered structure (Kuznetsow *et al.,* 1975; Lin and Koeinig, 1976; Oakes, 1976).

In mammalian cells, nonlethal heat shock produces changes in gene expression and in the activity of expressed proteins, resulting in cell stress response (Jaattela, 1999). This response characteristically include an increase in thermotolerance (i.e., the ability to survive subsequent, more severe heat stresses) that is associated with increased expression of heat shock proteins (HSPs). Heat shock proteins belong to a larger class of proteins called stress proteins. Some conditions that are known to induce heat shock protein synthesis at normal temperature include drugs, amino-acid analogs, ethanol, heavy metals, ionizing radiations and viral infections. Induction of HSPs expression starts within minutes after the initiation of thermal stress, with peak expression occurring up to several hours later (Lindquist, 1986). Hence, heat stress provokes metabolic adaptation in the whole organism and heat shock proteins (HSPs) are one of that adaptation.

## MATERIALS AND METHODS

Blood samples of steel foundries workers, working in high temperature (80-100°C), were collected from Tayyab Steel Mills & Barkat Steel Mills Lahore, whereas, blood samples of control subjects working in normal temperature were collected from Punjab University, Quaid-e-Azam campus, Lahore. These samples were processed for serum separation. Twelve controls and 24 confirmed subjects working in extreme temperature on furnace were selected for the study.

12% polyacrylamide gel was prepared for studying the low molecular weight protein fractions using the method of Laemmli (1970). Serum was diluted with distilled water and proteins were denatured by heating with loading dye. Protein size marker and each of the samples were loaded in separate wells and gels were electrophoresed at a

current supply of 30 mA and voltage of 200 V, in a cooling chamber maintained at 4°C until the tracking dye reached the lower end of the gel. Following electrophoresis, the gels were stained with coomassie brilliant blue for a duration of 30 minutes and destained afterwards until the protein fractions of different molecular weights became visible in the form of blue bands on a transparent background.

Photographs of stained gels and quantification of separated protein fractions were carried out by Gene Genius Bio-imaging Gel Documentation System that provided the data of molecular weight and percent area covered by each of the fraction. The data was analyzed statistically using Student 't' test and employed in finding the enhancement or reduction and appearance or disappearance of particular protein fraction for comparison among control and heat stressed subjects.

## RESULTS

An overall view of protein profile pattern resulted in the detection of four low molecular weight protein fractions ranging between 28-17 kDa in control subjects and five low molecular weight protein fractions ranging between 28-14 kDa in heat-stressed subjects because protein fraction of 14 kDa was only expressed in heat stressed subjects with average percent raw volume of 0.66±0.12 and was not found to be expressed in control subjects.

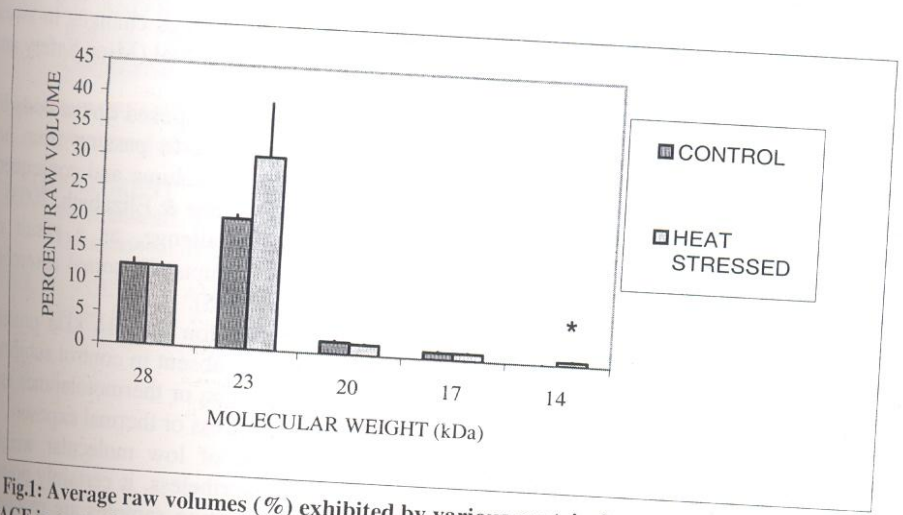


**Fig.1: Average raw volumes (%) exhibited by various protein fractions resolved by SDS-PAGE in control and heat stressed subjects. Values are mean±SEM.* Significance at P<0.05.**

In comparison of control with heat stressed subjects, protein fraction of 28 kDa did not exhibit any significant change with average percent raw volume of 12.60±0.90 in control and 12.53±0.47 in heat stressed subjects.

Protein fraction of 23 kDa exhibited non-significant elevation of 48% in heat stressed subjects indicating an average percent raw volume of 20.63±0.63 in control subjects and 30.6±8.6 in heat stressed subjects.

Another protein fraction of 20 kDa showed non-significant reduction of 13% in heat stressed subjects with average % raw volume of 1.98±0.20 in control and 1.72±0.092 in heat stressed subjects.

Protein fraction of 17 kDa remain unchanged with average % raw volume of 1.17±0.13 in control subjects and 1.17±0.08 in heat stressed subjects (Fig.1).

## DISCUSSION

Operations involving high air temperatures, radiant heat sources, high humidity, direct physical contact with hot objects, or strenuous physical activities have a high potential for inducing heat stress in employees engaged in such operations. A person's sensitivity to heat is affected by age, weight, degree of physical fitness, degree of acclimatization, metabolism and a variety of medical conditions such as hypertension (Occupational Safety & Health Administration, 1999).

Heat refers to the total heat related load on the individual from both environmental and metabolic sources. An increasing environmental heat stress causes changes in sweat rate, heart beat rate and body core temperature of the affected individual (Mine Safety and Health Administration, 1976).

Larry Thurston (2004) define heat stress as "the total heat imposed on the body by the combined effect of environment and metabolic heat factor". In passing from hot climate the first thing that occurs is the expansion of the plasma volume and consequent fullness of the vessels is constantly observed to take place" (Suzanne & Elizabeth, 2003).

Protein synthesis is inhibited after extreme heat challenge, as a result of phosphorylation of initiation factors such as elF2 α, which disrupts ribosomal assembly and inactivates cap-binding proteins (Benjamin & McMillan, 1998).

In present study the most evident result is the expression of a 14 kDa protein fraction in heat stressed subjects which was found to be totally absent in control subjects. This 14 kDa protein fraction may have a role in thermoregulation or thermotolerance and may be taken as a probable marker for the assessment of heat stress or thermal exposure.

Hence, it is a preliminary study regarding the role of low molecular weight proteins in heat stressed or thermally exposed subjects. Nevertheless, it certainly points out that further investigations on heat stress with large population samples are required which may provide better understanding about the roles of low molecular weight proteins in thermoregulation. To make the study more valuable and to characterize the marker proteins, protein sequencing may also be performed.

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# UPDATED EVIDENCES OF THE SIWALIK *HIPPARION* WITH SPECIAL EMPHASIS ON PALEOZOOGEOGRAPHY AND BIOCHRONOLOGY

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**Abstract:** 500 specimens of the Siwalik hipparions collected from the last four decades, present in the Fossil Display and Research Centre, Department of Zoology, University of the Punjab, Lahore, Pakistan, are studied to find out the oldest occurrence in this Continent. A very few specimens belong to the upper limits of the Nagri Formation, indicating that the *Hipparion* might have occurred in the Nagri Times, which major proportion of these specimens belong to Dhok Pathan Formation and non of them from Chinji Formation.

**Key words:** *Hipparion,* Nagri Formation, Dhok Pathan Formation and Chinji Formation.

## INTRODUCTION

The Siwalik equids are of great importance in establishing a framework for Holarctic Biochronology and Paleozoogeogrphy. The Siwalik equids are mostly known from isolated teeth and cranial fragments. Different extinct species of the family Equidae are important markers in faunal correlation. Their appearance is normally taken to define the boundary between Miocene and Pliocene in continental deposits (Koenegswald, 1956).

Falconer and Cautley (1849) described one species of *Hipparion* as *H. antilopinum,* while Lydekker (1882) added a new species to the same genus as, *H. theobaldi*. Colbert (1935) reviewed the genus and also described the above mentioned two species. Hussain (1971) added one more species to the genus i.e. *H. nagriensis* announcing three species of the Siwalik *Hipparion*, Macfadden and Wood brune (1982) presented a two fold systematic scheme based on dental and skull morphology. The scheme using dental morphology discriminated three species similar to Hussain's (1971) interpretation; where as their studies of cranial morphology resulted in the discrimination of five species. Bernor and Hussain (1985) recognized four species of the Siwalik hipparions based on the dental, cranial and postcranial materials. But most recently Ghaffar *et al.* (2003) working on the percentage ratio of the Siwalik equids, noted that there are four species, belonging to the genus *Hipparion* and only one species belonging to

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the genus *Equus*. The happarions are characterized by the isolated protocones in the upper molars with tridactyl feet. Most palaeontologists have come to believe that *Hipparion* evolved in the New World from an unidentified species of *Merychippus*. Subsequently, *Hipparion* underwent a vast late Miocene evolutionary radiation throughout the Old World.

## DISCUSSION

500 specimens of the hipparions collected during the last 40 years were studied to find out the range of fossils in different formations and also to find out the age of the first immigrant in the Siwaliks. From the cataloguing of these specimens, it is clear that a few specimens belong to the Nagri Formation while all other come from the Dhok Pathan Formation and none of them from the Chinji Formation. Confirming that the hipparions might have occurred in the Nagri Formation, as also stated by Hussain (1971). In the phylogeny of the Siwalik hipparions, he stated that one intermediate sized species; *H. nagriensis* was involved in *Hipparion* Datum. Recent biostratigraphic studies in the Potwar Plateau has set the oldest occurrence of hipparionine horses circa 9.5 Ma. Macfadden and Wood brune (1982) and Hussain (1971) have suggested that *Hipparion nagriensis* is the first immigrant in the Siwalik Continent. The biostratigraphic correlation of the Nagri Formation fauna with chronologically controlled sequence of Western Asia and Europe indicates 9 to 11 mya (early vallesian), (Pilbeam *et al.*, 1977). He also recorded the *Hipparion* fossils from this Formation. The Nagri Formation is conformable with the underlying Chinji Formation and overlying Dhok Pathan Formation. Pilbeam *et al* (1977) also observed that the Nagri Formation fauna for the most part is similar to the Dhok Pathan Formation and in the Dhok Pathan Formation there are two species of *Hipparion* as *H. antilopinum* and *H. theobaldi*. *H. nagriensis* from the Nagri Formation is no more primitive than the two Dhok Pathan species in dental pattern or size but it does differ in cheek teeth hypsodonty and especially in certain functionally important features in the structure of foot (Hussain, 1973). Infact, both of these Siwalik hipparions (*H. antilopinum* and *H. theobaldi*) are on present evidence restricted to the Dhok Pathan Formation, but they are separated by size differences and both seem to be derived from *H. nagriensis*, confirming that *H. nagriensis* is the first immigrant in the Siwalik Continent not before the Nagri Formation.

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# PATTERN OF DEHYDROEPIANDROSTERONE IN ACNE AFFECTED LOCAL YOUNG FEMALE POPULATION

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**Abstract:** The role of dehydroepiandrosterone sulfate (DHEA-S) has been studied in the young acne affected girls in comparison with the control subjects. Blood samples of acne affected subjects, diagnosed by the physician, were obtained from Mayo Hospital Lahore. The control samples were collected from the general population. Enzyme-linked immunosorbent assay (ELISA) was performed to estimate the concentration of DHEA-S in the control and the affected subjects. The levels were analyzed statistically by applying Student t-test to evaluate the significant variations in the comparable groups. Non significant reductions of 11.5 and 18.8%, for DHEA-S level, were exhibited by mild and severe acne affected subjects, respectively, when compared to controls. A similar trend with a non significant reduction of 7.5% was observed when severe acne affected subjects were compared with the mild acne affected subjects.

**Key words:** Dehydroepiandrosterone sulfate, acne.

## INTRODUCTION

Acne is a disorder of hair follicles and sebaceous glands. Sebaceous glands are located in the dermis, middle layer of skin, and secrete oil (sebum) onto the skin. With acne sebaceous glands are clogged, which leads to pimples and cysts. Acne results, mainly, from the action of hormones on the skin oil glands, which leads to plug the pores and outbreak of lesions commonly called pimples or zits (Questions and answers about acne, 2001).

Nearly 17 million people in USA are affected by acne. Acne can have a short-term, potentially lasting psychological effect. Decreased self-esteem and self-confidence can lead to social withdrawal and even depression (Russell, 2000). In case of extreme disfiguration, acne can have severe consequences for the personality development of young people and is associated with a relatively high prevalence of depression and suicide (Jappe, 2003).

Acne can be classified on the basis of degree of severity. Mild acne comprises comedones, few to several papules or pustules, whereas, few to several nodules define moderate acne. Severe acne consists of comedones numerous to extensive papules, pustules and nodules leading to disfiguring and permanent scarring (Lucky *et al.,* 1997).

Androgen stimulation of sebaceous glands is an important factor in the development of acne. Androgens act in two ways, firstly they enlarge sebaceous glands or

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sebocytes differentiation and secondly they cause the sebaceous glands to increase sebum production. The increased sebum leads to plug formation (Beylot, 2002).

Puberty is a time when hormones stimulate changes in all parts of the body. Sometimes, before adolescence, around the age of 9 and 10, the adrenal gland begins to produce dehydroepiandrosterone sulfate (DHEA-S), an androgen. Other hormones such as testosterone and dihydrotestosterone (DHT) join in at the onset of puberty. All these hormones stimulate sebaceous glands (Shalita, 2001). Sebocytes have nuclear androgen receptor and also have 5α reductase enzymes that convert testosterone to more potent dihydrotestosterone (Thiboutot *et al.,* 1995). DHEA-S binds to a specific androgen receptor to form complex that can regulate the gene expression and stimulate the terminal sebocytes differentiation and sebum production, playing terminal role in development and maintenance of acne vulgaris.

Adrenally derived androgen, DHEA-S may trigger follicular cell epidermis (Thiboutot *et al.,* 1999). DHEA-S secretion begins at age 10, peak at age 20 and then wanes (Spark, 2002). It is significantly associated with the initiation of acne in young girls (Lucky *et al.,* 1994). Girls with severe comedonal acne had significantly high level of serum DHEA-S and longitudinal study analysis show somewhat high level of testosterone (Lucky *et al.,* 1997).

The present investigation is, therefore, designed to study the role of DHEA-S in acne development in our local young female population.

## MATERIALS AND METHODS

Sampling facility was available at "Mayo Hospital", Lahore. The blood samples of young girls having acne, diagnosed by physician of respective hospital were obtained. The control samples (N=17) were collected from girls of general local population.

Acne-prone females were further categorized on the basis of mild (N=16) and severe (N=29) acne development and compared with the controls for dehydroepiandrosterone sulfate level. The subjects were diagnosed on the basis of acne lesions present on the face and neck region. The experimental and technical analysis were performed on the basis of acne condition. The control subjects were normal and healthy. Enzyme-linked immunosorbent Assay (ELISA) was performed to evaluate the concentration of dehydroepiandrosterone sulfate (DHEA-S) in serum.

The assay was based on competitive interaction of DHEA-S and the hormone-enzyme conjugate for a limited number of immobilized anti DHEA-S antibodies (rabbit). Thus the amount of bound hormone-conjugate was inversely proportional to the concentration of dehydroepiandrosterone sulfate in the specimen. After incubation of specimen and hormone-enzyme conjugate in the well, unbound conjugate was removed by washing. When substrate solution was added a blue color developed changing to yellow after stopping the reaction. The intensity of the colors was inversely proportional to the amount of DHEA-S in the specimen. The absorbance of calibration and specimen was determined by using an enzyme-linked immunosorbent assay microplate reader.

Concentration of unknown specimen was interpolated from a dose response curve generated by utilizing serum calibrators of known DHEA-S concentrations. The data was analyzed statistically using Student t-test to work out the significance of results in comparable groups.

## RESULTS

The concentrations of dehydroepiandrosterone sulfate (DHEA-S) in μg/ml, were determined, by ELISA, in control and acne affected young female population. Acne affected group was further classified into mild and severe acne affected subjects (Fig. 1).

The average levels of DHEA-S were found to be estimated at 2.02±0.26 and 2.28±0.28 μg/ml in mild acne affected and control subjects, respectively. A non significant decrease of 11.5 % in DHEA-S concentration was observed in mild acne affected subjects when compared with controls.

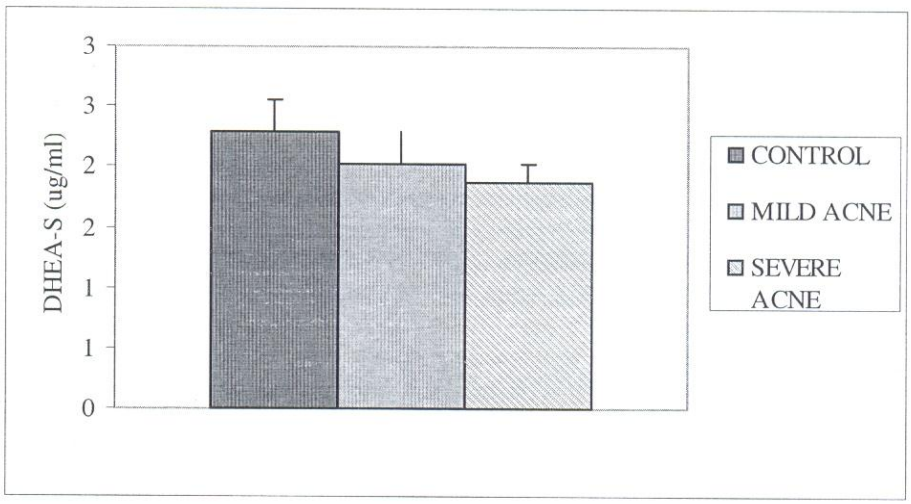


**Fig.1: Average dehydroepiandrosterone-sulfate (DHEA-S) levels exhibited by control, mild and severe acne affected subjects. Values are mean ± SEM.**

Severe acne affected subjects also indicated a non significant reduction of 18.02% in DHEA-S concentration with an average value of 1.87±0.15 μg/ml as compared to control subjects with an average level of 2.28±0.28 μg/ml.

A similar trend of non significant reduction of 7.47% was indicated when severe acne affected subjects with an average DHEA-S concentration of 1.87±0.15 μg/ml were compared with mild acne affected subjects exhibiting a mean DHEA-S level of 2.02±0.26 μg/ml.

## DISCUSSION

The pathogenesis of acne is complex and dependent on the interplay of multiple factors (Harper and Thiboutot, 2003). The sebum is considered to be a prime suspect in the crime of acne (Downing *et al.,* 1987). Sebum secretion is under the hormonal control (Holland *et al.,* 1998). The sebaceous gland is the target of androgens. Acne may be related to extensive sensitivity of sebaceous end-organs to androgens (Beylot, 2002).

Hyperandrogenism must be considered in any girl with premature pubarche, unusual acne, hirsutism, or androgenetic alopacia. The most common causes of hyperandrogenism presenting in a teenage girls are functional adrenal hyperandrogenism, which usually seems to be due to an exaggeration of adrenarche (Rosenfield and Lucky, 1993).

According to Lee (1976) acne occurred when androgen-produced pubertal changes began. The present study was designed to investigate the correlation between androgen, particularly, dehydroepiandrosterone sulfate (DHEA-S) and acne. The comparison made between the control and mild acne affected subjects revealed an 11.5% non-significant reduction of dehydroepiandrosterone sulfate level in the mild acne affected subjects. This result coincides the finding of Lee (1976) where DHEA-S level did not differ, significantly, in the control and mild acne affected subjects. The role of androgen in acne is permissive and plasma androgen measurements usually have no place in its management (Levell *et al.,* 1989).

Further investigation was done to find the relationship between concentration of dehydroepiandrosterone sulfate and severity of acne. Non-significant decline of 18.8% in DHEA-S level was observed in severe acne affected subjects when compared to controls. Aizawa *et al.* (1995) illustrated that there was no corrlation between androgen levels and acne severity. Thus it is unlikely that serum androgens play a principal role in women with adolescent acne.

Severity of acne, based on the acne grade was highly correlated with the inflammation count and less correlated with the sebum excretion rate (Walton *et al.,* 1995). The comparison of severe acne affected subjects with the mild acne affected subjects indicated 7.5% non-significant decrease of DHEA-S level in severe acne affected subjects. Aizawa and Niimura (1993) stated that there was no significant difference between the severe and mild acne affected subjects in any of the androgen level.

Conclusively, this study is initial or preliminary study regarding the role of dehydroepiandrosterone sulfate in acne development. Multiple factors are responsible for the development of acne. Keeping in view our results, it is analyzed that acne affected subjects exhibit non-significant changes in dehydroepiandrosterone sulfate level and its concentration remains within the normal range. It is suggested that this parameter may be worked out for longitudinal study on a large population for further elucidation of the correlation of DHEA-S with acne development.

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# POTENTIAL OF A PIGMENT PRODUCING MALATHION RESPONSIVE BACTERIUM FOR ENVIRONMENTAL ASSESSMENT OF THE INSECTICIDE IN ENVIRONMENTAL SAMPLES

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**Abstract:** A Gram positive bacterium was isolated on a selective medium containing malathion as sole carbon source from a soil sample collected from vicinity of an industry involved in insecticide formulation. The bacterium produced a green pigment in nutrient broth. The cell-free greenish culture fluid had maximum light absorption at 625 nm. Following the inoculation in nutrient broths containing 2, 4, 6, 8 and 10 mg of malathion/ml, the bacterial isolate yielded a dose dependent reduction in the amount of the pigment production up to the third dose. It is concluded that the microbe can be exploited as monitoring agent for biological assays of the insecticide in natural samples.

**Key words:** Bacterial production of green pigment; microbial assay of insecticide; malathion degrading bacteria.

## INTRODUCTION

Organophosphorus pesticides have been used in large quantities throughout the world since the first introduction of a synthetic insecticide, parathion for use in crop protection in 1944 (Saunders, 1957). Problems of contamination resulting from surplus pesticides and wastewater from pesticide factories have become obvious. The transformation of pesticides in environment results from physicochemical reactions as well as activity of cellular or extracellular components of the biota of a given habitat. However, principal biological pathway is microbial degradation of such pollutants (Karns *et al.*, 1983; Kadam *et al.*, 2003).

Frequent use of synthetic pesticides for over six decades has made them and their metabolites a permanent feature of the modern environment (Frank, 1987; Hind and Evans, 1988; U.S. EPA, 1988). Intact malathion is most commonly detected pesticide in food products. In one report, 18% samples appeared positive for malathion residues out of the 936 food items tested (FDA, 1992). This indicates widespread use of the insecticide in many crops.

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The contaminated food, water and air has generated a long list of health hazards associated with presence of varying levels of the xenobiotics (Greenberg and Laham, 1969; Mufti and Nazir, 1988; Machin and Mcbride, 1989; Asmatullah *et al.*, 1993; Asmatullah and Ijaz, 2004). The contaminated environment has necessitated the monitoring of foods, water and air for the presence and concentrations of primary and secondary pollutants. In this regard chemical analytical methods have progressed from simple reaction based changes in the physicochemical features of notorious substances to the contemporary highly refined techniques such as HPLC and gas chromatography (Jain and Imran, 1997; Hashmi *et al.*, 2002). Besides much expensive nature of the modern chemical analytical facilities these may not bring into our notice the presence of a modified chemical pollutant available to biological system but unapproachable by the analytical methods. On the other hand, biological/ microbiological assays can sense a biologically available pollutant or its derivatives. Sometime these methods might prove considerably sensitive, whilst they throw light on ecotoxicological impacts of the pollutants.

Microbial assays of the environmental samples is a growing field of interest both among ecotoxicologists as well as microbiologists (Gillespie and Guttman, 1993). Qazi and Hassan (2003) have isolated heavy metal responsive bacteria and reported their potential of monitory varying levels of Cr, Cu, Ag and Hg in polluted waters. The present study reports the response of a gram +ve bacterium to the presence of different concentrations of malathion in terms of production of a green pigment, while growing in nutrient broth. Change in amount of pigment production correlated to the amount of insecticide. The bacterium has the potential to design ecotoxicological malathion monitoring facility, easily judgeable by a layman.

## MATERIALS AND METHODS

*Isolation and Characterization of the Isolate*

Soils influenced by drainage of a factory involved in packing and formulation of insecticides were sampled in autoclaved bottles and processed on the same day by suspending 20 g of a sample in 50 ml autoclaved tap water. Containers were kept on orbital shaker at 100 rpm for overnight period. A selective medium which contained 0.5% malathion (commercial grade) as sole source of carbon was prepared by incorporating 0.1% $K_2HPO_4$, 0.5% $NH_4NO_3$, 0.02% $MgSO_4$ and 1.5% agar in distilled water. To this solution 20 µl of a mineral solution containing $FeSO_4H_2O$ 10%, $CaCl_2$ 10%, copper nitrate 0.5%, Zinc powder 0.5% and $MnCl_2$ 0.5% was added. The ingredients were autoclaved routinely and poured in pre-sterile petri plates.

Each processed sample, 0.5 ml was spreaded over the agar plates with the help of a glass spreader. The inoculated plates were subsequently incubated at room temperature for 48 hours. The growth was streaked for pure culturing on nutrient agar and then on the selective medium. Growth was preserved on nutrient agar slants as well as in glycerol stocks. The bacterium yielding green pigment in nutrient broth was used in this study. It

was optimized for growth conditions viz., temperature, pH and aeration by growing the bacterium at RT, 37°C and 50°C, at pH 5, 7 and 9, and incubating at 100 rpm and without shaking. Similarly, the bacterium was optimized for inoculum size by initiating growth with 1, 5 and 10% inocula. The growth was measured by taking O.D. of 24 hours old nutrient broth cultures at 600 nm.

Various morphological and biochemical characters of the isolate viz., catalase, oxidase and motility tests and Gram's and endospore stainings were determined as described by Benson (1994).

*Exposure to Malathion*

The bacterium was grown under the optimized conditions in control as well as nutrient broths containing 2, 4, 6, 8 and 10 mg/ml of malathion. Growth and level of pigment production were noticed daily up to 96 hours, following inoculations. Concentration of the pigment was recorded first without and then after shaking the culture fluids within the test tubes. The observations were photographed. At the completion of fourth day the culture fluids were centrifuged at 10000 rpm for 10 minutes in a refrigerated centrifuge adjusted at 15°C. Then optical density of the cell free culture fluids was measured at 625 nm by using spectrophotometer. These cell free culture fluids were then exposed for 10 minutes at 40°C, 60°C, 80°C and then 100°C in a water bath. After each temperature shock optical density was measured at 625 nm.

## RESULTS AND DISCUSSION

Growth of the isolate appeared after 24 hours on nutrient agar and the parallelogram shaped yellowish off-white colonies measured up to 0.6 x 1.4 cm (Fig.1A). The colonies were translucent and convex having wavy with scalloped margin. The isolate was found positive for catalase and oxidase tests. The Gram positive diplobacilli rods did not yield endospores even in 10-days old culture.

*Optimization of Growth Conditions*

The bacterial isolates grew best at pH 7 with 1% inoculum size when it was incubated at 37°C in nutrient broths. The aeration also significantly enhanced the growth (Table 1).

**Table 1: Growth conditions' optimization of the isolate**

| | Temperature | | | Aeration | | pH | | | Inoculum size | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | R.T | 37°C | 50°C | Rest | Shaking | 5 | 7 | 9 | 1% | 5% | 10% |
| X ± S.E.M. | 0.384 ± 0.0169 | 0.454 ± 0.026 | 0.017 ± 0.008 | 0.181 ± 0.028 | 0.559 ± 0.013 | 0.383 ± 0.043 | 0.439 ± 0.040 | 0.269 ± 0.046 | 0.57 ± 0.009 | 0.55 ± 0.013 | 0.482 ± 0.005 |

Values indicate optical densities of cultures and are means of four replicates ± SEM.

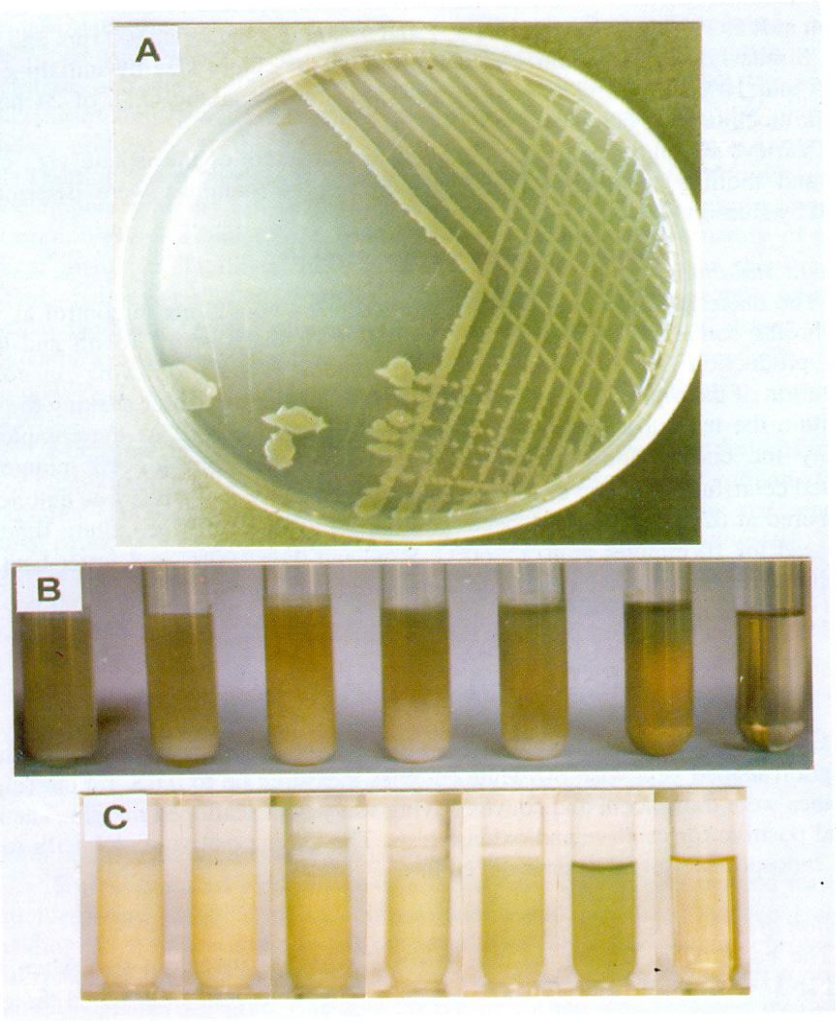


Fig. 1: Colonial appearance of the bacterium on nutrient agar (A). Appearance of green pigment production by the bacterial isolate in control and nutrient broths having different amounts of malathion. The test tubes from the right to left indicate blank (uninoculated broth), control and the cultures containing 2, 4, 6, 8 and 10 mg of malathion/ml (B). Appearance of the pigment in the cultures described above after shaking (C).

*Effects of different concentrations of Malathion*

Nutrient broths containing different concentrations of malathion gave milky appearance, probably due to carrier fluid/substance in the commercial grade of the insecticide used in this study. This isolate produced green pigment in nutrient broth. Different concentrations of malathion in the nutrient broth cultures resulted into dose dependent reduction in the production of pigment following the incubation period. The green pigment appeared to the upper 1/3 of the culture volume in the test tubes with a decreasing gradient downward (Fig.1B). Following the shaking of the contents of the test tubes the green pigment dispersed into the whole fluid but soon it started to resume its preferred presence in the upper layer (Fig.1C). Similarly, following the heat shock the pigment not only darkened but took a uniform look in the fluid column within the test tubes. These observations indicate the oxygen requirement of the pigment to be visualized. As in the unshaken and non-heat treated cultures the bacterial cells continued to consume the dissolved oxygen for their respiration, which was then not available to the pigment to render it green look, except for the upper one third portion wherein the higher oxygen content sufficed to provide oxygen requirements both for the pigment and the respiring aerobic bacteria. Further growth was accompanied by decrease in the milkiness of the medium. These observations have been recorded in Table 2.

**Table 2: Visual assessment of intensity of the pigment produced and reduction of milkiness in control and the cultures containing different concentrations of malathion**

| Experiments | After: | | | |
|---|---|---|---|---|
| | 24 hrs | 48 hrs | 72 hrs | 96 hrs |
| Control | +++[a] | ++++ | +++++ | ++++++ |
| | (-)[b] | (-) | (-) | (-) |
| 2 mg/ml | + | ++ | +++ | ++++ |
| | (++++) | (+++) | (++) | (+) |
| 4 mg/ml | - | + | ++ | +++ |
| | (+++++) | (++++) | (+++) | (++) |
| 6 mg/ml | - | - | + | ++ |
| | (++++++) | (+++++) | (++++) | (+++) |
| 8 mg/ml | - | - | - | - |
| | (++++++) | (+++++) | (++++) | (++++) |
| 10 mg/ml | - | - | - | - |
| | (++++++) | (++++++) | (+++++) | (++++) |

a: No. of + sign corresponds to intensity of the pigment.
b: Signs in parenthesis are indicative of the level of milkiness.

As can be seen from Table 2 the pigment intensity decreased in media containing higher concentration of malathion and it did not appear at all in media having highest contents of the insecticide. The water soluble green pigment appears a direct response of

growth and its intensity might be proportional to growth. Moreover, the insecticide delayed appearance of growth and the pigment.

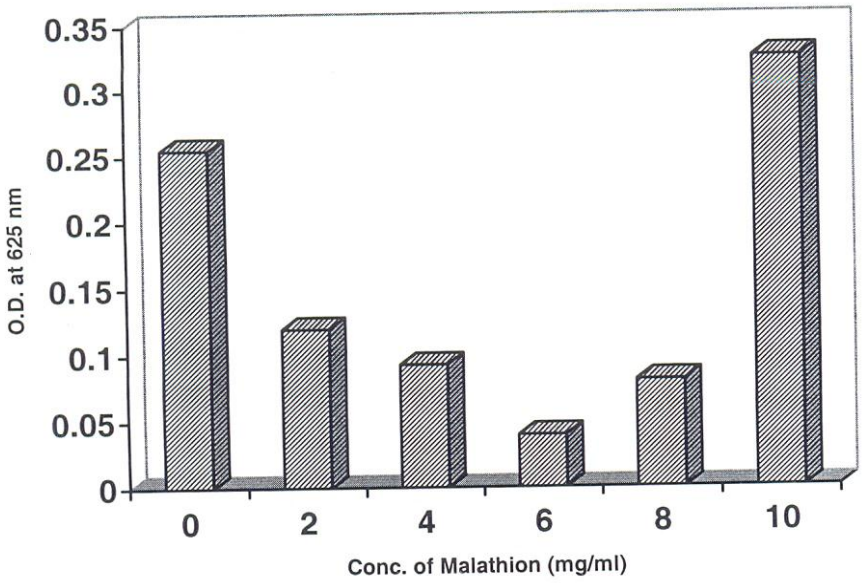


**Fig. 2: Optical densities of the cell free fluids of 4-day old bacterial cultures in nutrient broths containing varying amounts of malathion indicating, in general, a dose dependent decrease in the pigment production (the trend appeared diagrammatic up to 6 mg/ml dose).**

The green pigment appeared in control and 2 mg malathion/ml cultures after 24 hours. While in case of 4, 6, 8 and 10 mg/ml doses it was not visible after one day (Table 2). In control as well as experimental cultures the greenish colour appeared in the form of a surface band of about 0.8 mm in height having colour intensity gradient from surface to bottom. A such very thin green band was observable at the surface of the culture in the test tubes containing 4 mg dose of malathion after 48 hours of incubation. After 96 hours this green colour became more intensed in control and first and second experimental cultures, while in the cultures containing 6 mg/ml malathion it was very light and did not appear in the medium containing 8 and 10 mg of the insecticide. The colour did not appear even at the end of experiment. The milkiness remained consistent up to 48 hours of inoculation and it decreased after 72 hours (Fig.1B).

Heat shock experiment indicated heat stable nature of the pigment produced. In control culture the O.D did not change even after the exposure to 100°C. However, in

insecticide containing cell free culture fluids the O.D. decreased at higher temperature (Table 3).

**Table 3: Optical density of the green pigment in the cell free culture fluids of control and the nutrient broths containing varying concentrations of malathion, exposed at different temperatures for 10 minutes.**

| Experimental Group | Exposure temperature (°C) | | | | |
|---|---|---|---|---|---|
| | 30 | 40 | 60 | 80 | 100 |
| Control | 0.255 ± 0.0038 | 0.249 ± 0.0035 | 0.245 ± 0.00165 | 0.241 ± 0.00413 | 0.232 ± 0.008 |
| 2 mg/ml | 0.120 ± 0.0116 | 0.103 ± 0.0085 | 0.098 ± 0.0074 | 0.09 ± 0.009 | 0.091 ± 0.017 |
| 4 mg/ml | 0.093 ± 0.0075 | 0.0064 ± 0.0029 | 0.0875 ± 0.01128 | 0.057 ± 0.00075 | 0.084 ± 0.0075 |
| 6 mg/ml | 0.397 ± 0.0145 | 0.0029 ± 0.0083 | 0.01225 ± 0.01225 | 0.018 ± 0.012 | 0.01125 ± 0.006 |
| 8 mg/ml | 0.0812 ± 0.009 | 0.0253 ± 0.022 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.008 ± 0.0064 |
| 10 mg/ml | 0.326 ± 0.019 | 1.01 ± 0.0355 | 1.25 ± 0.1105 | 1.00125 ± 0.00825 | 0.963 ± 0.018 |

Values are means of four replicates ± SEM

The aerobic mesophilic and neutrophilic bacterium has mild growth conditions requirements and it yielded the green pigment intensity retrogressively up to 6 mg of the insecticide/ml. Disturbing this trend the higher values of optical density obtained for the cultures containing higher concentrations of malathion did not correspond to the apparently low amount of the pigment produced, but rather it occurred probably due to higher levels of turbidity in such cultures. As the milkiness of these media persisted owing to bacteriostatic effects of the higher concentrations of malathion.

In the reported medium the bacterial growth and its greenish product can detect and differentiate malathion concentrations ranging from 2 to 6 mg/ml. The differences between the study points are prominent and can allow to reduce the interclass interval and thus obtaining precise information about the amount of the toxicant in a sample. This bacterium by virtue of its growth and differential pigment production ability while responding to varying concentrations of malathion provide a valuable model for microbiological assay of the pollutant. The dose-response curve is the basis of such

biological monitoring strategies. Landis and Yu (1999) have explained that a graph describing the response of an enzyme, organism, population or biological community to a range of concentrations of a xenobiotic is the dose-response curve. These authors also described that bioassays for toxicity identification, evaluation and toxicity reduction evaluation programs have attained ongoing trend in the use of toxicity test designed for the monitoring of effluents. In fact, biological monitoring has been favoured over physico-chemical methods for obvious reasons. For example, Beeby (1993) described that simple physical or chemical determinations can be used to measure the rates of pollutants' input, distribution and dispersal in the environment and their assimilation into living tissues. But the total concentration measured in an individual can easily overestimate its biological significance: high levels of surface contamination, or the binding of the pollutant at inert sites may mean that the effective dose is much lower. Biological monitoring, on the other hand, aims to assess the significance of a pollutant for an organism and monitor species are used to detect pollution in this way.

Besides other monitoring species, microorganisms have long been considered appealing candidates for the simple reasons of ease of their culturing and quick response to pollutants (Bulich and Isenberg, 1980; Dutka and Kwan, 1982; Bitton and Dutka, 1986). Such efforts have progressed to the development of microtox toxicity test systems. Qureshi *et al.* (1998) have described that Microtox test is based on measuring changes in the light emitted by a non-pathogenic naturally luminescent bacterium (*Vibrio fisheri*, NRRLB-11117) upon exposure to a toxic substance or sample containing toxic materials. And that the microtox test is a short-term acute toxicity bioassay that combines the advantages of a biological test with the speed and ease of use of a laboratory instrument. Inasmuch, such sophisticated toxicity test facilities are not available in this country. Therefore, the present effort aimed at establishing a vivid response of the bacterium to varying concentration of the pollutant reported here. The same bacterium can also be tested for other pollutants such as heavy metals and other xenobiotics.

In the present model the results appeared after the incubation of at least overnight period. For rapid results the bacterium has to be worked out otherwise. For instance, the bacterium can be grown first in bulk following by centrifugation and suspending the cells in a sterile liquid that might be physiological saline or standard/dilute broth. Thus the metabolically active higher densities of the cells could be exposed to different amounts of pollutants and the resultant changes in the efficacy of pigment production noted.

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# PROVISION OF NUTRIENTS ENHANCES $Cr^{+6}$ REDUCTION POTENTIAL OF MICROBIAL COMMUNITY INDIGENOUS TO TANNERIES' EFFLUENTS

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**Abstract:** Chromium containing tanneries' effluents were used in these experiments to check the heavy metal resistance and degradation/biosorption ability of indigenous microbial community. The microbial community was found to reduce all the $Cr^{+6}$ in the original effluents within one week. When the effluents were supplied with additional 100 to 500 $\mu$g/ml of $Cr^{+6}$ and 0.1 % each of molasses and peptone, it was found that in presence of these growth promoting substances the heavy metal reduction occurred significantly higher. The results suggest that the indigenous microbiota can be activated for bioremediation process. Three bacterial strains isolated from the effluents were found to resist $Cr^{+6}$ up to 10mg/ml in a medium comprising of 1% each of molasses and peptone solidified with 1.5% agar. The microorganisms can reduce/biosorb the metal better in presence of these nutrients and it is suggested that *in situ* bioremediation may help in reducing ecotoxicological effects of $Cr^{+6}$.

**Key words:** $Cr^{+6}$ resistant microbes, bioremediation of heavy metals, augmenting growth of microbial community.

## INTRODUCTION

Drastically increasing pollution resulting from daily human activities is now becoming a threat for human life as well as indigenous microbiota at some places. No practical measures are taken into account while discharging industrial effluents especially in developing countries. Water pollution is mainly due to disposal of sewage and industrial effluents (Bonaventura and Johnson, 1997; Qazi *et al.,* 1997). Heavy metals directly influence the biota in fragile wetland ecosystem. As the metals leach down in the presence of strong binding legends, they are affected by several processes like complexing reactions, redox processes, adsorption, precipitation and coprecipitation. Several heavy metals are known to interact with nuclear component eliciting a toxic cellular response *e.g.* Cd binds DNA and causes cell death and Cu produces reactive oxygen leading to DNA damage (Gupta and Florence, 1981; Ogawa *et al.*, 1989; Fernandez and Novillo, 1994; Madoni *et al.,* 1996).

Chromium exists in hexavalent and trivalent forms. While reducing from hexavalent to trivalent form it passes through two highly reactive, carcinogenic, mutagenic and teratogenic states *i.e.,* pentavalent and tetravalent. The metal is a micronutrient in

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living systems and metabolizes fats and carbohydrates but its higher amounts are highly fatal (Cary, 1982; Anderson *et al.,* 1983; Nieto *et al.,* 1989; Nair and Krishnamurthi, 1991; Chakraborty and Mishra, 1992; Ohtake and Silver, 1992; Vishnyakov *et al.,* 1992; Mertz, 1993; Yamamoto *et al.,* 1993; Landsdown, 1995). Solubility, oxidation state or the type of complex formed, affects the potential for toxicity. Derivatives of $Cr^{+3}$ are water insoluble at neutral pH and can be removed from the medium in the form of $Cr(OH)_3$, while $Cr^{+6}$ forms are highly soluble thus impact much more toxic effects on exposure (Laziinsky,1990; Vishnyakov *et al.*, 1992; Anderson, 1995; Stoecker, 1996).

In Pakistan, leather industry is continuing to grow and there are several hundreds tanneries in Faisalabad, Gujranwala, Karachi, Kasur, Lahore, Multan and Sialkot. Amongst these cities, Kasur is now becoming main leather production center. Although recently a treatment plant at the said site has been working, mainly employing chemical processes. Before the installment of this facility $Cr^{+6}$ being used in tanning process had been discharged in the effluents, without any treatment that contained toxic chemical wastes with high values of COD. This resulted into accumulation of the effluents into shallow ponds and conversion of agricultural land into barren areas (Fig 1, A-C).

Very sad aspect of this environmental deterioration is that with the help of underground water sucking system and constructed canals (Fig. 1D), chromium and solid wastes were discharged into natural water system that finds its ultimate way to river Sutluj. Due to continuous leaching the toxic matter has started mixing with the water table of Kasur area. Inhabitants of such areas may face the danger of suffering from physical and mental retardations (Cary, 1982). It is well known that some microorganisms are helpful in cleaning up the environment by the process of bioremediation. Microorganisms can metabolize many aliphatic and aromatic organic compounds for obtaining energy for growth, or to use them as co-substrates, thus converting them to products such as $CO_2$, $H_2O$ and biomass. These biotransformations can be exploited for treatment of contaminated soils and ground water (Shanker *et al.,* 1990; Morgan *et al.,* 1993; White *et al.,* 1995; Wilson and Bouwer, 1997). Pretreatment of effluents could also help in reducing the toxic effects of heavy metals (Petrilli and DeFlora, 1977; Parsek *et al.,* 1995). Microorganisms surviving in chromium contaminated environments have been considered to adsorb or reduce $Cr^{+6}$ to $Cr^{+3}$ (Ogawa *et al.,* 1989; Bender *et al.,* 1995).

This study was intended to biostimulate indigenous microbiota of the stagnant tanneries' effluents of the study area to enhance its $Cr^{+6}$ reduction/removal potential. Bio-augmentation was achieved by adding peptone and molasses. The latter growth promoting substance is waste of sugar industries. Its disposal is another problem, leading to water and soil pollution. One major concern of this investigation was to design integrated industrial set up so that waste of one industry, for example organic compound(s), might promote bioremediation activities of microbes developing in effluents of other industrial origins containing heavy metals and/or other xenobiotic substances. Such efforts might solve industrial pollution problems of varied nature in a unit *in situ* bioremediation process. The results are helpful in designing *in situ* bioremediation plants for $Cr^{+6}$ contaminated environments.

## MATERIALS AND METHODS

Samples were collected in sterile containers from ponds receiving tanneries' effluents near Kasur city and brought to the laboratory. They were observed under microscope for the presence of microorganisms. A sample that contained protozoa as well as bacteria was selected for further studies. The effluent was filtered through sterile glass wool and then transferred into 100 ml pre-sterile conical flasks in an amount of 20 ml/ flask. A portion of filtrate was processed for the colorimetric method of $Cr^{+6}$ estimation (Petrilli and DeFlora, 1977).

The flasks were categorized into two groups. The group1 contained two types of cultures viz. untreated control and the experimental ones; exposed to 100, 200, 300, 400 and 500 $\mu$g of $Cr^{+6}$/ml. In group 2, the sample was processed similarly as for group1, except that 0.1% each of molasses (v/v) and peptone (w/v) was added as growth promoting substances. All the flasks were cotton plugged, kept at room temperature (21±2°C) and observed daily under microscope for microbial population dynamics up to 8 days. Size and number of protozoa was daily counted at 100X. For this purpose 2 $\mu l$ of a sample was taken on a clean slide and very little 1% methylcellulose was mixed to the droplet with the help of a toothpick. Number of protozoa was calibrated as / ml of culture. Size was measured with the help of an ocular micrometer, calibrated as µm at 400X. Both length and width were then translated into volume ($\mu m^3$).

At the end of experimental period the effluents were made microbe free by centrifugation at 5000 rpm for 10 minutes and $Cr^{+6}$ contents were estimated by the method, referred above. Statistical comparisons between two respective groups of each experimental period were made by employing Student's t-test.

*Isolation, growth optimization and $Cr^{+6}$ resistance of bacteria*

Bacteria were isolated from the field sample by streaking on nutrient agar. After isolating into pure cultures their colonial characteristics were observed with the help of a colony counter. They were optimized by growing in 0.1, 0.5 and 1.0 % each of molasses and peptone solutions with initial pH values of 7, 8 and 9 and incubating at 45°C, 37°C and the room temperature . After 24 hours optical density of the cultures was noted at 600 nm. For $Cr^{+6}$ resistance testing, molasses peptone agar was prepared. The medium containing 1.5 % of agar and 1% each of molasses and peptone was supplied with 5, 10 and 15 mg of $Cr^{+6}$/ml in the form of $K_2Cr_2O_7$. Photographs of the bacterial colonies grown on the molasses-peptone agar media were made. While photomicrographs of stained microbes (Gram's reaction) were taken with the help of a camera-fitted microscope.

## RESULTS

*Bio-physical features of the waste water*

Effluents in the pond was green in appearance with a pungent smell, having about 8 pH and 22°C temperature. Chemical analysis indicated that the sample contained

0.12 µg/ml of $Cr^{+6}$. Microscopic observation of the sample revealed that microbial community was comprised of ciliate and flagellate protozoa and bacteria. Only one kind each of ciliate and flagellate protists was observed. Ciliates were more or less elliptical in shape and measured about 62.37 $(\mu m)^3$. The flagellates were found in general similar to the ciliates but were more conical at rear end and of too minute dimensions, to be measured microscopically. However, under laboratory conditions they grew and became measurable at 2nd day.

*Survival of protozoa in the original sample under laboratory conditions*

Number of the protozoans increased progressively up to one-week period followed by a considerable decrease at day 8. In other words, the field sample in glass containers could support the growth of the protozoa up to one-week period which was followed by the decline phase (Tables 1, 3). With day-to-day fluctuations, the overall trend in case of ciliates was of decrease in volume from the start to the end of the experiment (Table 2). But in case of flagellates, increase in their volume occurred at 6th day followed by a gradual decrease (Table 4). From these trends it could be inferred that best growth periods of the protozoans were 5th and 6th days for the ciliates and 6th and 7th days for the flagellates. Therefore, the original effluents as well as all the experimental ones were analyzed for $Cr^{+6}$ reduction efficiency of the microbes at end of the study period.

*Growth of protozoa in effluents supplemented with growth enhancing substances*

Addition of 0.1 % each of molasses and peptone to the effluents resulted into increases in growth, size and activities of the protists. The number of ciliates was found, in general, higher than those in the original sample during the study period. The differences turned out statistically significant so that at days 1,3,4 and 7 the elevations were 33.33, 15.24, 32.85 and 64.54 %, respectively (Table 1). Volume of ciliates grown in the presence of molasses and peptone showed significant increase over those in original sample at day 1st and then the parameter decreased compared to the controls (Table 2).

In case of flagellates, their number and volume remained higher almost throughout the study period in the molasses and peptone supplemented cultures than the figures for the cells in the original effluents and the differences were found statistically significant (Tables 3, 4). As can be observed from the table No.3, the flagellates' number increased several folds when they were supplied with the nutrients. Concerning the volume, except a decrease at day 4, the parameter turned out significantly higher for the growth promoting substances supplied effluents than the controls (Table 4).

*Growth of protozoa in original and the nutrients supplied effluents in the presence of additional amounts of $Cr^{+6}$.*

Study of the protozoa in the original effluents as well as those supplemented with molasses and peptone in the presence of additional amounts of $Cr^{+6}$ indicated dose allied toxicity of the heavy metal. The ciliates were not observed in the effluents provided with 400 and 500 µg of $Cr^{+6}$ /ml right from the start of observational period. However, in case

of 100 µg of the metal/ml the protozoa remained observable up to the end of the experiment. While in case of 200 µg concentration they managed to thrive up to 5th day. For 300 µg dose the protists appeared only at first day both in the original as well as the nutrient supplied effluents (Tables 1, 2).

Even in case of the lowest exposure (100µg/ml) the ciliates except for the increase at earlier days, could not attain the level of growth observed in the original effluent. However, provision of molasses and peptone appeared to retard the metal toxicity so that the number of ciliates at 3rd and 4th days in such cultures turned out significantly higher than the respective metal containing effluents (Table 1). Volume of the ciliates in the presence of 100µg of the metal remained comparable to those in the original effluents up to day 4, thereafter the parameter decreased about two folds around day 5 and 6 in the experimental effluents. Comparisons of the parameter between the $Cr^{+6}$ exposed original effluents and those supplied with the nutrients indicated that except significant increases at days 1, 5 and 6 the volume of the protists turned out significantly less in the nutrient supplemented ones (Table 1). As number and volume of ciliates were significantly decreased, it can be inferred that these eukaryotes could not tolerate higher concentrations of heavy metals. At higher concentration *i.e* 200 µg/ml the number and volume were affected on day 5. Significant differences were observed in the volume of the ciliates thriving in the effluents without growth enhancing substances. More or less similar was the case with flagellates i.e. they showed optimum growth at days 5 to 7 that was followed by a decline phase (Tables 3, 4).

*Chromium contents*

Chromium was completely reduced/ removed from the effluents by the microorganisms after one week in the original sample, both with and without growth enhancing substances. The original sample itself contained 0.12 µg of the heavy metal/ml. Effluents with additional 100µg dose reduced the heavy metal up to 30 and 50% in samples without and with growth enhancing substances, respectively. In case of 200 to 500µg/ml of the metal the samples containing growth enhancing substances showed much better reduction than the ones without them (Fig.2). These differences were found statistically significant at all the concentrations.

*Bacterial isolates and their $cr^{+6}$ resistance*

Three bacterial strains were isolated from the field sample designated as MBZ-I, MBZ-II and MBZ-III (Fig.3). As can be seen from the figure, MBZ-I and MBZ-II were gram-positive diplobacilli and streptobacilli, respectively. MBZ-III depicted gram-negative diplobacilli morphology. Isolated colonies of these bacteria grown on nutrient agar were off-white with mucoid consistency and raised elevation. Diameter of MBZ-I and MBZ-II bacterial colonies measured as 0.5 and 2.0 mm, respectively. While MBZ-III colony was not measurable, due to spreading nature of the growth. Margin of the bacterial colonies were smooth for the MBZ-I and MBZ-II, but irregular for the MBZ-III isolates.

**Table-1:** **Effect of $Cr^{+6}$ and 0.1 % each of molasses and peptone (MP) on the number x ($10^{3}$/ml) of ciliates inhabitants of original tanneries effluents.**

| Experimental group | | Days of observation | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Control | A[a] | [c] | | | | | | | |
| | B[b] | *** | | *** | *** | | | ** | |
| $Cr^{+6}$ 100 | A | | | | | | | 0.88 | |
| | | | | | | | | | |
| $Cr^{+6}$200 | | | | | | | [d] | - | - |
| | | | | | | | | | |
| $Cr^{+6}$300 | | | | | | | | | |
| | | | | | | | | | |

a: original sample; b: original sample supplemented with MP; c: mean of three replicates detected.

Values with asterisk(s) are significantly different from those in respective rows A. * = P<0.05; ** = P<0.01; *** = P<0.001 (Students t-test).

Table-2: Effect of $Cr^{+6}$ and 0.1 % each of molasses and peptone (MP) on volume x 3.85( $m)^3$ of ciliates inhabitants o original tanneries effluents.

| Experimental group | | Days of observation | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Control | A[a] | [c] | | | | | | | |
| | B[b] | *** | | *** | | *** | *** | | *** |
| $Cr^{+6}$100 | A | | | | | | | | |
| | | | | | | | | | |
| $Cr^{+6}$200 | | | | | | | [d] | - | - |
| | | | | | | | | | |
| $Cr^{+6}$300 | | | | | | | | | |
| | | | | | | | | | |

a: original sample; b: original sample supplemented with MP; c: mean of three replicates
detected.
Values with asterisk(s) are significantly different from those in respective rows A . *** = P<0.001 (Students t -test).

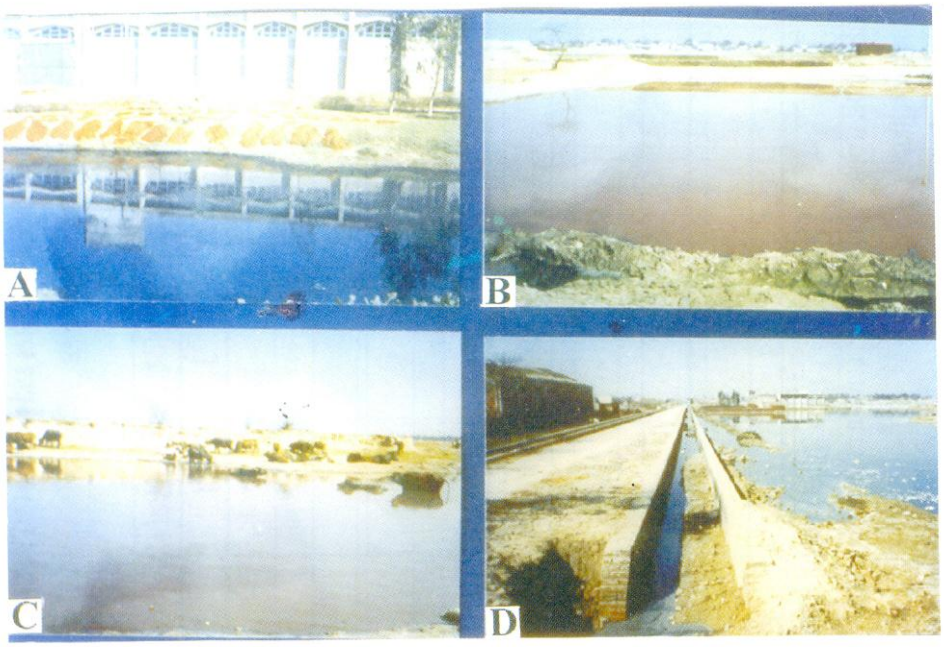


Fig. 1:

A: A view of a tannery. Under processing hides and effluents deposition are visible.
B: An open shallow pond retaining tanneries' effluents.
C: Another pond and land area. Cattle grazing and drinking the effluent water can be seen.
D: A panoramic view of the effluents contaminated area with a drainage canal.

Table-3: Effect of $Cr^{+6}$ and 0.1 % each of molasses and peptone (MP) on the number x ($10^3$/ml) of flagellates inhabitants of original tanneries effluents.

| Experimental group | | Days of observation | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Control | A[a] | [d] | | | | | | | |
| | B[b] | [c] | | * | *** | ** | ** | *** | *** |
| $Cr^{+6}$100 µg/ml | A | | | | | | | | |
| | B | | | | *** 8 | * | *** | * | * |
| $Cr^{+6}$200 µg/ml | A | | | | | | [d] - | - | - |
| | B | | | | * | - | - | - | - |

a: original sample; b: original sample supplemented with MP; c: mean of thre e replicates

e: no viable organisms were detected.

Values with asterisk(s) are significantly different from those in respective rows A. * = P<0.05; ** = P<0.01; *** = P<0.001 (Students t-test).

Table-4: Effect of $Cr^{+6}$ [3] of flagellates inhabitants of original tanneries effluents.

| Experimental group | | Days of observation | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Control | A[a] | [d] | | | | | | 9. | |
| | B[b] | [c] | * | *** | *** | *** | *** | *** | *** |
| $Cr^{+6}$ 100 µg/ml | A | | | | | | | | |
| | B | | *** | ** | *** .04 | * | *** | *** | *** |
| $Cr^{+6}$ 200 µg/ml | A | - | | | | | [e] - | - | - |
| | B | | *** | *** | *** | - | - | - | - |

[a]: original sample; b: original sample supplemented with MP; c: mean of three repli cates
e: no viable organisms were detected.
Values with asterisk(s) are significantly different from those in respective rows A. * = P<0.05; ** = P<0.01; *** = P<0.001 (Students t-test).

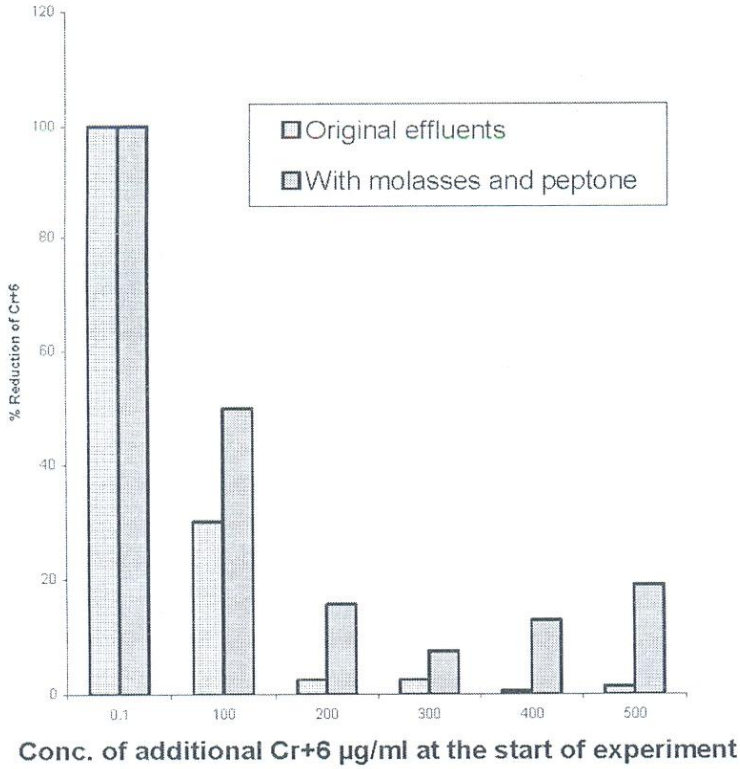


**Fig.2: Percent reduction of $Cr^{+6}$ after one week, in the original tanneries effluents and those supplied with molasses and peptone (each 0.1%) containing varying added concentrations of $Cr^{+6}$/ml.**

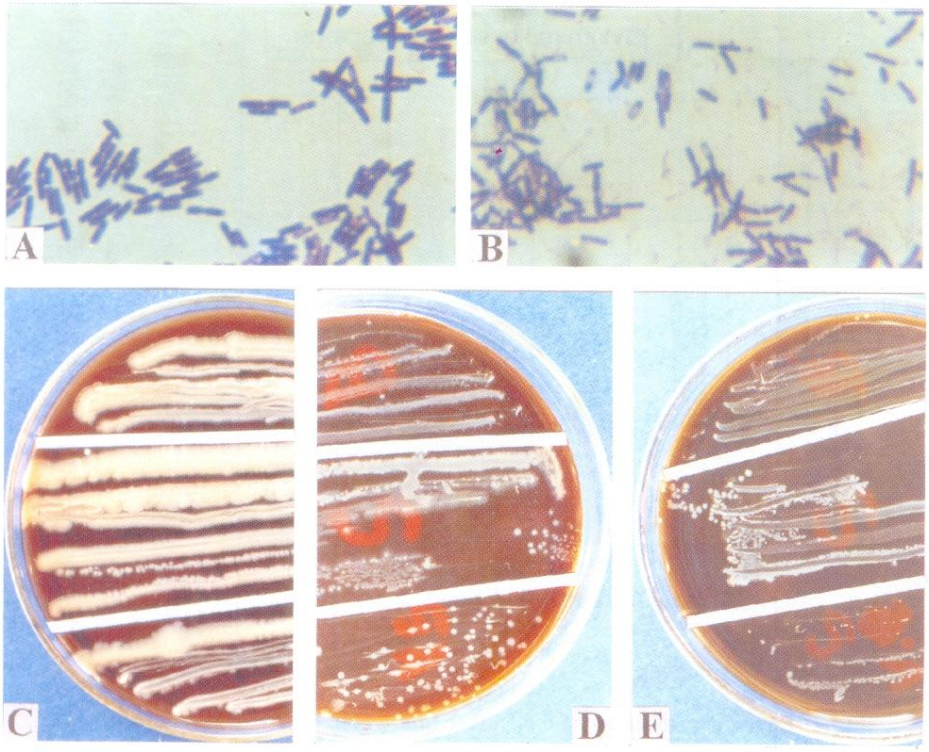


Fig. 3:

A: Bacterial isolate # MBZ-I. Gram staining x 1000.
B: Bacterial isolate # MBZ-II. Gram staining x 1000.
C: Growth of the isolates, MBZ-I, MBZ-II and MBZ-III (from top to bottom, respectively) on 1% each of molasses and peptone agar plates.
D: Growth of the isolates MBZ-I, MBZ-II and MBZ-III on the medium supplied with 5.00 mg of $Cr^{+6}$/ml.
E: Growth of the isolates MBZ-I, MBZ-II and MBZ-III on the medium supplied with 10.00 mg of $Cr^{+6}$/ml.

One percent solution each of molasses and peptone, 37°C incubation temperature and 9 initial pH were found optimum conditions for the growth of the three bacterial isolates. All the strains showed apparent growth up to the concentration of 10mg of $Cr^{+6}$/ml on the molasses- peptone agar (Fig. 2). In the medium containing 15mg of $Cr^{+6}$/ml, the bacterial strains MBZ-II and MBZ-III expressed very weak growth, while MBZ-I failed to manifest its growth.

## DISCUSSION

The industrial effluents of the study area contaminated with the heavy metal chromium harboured various protozoa and bacteria. The microbial community was found not only able to remove the toxic metal from the effluent within the period of one week under laboratory conditions, but it also showed a potential to reduce/remove extra load of $Cr^{+6}$ when the effluents were added with the metal up to 500µg/ml. Potential of these microorganisms to turn down the level of toxic metal in the effluents was found to increase significantly in the presence of nutrients like molasses and peptone. In fact, the presence of microorganisms in the environment contaminated with heavy metals, which have developed resistance mechanisms and are capable of rendering these metals to less toxic forms, has opened up the possibility for use of these microbes in biological treatment processes for such effluents (Cervantes, 1991; Liu and Suflita, 1993; Thomas, 1996).

The protozoans at higher exposure of $Cr^{+6}$ *i.e.* 300µg/ml were unable to survive for more than two days. Protozoa have been found sensitive to the heavy metal toxicity such as Cd, Cu, Pb, Zn and Cr. Results of this study indicate possible potential of eukaryotic microorganisms for biological metal remediation processes essentially at lower concentration of the heavy metal. But at higher concentrations the $Cr^{+6}$ is toxic for these models. This notion supports findings of earlier workers indicating toxicity of the heavy metals for the eukaryotic organisms (Nieto *et al.,* 1989; Cervantes, 1991; Turick *et al.,* 1996).

Bacterial isolates from the effluents were found to tolerate very high level of the metal as they thrived in the presence of 10 mg/ml of $Cr^{+6}$. Although in this study they were not tested for reduction potential. However, it is well known that the prokaryotes are more efficient in their roles in such bioremediation strategies including heavy metals (Higgin *et al.,* 1988; Stroo and Madison, 1992; Bender *et al.*, 1995; Warng and Sheu, 1995).

From the findings of this study it appears that the concentrated effluents, both in terms of the level of heavy metal as well as organic contents (nutrients) could be treated primarily by prokaryotic organisms and then for a secondary treatment, indigenous eukaryotic microorganisms may be employed with a subsequent generation of less toxic effluents for a healthy environment. Nutritional organic industrial waste such as molasses may be consumed to activate bioremediation process of microbes and thus to get simultaneous rid of heavy metal toxicity and organic pollution. This notion is suggestive for integrated industrial set up wherein wastes of the industries may be treated

microbiologically with less expenditure leading to the development of economically feasible bioremediation strategies.

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**Short Communication**

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# ABNORMAL FINS IN SOME FISHES

**Abstract:** Some abnormalities in fishes were observed. In two specimens of *Garra rossica* the pelvic fins were totally missing. In a specimen of *Puntius chola* the two pelvic fins were fused to form a sort of sucker. In two specimens of *Bagarius bagarius* the filamentous parts of the caudal fin of one fish were fused with filamentous parts of the caudal fin of other fish. The origin of this abnormality is briefly discussed.

## INTRODUCTION

Among bony fishes there are two pairs of paired fins: pectoral and pelvic fins. In some cases the one or both the pelvic fins are missing. This is a normal feature in some genera of teleost fishes. But normally the two pelvic fins are present. Hora (*Rec. Indian* Mus., **22**: 27-32, 1921) recorded the absence of a pectoral fin in *Rita rita* and both the pelvic fins in *Barilius barila*. He discussed the absence of pelvic fins in *Channa* and some other fishes also.

## OBSERVATIONS

On 2nd October 2003 a pair of catfishes belonging to *Bagarius bagarius,* commonly known as Fauji Khagga, were purchased from a local fish market. In this species the caudal fin is deeply forked and the outer rays of the two lobes are elongated in the form of two filaments. It was observed that the filaments of the caudal fin of one specimen were fused with the filaments of the caudal fin of the other specimen. Otherwise the two fish specimens were normal in all respects. It appeared that the specimens were twins. During development, they continued their growth as independent individuals but the tissues in one specimen developing into the caudal filaments remained attached to the tissues developing into the caudal filaments of the other individual. The two specimens were nearly alike having 29 cm standard length and 372 g weight in one specimen and 27.9 cm in standard length and 342 g in weight in other specimen. In the stomach of this specimen a semi digested *Bagarius bagarius* 15 cm stl and 22 g weight was discovered along with a small specimen of *Gagata cenia* (another catfish).

Previously, we have observed two specimen of *Garra rossica* collected from Barshore stream in Balochistan without the pelvic fins. In a specimen of *Puntius chola* collected from Ghazi (NWFP), the two pelvic fins were fused together to form a sort of sucker.

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# INSTRUCTIONS TO CONTRIBUTORS

Punjab University Journal of Zoology publishes articles in English on all aspects of animal life. Generally these articles will be in, or related to one of the following subject area: Anatomy, Physiology, Cell Biology, Molecular Biology, Genetics, Toxicology, Developmental Biology, Entomology, Parasitology, Microbiology, Pathology, Palaeontology, Taxonomy, Environmental Biology, Endocrinology, Wildlife, Fisheries, Vertebrate and Invertebrate Morphology.

The manuscripts can be submitted as full length papers or as short communications. All manuscripts should be submitted in triplicate (an original and two copies) complete in all respects and typed strictly according to the style of PJZ to **Prof. Dr. Tanveer Akhtar, Editor, Punjab University Journal of Zoology, Department of Zoology, University of the Punjab, Quaid-e-Azam Campus, Lahore 54590, Pakistan**.

The manuscript should have a uniform style, typed in double spacing on one side of the paper, and consist of sub-sections in the following sequence: (1) Title page, (2) Abstract, (3) Text (to be subdivided into Introduction, Methods, Results and Discussion), (4) References, (5) Acknowledgements, (6) Footnotes, (7) Tables, and (8) Legends.

Each section should start on a new page.

**Title page**

The first page of manuscript should include the following:

Complete title of paper.

Author's or names.

Institution from which paper emanated with complete address.

Total number of figures included.

Running title.

Name, address and telephone number of person to whom the proofs are to be sent.

**Abstract**

An abstract of not more than 300 words for full length papers and 150 words for short communications should be prepared. It will serve in lieu of a concluding summary and when published will precede the introductory section of the text. The abstract should be written in complete sentences, and should state succinctly the objectives, the experimental design, the principal observations and conclusions. Do not include references in Abstract.

Suggest Key words at the end of Abstract.

**Text**

Introduction, Material and Methods, Results and Discussion will be the only central headings, which will appear in bold capital letters. All other headings will be treated as side headings and will appear in low case italics.

Nothing in the text except for scientific names, should be underlined.

**References**

References should be cited in the text by giving the author's name followed by the year (e.g., Ahmad, 1990). Same principle will be followed for two authors (e.g., Khan and Haseeb, 1984). In the case of three or more than three authors, the references should be cited as Tanveer *et al.* (1989). All the references mentioned in the text should be listed alphabetical order at the end of text as follows. Names of the Journals should be abbreviated according to the latest edition of the World List of Scientific Periodicals.

TANVEER, A., 1992. Response to temperature and heat tolerance of some gastropod snails. *Bangladesh J. Zool.*, **20**: 257-268.

SCHWABE, C.W., 1986. Current status of hydatid disease: A zoonosis of increasing importance. In: *The Biology of Echinococcus and Hydatid Disease* (Thompson, R.C.A., ed.), pp.81-113. George Allan and Unwin, London.

SMITH, J.D., 1966. *The physiology of trematodes*. Butterworth, Edinburgh and London.

Do not list articles that have been submitted but not accepted for publications. References to such work should be noted in the text as "unreported data".

In short communications, references should not be listed at the end but instead complete citation be given inside the text, where reference has been made. The complete citation (consisting of name of Journal, volume, first page, last page and year of publication), should be given in brackets following name of the authors e.g., Anwar and Tanveer (Punjab Univ. J. Zool., 12: 139-150).

**General Manuscript Instructions**

The manuscript should be typed double spaced throughout on one side of bond or heavy-bodied paper 8½" x 11" (22 x 28 cm) with a 1" (2.5 cm) margin on all sides.

- Number all pages of the manuscripts consecutively.
- Corrections to the manuscript should be either typed of printed legibly in ink.
- Do not use abbreviations or mathematical numbers to begin sentences.
- The word "Figure" written in the text is not abbreviated excess when appearing in parenthesis.
- Spell non-technical terms according to the current Webster's International Dictionary.
- Always spell out numbers when they stand as the first word in the sentence. Numbers indicating times, weight and measurements are to be in Arabic numerals when followed by abbreviations (e.g., 2 mm; 1 sec; 3 ml). In general numbers one to ten should be written in the text. All higher numbers should be given in Arabic numbers.
- Dates should be written as follows: October 1, 1991.
- Sources of equipment and chemicals should include city and country.

**Illustration**

In general, to achieve greatest fidelity and rendition of detail it is preferable that the printer work directly from original photocopies or drawings with a little reduction as possible. Illustrations should be prepared for reproduction as halftones or line drawings. All illustrations are to be submitted in complete and finished form, with adequate labeling.

Illustrations should be drawn in Indian ink, preferably on bristole board. The author's name and the number of the figure should be written in pencil on the back side of each drawing. The size of the illustration after reduction will not exceed (6x3" or 8x6").

The photographs should be glossy black and white prints. The full cost of the blocks will be borne by the authors.

Page-proofs will be sent to the authors for correction, which should be returned within one week of their receipt. Authors may be required to pay for alterations in proofs other than those needed to correct printer's errors.

Authors will receive 20 offprints gratis. Extra copies may be ordered for purchase when proofs are returned to the Editor.

All editorial correspondence should be addressed to Dr. Tanveer Akhtar, Department of Zoology, University of the Punjab, Quaid-e-Azam Campus, Lahore 54590, Pakistan.