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INDIGENOUS *AGROBACTERIUM TUMEFACIENS*: GROWTH RESPONSES TO METALLIC SALTS, ANTIBIOTICS, TEMPERATURE AND pH

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Abstract: Sixty two *Agrobacterium tumefaciens* strains were isolated from local plants of Punjab, Pakistan. Their metallic salts and antibiotics resistance/sensitivity profiles, growth response at two different temperatures and at two different temperatures with various pH levels were investigated. The results of these experiments revealed that isolates shared many physiological characters. Majority of strains could tolerate different metallic salts but all of them could grow in the presence of $MnSO_4$, $PbNO_3$ and $ZnSO_4$ and all were sensitive to Cr salt. These strains could be divided into 15 groups on the basis of metallic salts resistance/ sensitivity profiles. While for different antibiotics these strains showed different behaviour and could be grouped into 10 clusters on the basis of their antibiotics resistance/sensitivity profiles. Results of bacterial growth experiments at 28° and 37°C and at different levels of pH (4, 5, 6, 7, 8, 9) were almost similar in some aspects. Most of the strains, initially, showed better growth rate at 37°C except some strains isolated from colder areas which exhibited better growth at 28°C. The experiments performed at different levels of pH revealed that all strains showed maximum growth rate at pH 6. However pH affect on the growth rate exhibited that all strains could grew better in extreme alkaline pH (9) as compared to extreme acid pH (4).

Key words: *A. tumefaciens*, metallic salts, antibiotics, growth rate, temperature, pH.

INTRODUCTION

The soil phytopathogenic bacterium *Agrobacterium tumefaciens* causes crown gall disease on a variety of plants (Hooykaas and Schilperoort, 1992; Shaw *et al.*, 1991). The soil dwelling phytopathogenic *A. tumefaciens* belongs to family Rhizobiaceae (Krieg and Holt, 1984; Lippincott *et al.*, 1986). The disease caused by *A. tumefaciens* can be recognized by the presence of tumors (galls) of different sizes, shapes and forms on stem, crown (at root-shoot junction) and roots of the infected plants. *A. tumefaciens* is found abundantly in soil as a soil inhabitant and the soil surrounding the infected plants is highly populated by the genus *Agrobacterium* (New and Kerr, 1972; Kerr, 1969; Bouzar and Moore, 1987). The number of the genus *Agrobacterium* around the roots of the infected plants is thousand fold more than the near by soil (Bouzar and Moore, 1987). The higher number of *Agrobacterium* around roots of infected plants is due to a highly sensitive chemotaxis system, responsive to a variety of sugars, amino acids and phenolics exuded by the plants roots and wounds (Ashby *et al.*, 1987; Loake *et al.*, 1988). For chemotaxis the organism must be motile

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(Shaw *et al.*, 1991). *A. tumefaciens* are motile and capable of attaining speeds upto 60 μm sec^{-1} and run in a straight line exceeds 500 μm (Loake *et al.*, 1988). Virulent strains of *A. tumefaciens* showed positive chemotactic response to compounds exuded by wounded tissues (Shaw *et al.*, 1988, 1991). Chemotaxis play an important role in virulence and non-chemotactic *A. tumefaciens* are impaired in virulence in certain conditions (Hawes *et al.*, 1988; Hawes and Smith, 1989). For *A. tumefaciens* crown gall tumors induced by the virulent strains are good source of isolation (Kerr, 1969). Different methods including biochemical and physiological analysis (Kersters *et al.*, 1973; Anderson and Moore, 1979; Holmes and Roberts, 1981), serology and intrinsic antibiotic resistance patterns (Nesme *et al.*, 1987), protein and genomic DNA digestion patterns (Alarcon *et al.*, 1987; Bouzar and Moore, 1987), or phage typing (Beadsley, 1955), have been used to characterize *Agrobacterium* strains. Generally these methods provide information and characterize chromosomally encoded attributes and at times provide precise information of Ti plasmids residing in these strains (Ponsonnet and Nesme, 1994). *A. tumefaciens* biovar I strains are 3-ketolactose positive (Lelliott and Stead, 1987; Hooykaas, 1988; Gerhardt *et al.*, 1994) and in the host tests experiments virulent strains causes crown gall disease on *Lycopersicon esculentum* Mill plants and *Daucus carota* L., root discs (Krieg and Holt, 1984; Holt *et al.*, 1994). In the need of efficient vectors for higher plants we are isolating indigenous *A. tumefaciens* strains from different localities of Pakistan. Isolation and characterization (morphological and biochemical) of 62 *A. tumefaciens* strains from plants of Punjab, Pakistan has been described earlier (Qazi and Hasnain, 1992). Physiological characterization of these strains, including metallic salts as well as antibiotics resistance/ sensitivity profile, growth responses to temperature and pH, is being described here.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Sixty two *A. tumefaciens* strains, isolated from different sources and localities (Table I) were used (Qazi and Hasnain, 1992). The bacterial cultures were generally grown at 28 ± 1°C. For growth of bacterial cultures potato dextrose agar (Lelliot and Stead, 1987) and potato agar (Gerhardt *et al.*, 1994) media were used. Different physiological experiments were performed on the *A. tumefaciens* strains to have a picture of their physiological attributes.

Resistance to metallic salts

For the selection of the metallic salts resistance markers medium was supplemented with the different metallic salts. The salts of following metals were used: 1) Ba ($BaSO_4$) 250 μg ml^{-1}, 2) Co ($CoCl_2$) 250 μg ml^{-1}, 3) Cr (Cr_2O_3) 50 μg ml^{-1}, 4) Cu ($CuSO_4$) 200 μg ml^{-1}, 5) Fe ($FeCl_3$) 250 μg ml^{-1}, 6) Mn ($MnSO_4$) 500 μg ml^{-1}, 7) Mo (Na_2MoO_4) 500 μg ml^{-1}, 8) Ni ($NiSO_4$) 200 μg ml^{-1}, 9) Pb ($PbNO_3$) 250 μg ml^{-1}, 10) Sn ($SnCl_2$) 200 μg ml^{-1}, 11) Zn ($ZnSO_4$) 350 μg ml^{-1}. Bacterial strains were streaked on the selective plates of the above mentioned metallic salts. These plates were incubated at 28 ± 1°C and results were recorded after 48 hours of incubation.

Table 1: **List of the parent plants, from which *Agrobacterium tumefaciens* strains were isolated and the different ecological and temporal regions of Punjab, Pakistan, in which these plants were growing.**

Sr. No.	Parent plant	Family	Strain	Locality
1.	*Acacia nilotica*	Mimosaceae	AN1, AN2	Murree
2.	*Acacia modesta*	Mimosaceae	AM1	Rai Wind
3.	*Bombax ceiba*	Bombacaceae	BC1, BC2	Lahore
4.	*Broussonetica papyrifera*	Moraceae	BP1, BP2	Islamabad
5.	*Cassia fistula*	Caesalpinanceae	CF1, CF2, CF3	Lahore
6.	*Cedrela toona*	Meliaceae	CT1	Lahore
7.	*Cedrus deodara*	Piaceae	CD1, CD2	Bhoor Ban
8.	*Cedrus deodara*	Piaceae	CD3	Islamabad
9.	*Celtis caucasia*	Ulmaceae	CC1, CC2	Lahore
10.	*Diospyros embryopteris*	Ebenaceae	DE1	Lahore
11.	*Erythrina suberosa*	Fabaceae	EI1, EI2, EI3	Lahore
12.	*Ficus virens*	Moraceae	FV1	Kala Shah Kaku
13.	*Fraxinus hookeri*	Oleaceae	FH1, FH2	Bhoor Ban
14.	*Hamelia patens*	Rubiaceae	HP1	Lahore
15.	*Mangifera indica*	Anacardiaceae	MI1	Lahore
16.	*Mangifera indica*	Anacardiaceae	MI2, MI3, MI4	Islamabad
17.	*Melia azedarach*	Meliaceae	MA1, MA2, MA3, MA4	Renala Khurd
18.	*Melia azedarach*	Meliaceae	MA5, MA6	Murree
19.	*Morus alba*	Moraceae	Ma1, Ma2	Charra Pani
20.	*Morus alba*	Moraceae	Ma3, Ma4, Ma5, Ma6	Murree
21.	*Morus serrata*	Moraceae	MS1, MS2	Murree
22.	*Phyllanthus emblica*	Euphorbiaceae	PE1	Lahore
23.	*Pinus roxburghii*	Pinaceae	PR1, PR2	Rawalpindi
24.	*Polyalthia longifolia*	Euphorbiaceae	PL1	Lahore
25.	*Pongamia pinnata*	Papilionaceae	PP1, PP2, PP3	Lahore
26.	*Psidium guajava*	Myrtaceae	PG1	Rai Wind
27.	*Psidium guajava*	Myrtaceae	PG2	Manga Mandi
28.	*Punica granatum*	Myrtaceae	Pg1, Pg2	Rawalpindi
29.	*Putrajiva roxburghii*	Euphorbiaceae	Pr1	Lahore
30.	*Salix tetrasperma*	Salicaceae	ST1, ST2, ST3, ST4	Lahore
31.	*Sapindus mukorossi*	Sapindaceae	SM1	Wah Cantt
32.	*Suaeda fruticosa*	Chenopodiaceae	SF1	Manga Mandi
33.	*Termanalia catappa*	Meliaceae	TC1, TC2	Rawalpindi

Antibiotics resistance profiles

For the selection of the antibiotic resistance markers, after autoclaving, medium was supplemented with 50 $\mu g\ ml^{-1}$ kanamycin (Km), 5 $\mu g\ ml^{-1}$ chloramphenicol (Cm), 300 $\mu g\ ml^{-1}$ ampicillin (Ap), 100 $\mu g\ ml^{-1}$ carbenicillin (Cb), 25 $\mu g\ ml^{-1}$ tetracycline

(Tc), after autoclaving the medium. Bacterial strains were streaked on these selective plates and incubated at 28±1°C. Results were recorded after 48 hours of incubation.

Effect of temperature on bacterial growth rate

A. tumefaciens strains were normally grown at 28±1°C. The growth rate of *A. tumefaciens* strains was determined at 28° and 37°C after different intervals of time. From overnight cultures inoculum was given to 50 ml of potato dextrose broth and incubated at 28° and 37°C with 150 rpm (revolution per minute) shaking. Samples were drawn periodically (after 2, 4, 6, 8, 12, 24, 28, 32 hours) and optical density was monitored on spectrophotometer (Model S200D, R&M Marketing, England) at 600 nm.

Effect of pH on bacteria

From overnight culture in potato dextrose broth (pH 6.5) inoculum was given to 50 ml of prewarmed potato dextrose broth, adjusted to different pH levels (pH levels 4, 5, 6, 7, 8 and 9), and incubated at 28±1°C with 150 rpm shaking. After 24 hours bacterial growth was monitored at 600 nm (spectrophotometer S200D, R&M Marketing, England).

RESULTS

Strains were characterized physiologically by exploring the metallic salts and antibiotics resistance/ sensitivity profiles, by comparing growth curve at different temperatures (28° and 37°C) and by monitoring growth rate at two different temperatures (28° and 37°C) with various pH levels (4, 5, 6, 7, 8, 9). All strains exhibited individual as well as similar responses reflecting its characteristics and attributes.

Metallic salts resistance/sensitivity profiles

The metallic salts resistance/sensitivity profiles revealed that majority of strains could tolerate metallic salts. All strains fall into 15 groups or clusters, isolated either from the same or different biotopes, depending upon their metallic salts resistance/sensitivity (Table II). The metallic salts profiles showed that all isolates could tolerate Mn (500 μg ml^{-1}, Pb (250 μg ml^{-1}) and Zn (350 μg ml^{-1}) in the medium, while all strains showed sensitivity to Cr (50 μg ml^{-1}) salt (Table II). After these Ba (61 strains), Mo (59 strains) and Fe (55 strains) salts were tolerated by majority of strains. Out of 62, 26 strains showed resistance to majority of (10 out of 11) salts tested and showed sensitive behavior to Cr salt. Next to Cr, most of the strains showed sensitive behavior for Ni (27 strains) and Co (21 strains) salts (Table II).

Antibiotics resistance/sensitivity profiles

Antibiotics (Ap, Cb, Km, Sm and Tc) resistance/sensitivity spectrum of all the strains was worked out. The strains which were exhibiting similar antibiotic resistance/sensitivity profiles were associated together. These strains fall into 10 groups on the basis of specific resistance/ sensitivity attributes for different antibiotics (Table III). The antibiotics resistance/ sensitivity spectrum revealed that different strains showed similarities as well as differences in the antibiotics resistance markers. The experimental data revealed that most of the strains could tolerate ampicillin (39 strains)

Table II: Results of metallic salts resistance/sensitivity profiles of *Agrobacterium tumefaciens* isolates.

Metallic salts concentrations			Strains														
			AN1 AN2	AM1.BC1.CF2.CT1. CC1.CC2.DE1.EI1. EI3.FV1.HP1.MI1. MI3.MA4.Ma1.Ma2. PR1.PR2.PL1.PP1. PG1.Pg2.Pr1.SM1. SF1	BC2.CF1. CF3.MI2 MI4.MA5. MA6.PE1. ST4.TC1, TC2	BP1	BP2 MA1 MA2	CD1	CD2 PG2	CD3 Ma6 ST1 ST2	FH1	FH2 ST3	MA3 PP2 PP3	Ma3 Ma5	Ma4	MS1 MS2	Pg1
1.	$Ba(BaSO_4)$	250μg ml^{-1}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
2.	$Co(CoCl_2)$	250μg ml^{-1}	-	+	+	-	-	-	-	-	-	+	-	-	-	-	+
3.	$Cr(Cr_2O_3)$	50μg ml^{-1}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4.	$Cu(CuSO_4)$	200μg ml^{-1}	-	+	+	+	-	+	+	+	-	-	-	-	-	-	-
5.	$Fe(FeCH_5O_7)$	250μg ml^{-1}	+	+	+	-	+	+	+	+	+	+	-	-	-	+	-
6.	$Mn(MnSO_4)$	500μg ml^{-1}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7.	$Mo(Na_2MoO_4)$	500μg ml^{-1}	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+
8.	$Ni(NiSO_4)$	200μg ml^{-1}	+	+	+	-	-	+	+	+	-	+	+	-	-	-	-
9.	$Pb(PbNO_4)$	250μg ml^{-1}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10.	$Sn(SnCl_2)$	200μg ml^{-1}	+	+	-	-	-	-	-	+	-	-	-	-	+	+	-
11.	$Zn(ZnSO_4)$	350μg ml^{-1}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ = Resistant
- = Sensitive

carbenicillin (41 strains) and kanamycin (41 strains) (Table III). While most of the strains were sensitive to streptomycin (48 strains) and tetracycline (53 strains) (Table III).

Temperature effects on bacterial growth

The results of the experiment reflect that in majority of the cases *A. tumefaciens* strains, initially, showed better growth response at 37° as compared to growth rate at 28°C (Fig.1). In few cases, the strains, AN1, AN2, FH1, MA5, MA6, MS1 and MS2, isolated from colder areas showed better growth response at 28°C (Fig.1). The experimental data showed that growth rate of the strains, in majority of cases, was almost equal after 24 hours of incubation. The strains isolated from colder areas had slow growth rate, except Ma1 and Ma2, and generally showed similar growth response at 28° and 37°C (Fig.1). Some of the strains isolated from different ecological and temporal regions showed similar growth rate. However some of the strains showed minor differences in growth rate, i.e., some strains showed almost equal growth rate at 28° or 37°C, while some strains showed very rapid growth rate at 37°C from 4 to 12 hours as compared to 28°C (Fig.1).

Strains AN1, AN2, FH1, MA5, MA6, MS1 and MS2 exhibiting better growth response at 28°C. These strains were isolated from colder areas (Table I), whereas none of the strain isolated from warmer areas gave better growth response at 28°C (Fig.1). AN1, AN2, MA5 and MA6 had a higher growth rate at 28°C as compared to 37°C even after 32 hours of incubation. While FH1, MS1 and MS2 showed initially better growth rate at 28°C which later on succeeded by the growth rate at 37°C and after twelve hours of incubation these strains showed better growth at 37°C (Fig.1). Some strains, CD1, CD2, FH2, Ma1, Ma2, Ma3, Ma4, Ma5 and Ma6, which were also isolated from colder areas did not show better growth rate at 28°C (Fig.1). However better growth rate was observed in some strains (Ma1, Ma2, Ma4, FH2 and Ma6) at 28°C at different time intervals. In case of Ma1, Ma2 and Ma4 better growth rate was observed at 28°C after 12 hours of incubation, which was again superseded by growth response at 37°C in strains Ma2 and Ma4. While FH2 and Ma6 gave better growth rate after 32 hours of incubation at 28°C (Fig.1). BC1, BC2, HP1, Ma1, Ma2, PL1 and PG2 showed better growth rate at 37°C, approximately, from 4 to 8 hours of incubation and later on these strains showed higher growth rate at 28°C after 8 hours of inncubation. However in Ma2 and PG2 growth rate at 28°C was again dominated by the growth response at 37°C after 32 hours (Fig.1). In majority of cases, after 32 hours, the growth rate was almost equal or even in some cases, i.e., AM1, CF2, CT1, CC2, FV1, FH1, MI4, MA1, Ma6, PE1, PR2, PG1, Pg2, Pr1 and TC1, was better at 28°C (Fig.1). Generally growth at 28°C and 37°C, increased rapidly upto 12 hours, except MA5, MA6 annd Pg1 which started growing after 8 hours (at both temperatures) and Ma1 which showed slow growth rate at 28°C (Fig.1). These results depict that majority of *A. tumefaciens* strains initially showed rapid growth rate at 37°C but after 24 to 32 hours this growth rate is almost equal at both temperatures (28°C and 37°C) and even in nsome cases it was better at 28°C (Fig.1).

Effects of pH on bacterial growth

The bacterial growth with different pH levels at 28° and 37°C showed that all *A. tumefaciens* strains grow better at pH 6 and 7 and with maximum growth was observed at pH 6. All strains, with few exceptions, gave higher population density at pH 8 and 9

Table III: Results of antibiotics resistance/sensitivity profiles of *Agrobacterium tumefaciens* isolates.

Antibiotic concentrations	Strains									
	AN1,CD2,FH1, FH2,MH1,MA2, Ma3,Ma5,Ma6, MS1,MS2,PE1 PR2,PL1,PG1, SF1	AN2,BC1, CT1,HP1, MA4,PP3, SM1	BP2,CD1, EI2,EI3, Ma4,TC1	BC2,CD3,CC1, CC2,PG2,Pg1, ST1,ST2	EI1	CF1,Ma1 Ma2	CF2	AM1,BP1,CF3, MI2,MI3,MI4, MA1,MA3,MA5, MA6,PR1,Pg2, ST3,ST4	DE1,FV1, PP1,PP2 Pr1	TC2
1. Ampicillin 300 μg ml^{-1}	+	+	+	-	+	+	-	-	+	-
2. Carbenicillin 100 μg ml^{-1}	+	+	-	+	-	+	+	-	+	+
3. Kanamycin 50 μg ml^{-1}	+	+	-	+	-	+	+	-	+	+
4. Streptomycin 300 μg ml^{-1}	-	+	-	-	+	-	+	-	+	-
5. Tetracycline 25 μg ml^{-1}	-	-	-	-	+	+	-	-	+	-

+ = Resistant
- = Sensitive

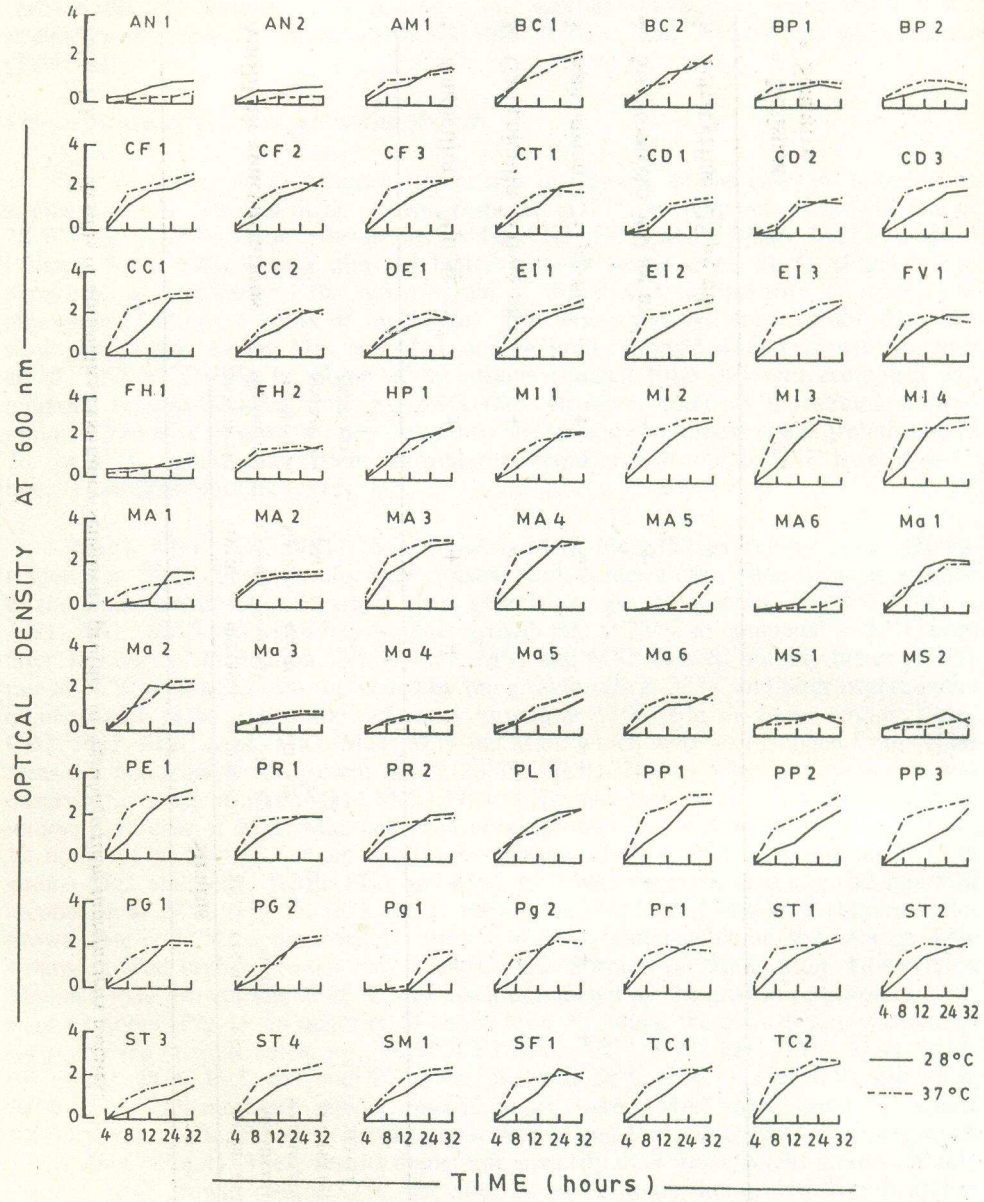

Fig. 1: Growth curves of *Agrobacterium tumefaciens* strains at 28° and 37°C.

as compared to cell density at pH 4 and 5. This shows that mostly *A. tumefaciens* survived better in extreme alkaline pH as compared to extreme acidic pH (Fig.2). Similar results were recorded as were observed when bacterial strains were grown at two different temperatures (28°C and 37°C) i.e., higher growth density was observed in all pH levels at 37°C as compared to 28°C, except for some strains (AN1, AN2, MA5, MA6, Ma1, Ma2, MS1 and MS2) which were isolated from colder areas as well as few strains (AM1, BC1, MI1, MA1, Pg2, SM1 and SF1) isolated from warmer areas (Table I, Fig.2).

AN1, AN2, MA5 and MA6 showed better growth in all pH levels at 28°C, whereas at 37°C very poor growth rate was recorded (Fig.2). The strain, MI3, showed almost similar growth pattern at 28°C and 37°C, however higher growth rate was observed at 37°C. MI3 showed slightly higher growth rate at 28°C with pH 7 as compared to pH 7 at 37°C, but at pH 8 and 9 growth rate again increased at 37°C (Fig.2). Some of the strains which were isolated from warmer areas (Table I) also showed higher growth rate at 28°C in all pH levels as compared to growth rate at 37°C. Whereas some strains, CD1, CD2, FH1, FH2, Ma3, Ma4, Ma5 and Ma6, isolated from colder areas (Table I) gave better growth response at 37°C at all pH levels (Fig.2). At pH 4 all strains showed either poor or no growth response, while at pH 9 somewhat better growth rate was noticed. These results reflect that these *A. tumefaciens* strains could grew better in extreme alkaline pH (9) as compared to extreme acidic pH (4). However these strainns gave maximum growth rate at pH 6 at both temperatures (28°C and 37°C).

DISCUSSION

The physiological characterization revealed that strains shared many aspects and attributes even though isolated from different ecological and temporal regions (Table I). The metallic salt resistance/ sensitivity profile revealed that many isolates showed similar characters, however some strains exhibited individual attributes which were different from the rest of the strains. Differences were observed in strains which were isolated from same parent trees growing in same ecological and temporal region (Table I), and vice versa i.e., similarities were exhibited by strains isolated from differet parent plants growing in different biotope (Table II, III). It has been observed that metallic salts effect the metabolism and growth of the bacterium (Jonas *et al.*, 1984) and decreased growth at elevated levels of metabolic salts have been attributed to impaired metabolic activities and reduced cell expansion (Wood and Wang, 1987). Some bacteria which survive the higher concentration of metallic salts could be genetically adapted or their survival could be due to the composition of the medium, because metallic salt toxicities to microbes also depends on the growth medium (Hughes and Poole, 1989). Bacterial growth medium components form complexes with metallic salts and remove them from solution, thus reduces its apparent concentration in the medium (Guffanti and Hicks, 1991). For Cr (50 μg ml^{-1}) metallic salt all strains showed negative growth response which might be due to impaired metabolic activities and reduced cell expansion of the strains. While all strains exhibited tolerance for metallic salts of Mn (500 μg ml^{-1}), Pb (250 μg ml^{-1}) and Zn (350 μg ml^{-1}) in the growth medium (Table II). All strains tolerated these metallic salts which might be due to the presence of resistance markers on the plasmids of these strains. Lead and zinc are toxic salts and tolerance of all strains to these salts indicate the presence of these salts in the environment, and is of serious health concern. Out of 62 strains, 26 strains exhibited resistance to majority of salts tested. Similarly for antibiotics resistance/ sensitivity spectrum different strains

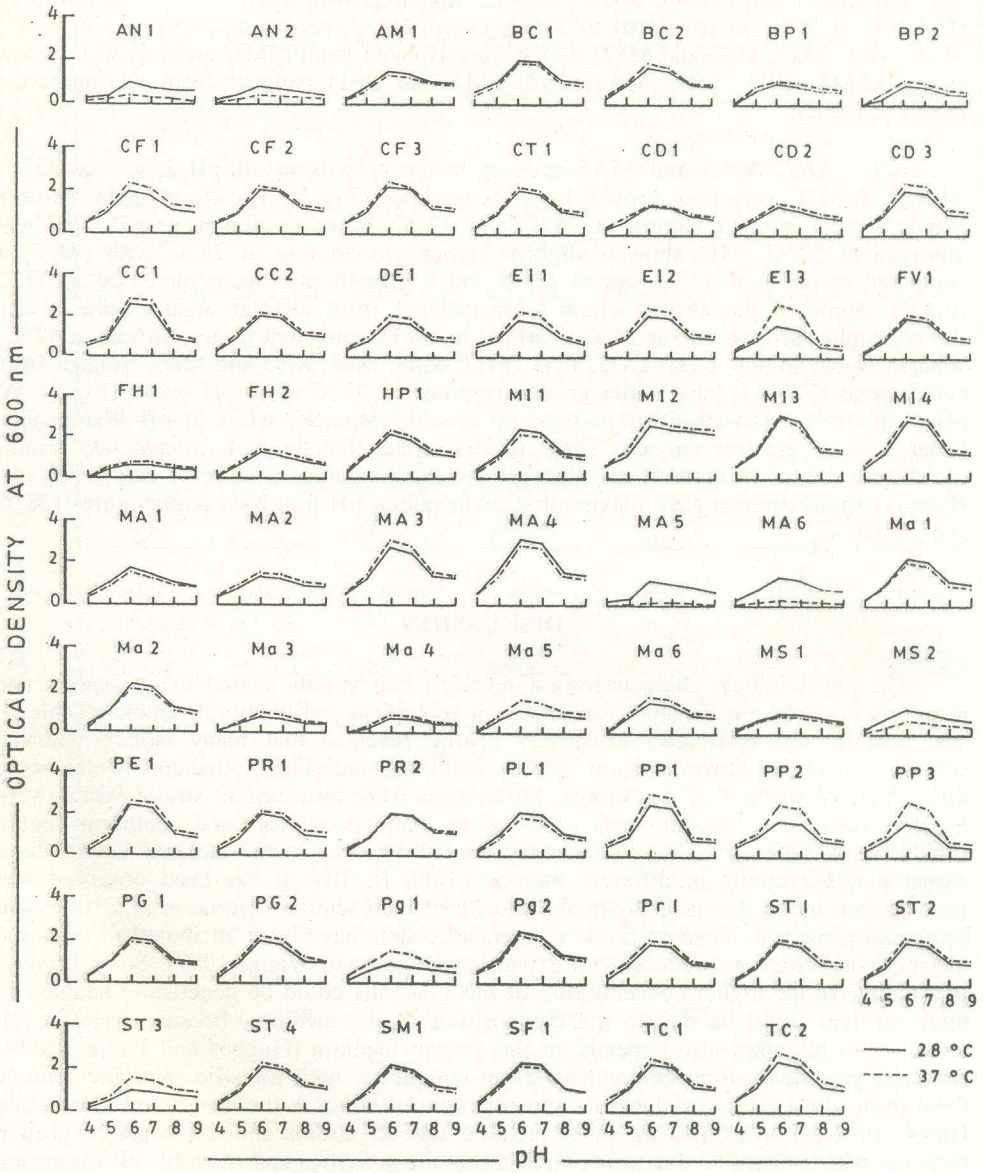

Fig. 2: Effects of different pH levels on the growth response of *Agrobacterium tumefaciens* strains.

exhibited individual as well as similar and common responses (Table III). Antibiotics resistance determinants may play a role in cellular metabolism other than the protection of the host. Majority of strains showed sensitive attribute to Tc (53 strains) and Sm (48 strains). Whereas many of them could bear Ap (39 strains), Cb (41 strains) and Km (41 strains). Fourteen strains showed sensitive behavior to all the antibiotics tested in the experiments (Table III). Expression of the antibiotics resistance genes some times may depend on the cytoplasmic background i.e., in some cases the gene is not expressed because it lacks a functional promoter (Davies, 1992). Therefore it might be possible that strains which showed sensitive behavior for some antibiotics might had resistance marker for that particular antibiotic but due to lack of functional promoter that gene was not expressed. However these results showed that many strains had resistance markers for various metallic salts and antibiotics, and these results could be utilized in selecting the transformed plants or tissues with that particular strains.

There are environmental factors that affect the physiology and chemistry of the living organism e.g., temperature and pH. Growth and metabolism of bacteria are related with temperature. Either at high (Patterson and Giltespie, 1972) or low (Inniss and Ingraham, 1978) temperature one or more reactions become rate limiting due to inability of cells to adjust to thermally induced changes (Araki, 1991). For growth and each biochemical reaction there is optimum temperature for a bacterial strain. Like temperature or other environmental conditions, pH also exerts noticeable influence on growth of bacteria. Some bacteria can survive in an environment with an extreme acid pH (*Thiobacillus thiooxidants*, *Acetobacter* sp. etc.), while some grow in extremely alkaline pH. For most bacteria, however, the optimum pH for growth lies between 6.5 and 7.5. A sever change in the pH can bring the growth of a microbial population to a halt. Bacterial growth experiments at different temperatures and at different levels of pH were almost similar in some aspects. Optimum temperature for *A. tumefaciens* growth ranges from 25° to 28°C (Holt *et al.*, 1994). The results of these experiments showed that majority of strains isolated from warmer areas showed rapid growth rate at 37°C (Fig.1, 2). According to Hooyakaas (1988) *A. tumefaciens* biotype I strains can grow at 37°C. While some of the strains CD1, CD2, FH2, Ma1, Ma2, Ma3, Ma4, Ma5 and Ma6, which were isolated from colder areas, initially, also showed somewhat better growth at 37°C. These strains were isolated from trees growing along road side in sunny area. This might be a factor in acclimatizing these strains to warmer temperatures. Whereas AN1, AN2, FH1, MA5, MA6, MS1 and MS2, isolated from colder areas showed better growth response at 28°C (Fig.1) and these strains were isolated from trees growing away from road side in a shady area. This showed that not only the temperature of biotope effect the characteristics of a strains but locality of the strain also effects its physiological characters. The experiments performed at different levels of pH also showed that all these strains had a specific affinity for pH 6 irrespective of temperature i.e., all strain showed maximum growth peek at pH 6 when grown at 28° or 37°C. Cell wall components of bacteria are important in determining the pH tolerance of a bacterium. Generally alkalophilic bacteria have more acidic amino acids and sugars in their cell walls (Aono *et al.*, 1993). The strains isolated from warmer areas showed higher growth rate at 37°C except few strains (AM1, BC1, MI1, MI3, MA1, Pg2, SM1 and SF1) which showed better growth response at 28°C (Fig.2). These results were similar to results observed at temperature effect on growth rate. Here the optical density was measured after 24 hours of incubation and growth curves showed that these strains had a higher growth rate at 28°C after 24 hours of incubation (Fig.1). The pH affect on growth rate exhibited that all strains could grew better in extreme alkaline pH (9) as compared to extreme acid pH (4). According to Guffanti and Hicks (1991) bacteria which can grow well in alkaline pH range 9-11, maintain an internal pH 1-2 units lower

than external pH. The bacterial cells also have the capacity to regulate the cytoplasmic pH (pH_{in}) at around neutrality regardless of their external pH (pH_{out}) (Booth, 1985). It can be concluded that bacterial strains were growing in the medium by shifting the pH towards their optima.

The ongoing discussion surmise that *A. tumefaciens* strains from different sources and localities may exhibit similar antibiotic/metallic salt profiles whereas strains from the same source may display variation in resistance/ sensitivity spectrum to antibiotics and metallic salts. *A. tumefaciens* strains from colder areas prefer 28°C for their optimum growth and those belonging to warmer regions prefer 37°C. Previously optimum temperature reported for *A. tumefaciens* growth is 25°-28°C (Holt *et al.*, 1994). Most probably those strains were isolated from colder regions. Reports on tumorigenesity of *A. tumefaciens* state that above 32°C virulence of the strains as well as conjugal transfer of Ti plasmid are hampered (Jin *et al.*, 1993). We anticipate that strains from warmer areas, which grow better at 37°C, might also show virulence as well as conjugal transfer of Ti plasmid at relatively higher temperature and further studies might reveal additional aspects in understanding of this bacterium.

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TERATOGENICITY OF DICHLORVOS IN CHICK EMBRYOS

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Abstract: Dichlorvos (DDVP), an organophosphorus insecticide, was tested for embryotoxic and teratogenic effects in chick. Different aqueous concentrations of Dichlorvos (6.25, 12.5, 25.0 and 50.0 µg/egg) were injected, in yolk sac of egg, before incubation. Embryo recoveries were made at day 3, 7 and 15 of incubation. At day 3, dose dependent developmental anomalies including undifferentiated and unfolded brain parts, patent neurocoel, incomplete somite formation, twisted spinal cord, abnormal heart position and under-developed eyes were found. At day 7, morphological, anatomical and morphometric studies revealed concentration dependent adverse effects of the insecticide. The developmental defects were reduction in CR length, microcephaly, microphthalmia, short beak, twisted spinal cord, micromelia, ectopia cardis and short and thick neck. Developmental anomalies in 15 day embryos were also found dose dependent, such as dwarfism, monopia, micromelia, amelia, turned beak, gastroschisis and ectopia cardis. The present study indicates that DDVP is potentially dangerous to avian development.

Key words: Developmental defects, organophosphates, chick embryos.

INTRODUCTION

Besides increased crop production possibilities, environmental and health side effects of the insecticides has rendered difficulties for living creatures (Wild, 1975), especially since many pesticides have been found to be mutagenic, carcinogenic and teratogenic (Axelson and Sandell, 1974; Wild, 1975; Shirasu *et al.*, 1976; Eto *et al.*, 1980; Gomez-Arroyo *et al.*, 1985, 1987, 1988). Acute pesticide poisonings are an important cause of morbidity and mortality. Although data are inadequate to quantify, with certainty, the extent of the problem, recent estimates suggest that each year worldwide, there are 3 million acute severe pesticide poisonings with 220,000 deaths (WHO report, 1986, 1990). Much of this burden is borne by developing countries, where 99% of fatal pesticide poisonings occur and where 25 million episodes of intoxication occur annually among agricultural workers alone (Jeyaratnam, 1985, 1990).

The organophosphorus compounds represent a broad class of insecticides and are widely used for the eradication of assorted household and agricultural pests. These compounds have been considered relatively safe, especially in the sense of these being biodegradable and thus non-cumulative (Durham and Williams, 1972; Jennings *et al.*, 1975; Harbison, 1978). Unfortunately, the effective application of these compounds is confounded by the frequent intoxication of beneficial non-target organisms, including

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numerous documented cases of human fatalities. Contributing to this selectivity is the broad range of sensitivity of different organisms to acute poisoning by OP compounds, with fish being relatively resistant and rodents and birds very sensitive (Kemp and Wallace, 1990).

When these organophosphorus insecticides are used in high concentrations and over a long period of time, they can prove to be potentially teratogenic and embryotoxic in mammals (Rosenstock *et al.*, 1991; Asmatullah, 2000). OPs are anticholinesterase agents and are among the most widely used neurotoxic pesticides. If humans or animals are exposed to OPs, their nervous system will always be affected more or less seriously (Papp and Desi, 1998). Many reports are available on OPs causing significant inhibition of the AchE *in vivo*. In view of the complexity of the nervous system, particularly the central nervous system (CNS), *in vivo* models have an important role in elucidating both the potential for, and the mechanism of, neurotoxic insults (Atterwill and Walum, 1989).

Dwivedi and Mathur (1999) tested different concentrations of fenvalerate, cypermethrin, dichlorvos and monocrotophos against the eggs of *Spodoptera litura* (Lepidoptera) for oviducal effect. The higher concentration (700 ppm) of fenvalerate and (500 ppm) of cypermethrin, dichlorvos and monocrotophos showed significant results and led to failed hatching and increased mortality to 100%. Nuvan mixed with piperonyl butoxide (PB) and Decis when studied, proved to be highly toxic to snail and was a strong inhibitor of AchE activity (Tripathi and Agarwal, 1998). In many other studies, the harmful effects of organophosphorus compounds, especially to avian embryos have been shown quite convincingly (Khera, 1966; Khera and Bedok, 1967; Meiniel *et al.*, 1970; Meiniel, 1976; Sternberg, 1979; Wyttenbach and Thompson, 1985). In most of these studies, it has been shown that even very small quantities of OPs induced gross embryonic malformation which included microcephaly, eye cataracts, ascites, hepatic degeneration, micromelia, ectrosyndactyly, microphthalmia, anophthalmia and many other musculo-skeletal abnormalities.

Virtually all the known chemical agents including insecticides have at one time or another been known to cause injury or death in man. Above mentioned studies have indicated that organophosphorus insecticides are toxic for non-target organisms and are also embryotoxic and teratogenic. Thus, the present study was planned to evaluate the embryotoxic and teratogenic potential of DDVP in developing chicks.

MATERIALS AND METHODS

Fresh eggs (White leghorn breed) were purchased from Government Poultry Farm, Lahore. The eggs were divided into 6 groups. Four groups were treated with different concentrations of dichlorvos (DDVP).

All the eggs in each group were selected randomly without considering the size and colour of eggs. Eggs were cleaned with a piece of cotton soaked in alcohol, and were marked according to their respective groups. DDVP was available as emulsifiable concentrate with trade name, Neovas 50 EC (ACMES International). The concentration of DDVP used in the study ranged from 6.25 to 50 μg/egg. These concentrations were prepared by dissolving insecticide in distilled water in such a way that 0.1 ml contains desired concentration.

A small window was made in the shell of each egg except control group eggs, with the help of a scalpel, provided shell membrane was not ruptured. 0.1 ml of each concentration of aquous solution of DDVP was injected, into the yolk sac of the eggs of respective groups, with microapplicator. In case of vehicle control group, only 0.1 ml distilled water was injected in each egg. All these treatments were applied in sterilized conditions. Following injection, the hole in the egg shell was sealed with liquid paraffin wax.

The embryos recovered on day 3 of incubation were fixed in freshly prepared Bouin's fixative and whole mounts of embryos were prepared on day 7 and 15 of incubation, 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. These observations included the studies of developmental conditions of brain, eyes, ear, limbs, beak etc. These organs were studied with the help of magnifying lens and with naked eye depending upon the size of the embryo. The data were analyzed by using student's 't' test. The embryos from 3rd day recovery were microphotographed with camera fitted microscope and embryos from day 7 and day 15 of incubation were macrophotographed by using camera, equipped with telephoto lens.

RESULTS

Three days incubation

Embryos of control and vehicle control group had CR length 6.75±0.95 mm and 5.55±0.02 mm, respectively (Table I) and had well developed embryonic parts (Fig.1a). The CR length of embryos was reduced dose dependently in insecticide treated groups (Table I). The delay in differentiation of different organs was very obvious (Table I, Figs.1b, 2a).

Table I: Developmental anomalies induced by different concentrations of DDVP in 3 days old chick embryos.

Dose (µg/egg)	CR length (mm±S.D)	Brain (%)	Spinal cord (%)	Eyes (%)	Cardiac position (%)	Limb buds (%)	Somites (%)
Control	6.75±0.95 (n=10)	Normal & distinct	Normal	Normal with lens vesicle prominent	Normal	Prominent limb buds	Quite distinguishable somite pairs
Vehicle control	5.25±2.02 (n=10)	Under developed (50)	Under-developed (50)	Unidentifiable (50)	Unidentifiable (25)	Under-developed (50)	Undistinguish-able (25)
6.25	4.02±1.50** (n=10)	Small and undifferentiated brain parts (50)	Twisted (15). Unidentifiable (15)	Small (25). Unidentifiable (25)	Unidentifiable (30)	Small & under-developed (50)	Primitive in development (70)
12.5	3.25±2.22* (n=10)	Small and under-developed (100)	Twisted (50). Unidentifiable (50)	Unidentifiable (75)	Ectopic heart (25). Unidentifiable (75)	Unidentifiable (75)	Primitive in development (75)
25	4.75±2.77 (n=10)	Unfolded brain parts (25). Unidentifiable (60)	Twisted (60). Unidentifiable (25)	Unidentifiable (50)	Unidentifiable (50)	Unidentifiable (70)	Primitive in development (50)
50	3.57±1.86* (n=10)	Partially differentiated brain parts (30). Undifferentiated brain parts (50)	Under-developed (25). Unidentifiable (50)	Under-developed (80)	Unidentifiable (80)	Not prominent (80)	Primitive in development (80)

Significant difference against controls * P 0.05; ** P 0.01.

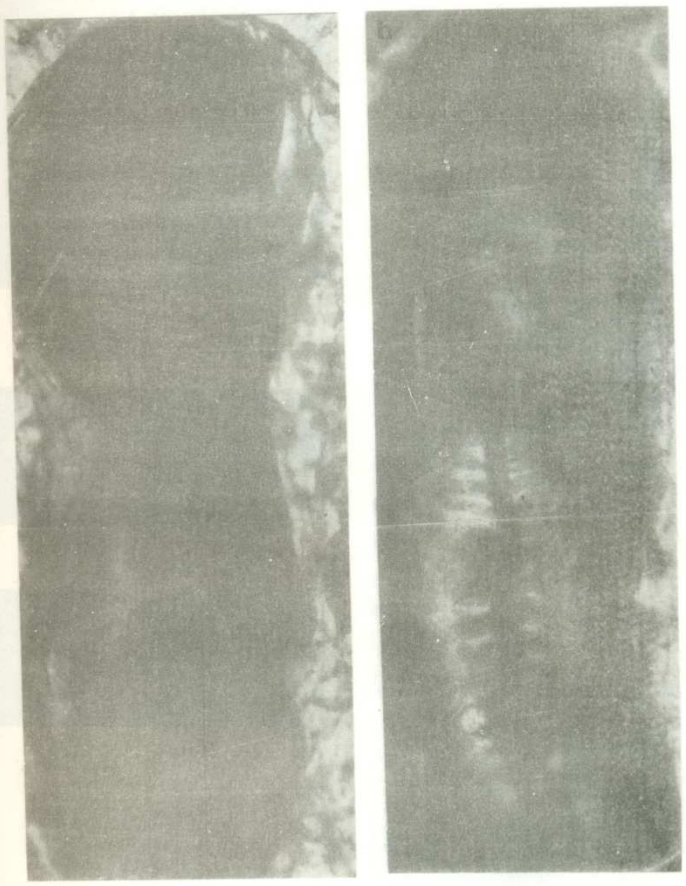

Fig. 1: Microphotographs of 3 days chick embryo: a, control embryo with normally differentiating body parts; b, an embryo from 6.25 µg/egg dose group with abnormally differentiated body parts including brain (arrow head) neural tube (p) and somites (s).

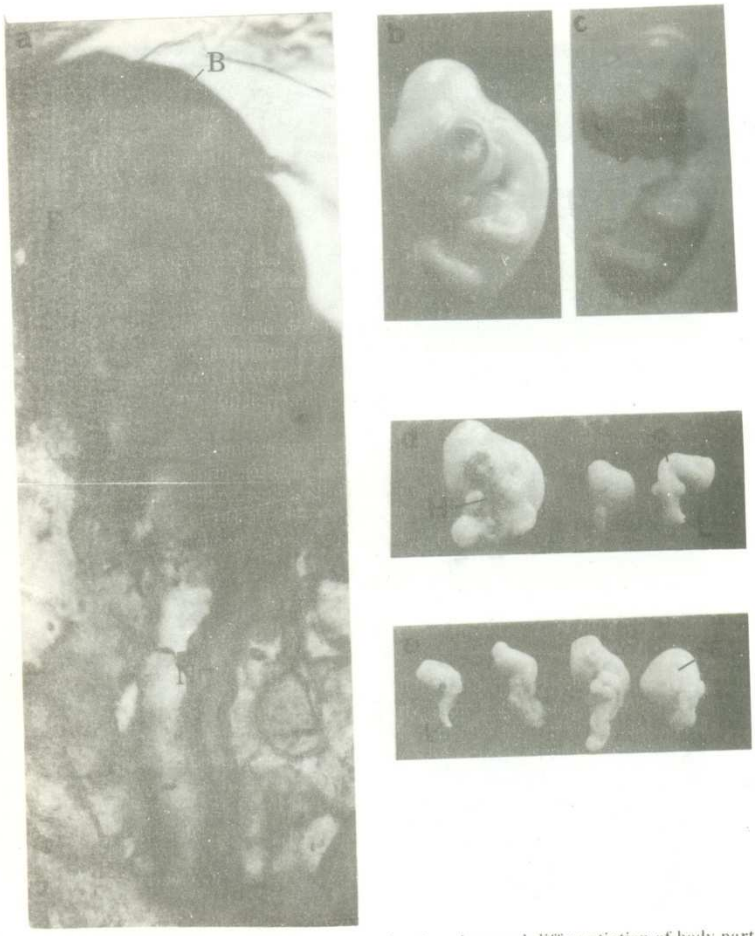

Fig. 2: a, an embryo from 50 µg/egg group showing abnormal differentiation of body parts including brain (B), eye (E) and patent neural tube (N); b-c, macrophotographs of seven days control and vehicle control chick embryos with normal development; d, embryos from 12.5 µg/egg dose group; and e, embryos from 50 µg/egg dose group with abnormally developed organs including ectopia cardis (H), hind limb (L), twisted spinal cord (S), small eye (SE).

Table II: Developmental anomalies induced by different concentrations of DDVP in 7 days chick embryos.

Dose (µg/egg)	Malformed embryos (%)	Resorbed embryos (%)	CR length (mm±S.D)	Head (%)	Eyes (%)	Beak (%)	Limbs (%)	Neck (%)	Cardiac position (%)
Control	00.0	7.69	13.3±1.13 (n=10)	All brain parts distinct	Normal	Normal	Normal, well developed	Normal	Normal
Vehicle control	00.0	00.0	12.75±0.35 (n=10)	All brain parts prominent	Microphthalmia (30)	Short (50	Micromelia (10)	Normal	Normal
6.25	3.125	96.87	9.50±0.50*** (n=10)	Small, brain parts, not distinct (100)	Not developed (100)	Not formed (100)	Not formed (100)	Not prominent (100)	Not prominent (100)
12.5	12.90	87.09	8.73±0.98*** (n=10)	Brain parts distinct but small (80) Normal, well developed (20)	Microphthalmia (75)	Not formed (80)	Not prominent (75), Under developed (25)	Short & thick (75)	Ectopic heart (50)
25	18.75	81.25	6.91±2.26*** (n=10)	Distinct brain parts (40), Small, not distinct (60)	Not formed (60) Microphthalmia (20)	Not formed (100)	Not prominent (80)	Not formed (100)	Ectopic heart (50) Not formed (30)
50	50.0	50.0	5.96±2.99*** (n=10)	Brain parts not distinct (100)	Microphthalmia (20), Undisting-uishable (80)	Not formed (100)	Not prominent (80)	Short & thick (80)	Ectopic heart (20) Not formed (60)

Significant difference against controls *** = P<0.001.

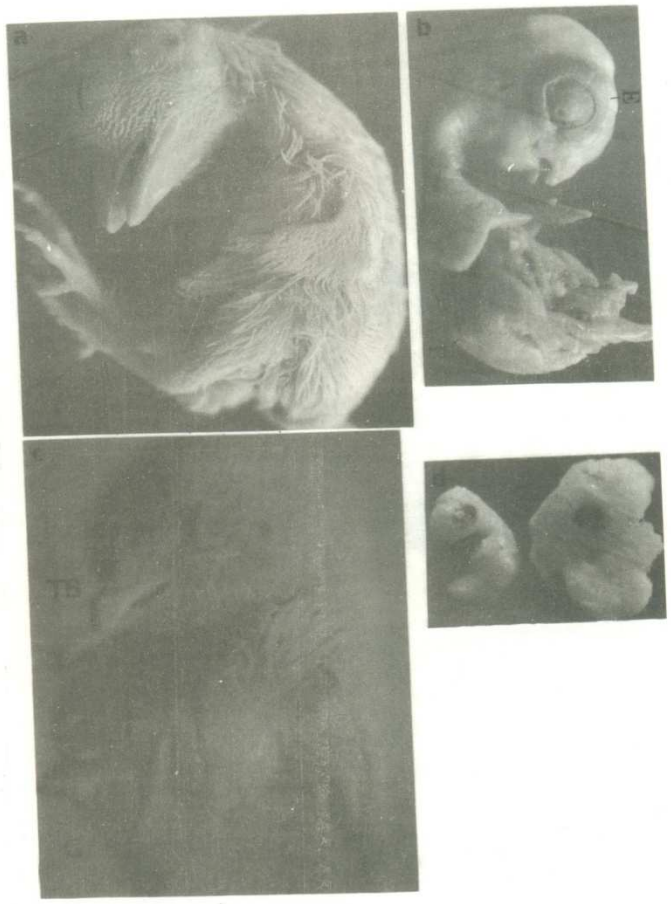

Fig. 3: Macrophotographs of 15 days chick embryos: a, normally developed control; b, a member of vehicle control group with some abnormalities including eye (E) and plumage (P); c, a member of 25 µg/egg dose group with turned beak (TB), gastroschisis (G) and anophthalmia (arrow head); d, resorbed embryos of 50 µg/egg dose group.

Table III: Developmental anomalies induced by different concentrations of DDVP, in 15 days chick embryos, injected before incubation.

Dose (µg/egg)	Malformed embryos (%)	Resorbed embryos (%)	CR length (mm±S.D)	Head (%)	Eyes (%)	Beak (%)	Heart position (%)	Limbs (%)	Plumage (%)
Control	0.00	37.5	43.60±2.88 (n=10)	Microcephaly (20)	Normal, closed	Smaller (20)	Normal	Micromelia (20)	Partially developed (20)
Vehicle control	80.0	10.0	24.39±2.83** (n=10)	Small but distinct brain parts (100)	Opened no eyelids (100)	Short (100)	Normal	Micromelia (90)	Under-developed (90)
6.25	44.44	55.55	6.62±1.37*** (n=10)	Not distinguishable (100)	Not formed (100)	Not formed (100)	Ectopic heart (25), Undistinguish-able (75)	Not formed (100)	Not formed (100)
12.5	11.11	88.88	7.50±1.50*** (n=10)	Microcephaly (100)	Anophthalmia (100)	Not formed (100)	Not prominent (100)	Not formed (100)	Not formed (100)
25	50.0	50.0	9.00±2.48*** (n=10)	Brain parts not distinguished (100)	Right eye is smaller than left (25), Not formed (75)	Not formed (100)	Not prominent (100)	Not formed (100)	Not formed (100)
50	37.5	62.5	8.5±2.12*** (n=10)	Microcephaly (33), Not formed (66)	Left side monopia (33), Under developed (66)	Turned beak (33), Not formed (66)	Not prominent (70), Ectopic heart (30)	Amelia of hind limb (33)	Patchy (33)

Significant difference against controls *= P<0.05; ** P<0.01; *** P<0.001.

Seven days incubation

The control and vehicle control groups embryos typically represented stage 31 described by Hamburger and Hamilton (1951). The CR length of the embryos was 13.3±1.13 and 12.75±0.35 mm, respectively (Table II). The insecticide treated group's CR length was significantly ($P<0.001$) reduced as compared to the controls (Table II). The developmental anomalies were found very severe in all insecticide treated embryos (Table II, Fig.2d,e).

Fifteen days incubation

At this stage, the control group embryos had attained the morphological features of the adult and embryos were at the stage 40, described by Hamberger and Hamilton (1951). The CR length was 43.60±2.88 mm (Table III, Fig.3a).

In case of vehicle group, the CR length was 24.39±2.83 mm which is significantly ($P<0.01$) less than controls (Table III). As compared to control group, embryos of this group had reduced body size and growth, with 10% resorption at the time of recovery (Table III). Head was small but with distinct parts. Eyes were smaller than controls. Beak was shorter, while neck was of normal size. Limbs and plumage were under-developed (Fig.3b).

In treated groups the fetuses were significantly ($P<0.001$) reduced in size (Table III). A higher rate of resorption was noted in all dose groups (Table III, Fig.3d). Some members of a higher dose groups, 25 µg/egg, showed severe developmental defects including twisted beak, patchy plumage, anophthalmia and gastroschisis (Fig.3c).

DISCUSSION

The purpose of the present study was to evaluate the developmental toxic effects of DDVP in avian systems which is comparatively more volatile than most of the organophosphorus insecticides, is highly toxic and rapid acting, having direct inhibitory effect on acetyl- and non-specific cholinesterase and is rapidly absorbed by any route (Jamil, 1989; Lindrigan *et al.*, 1999).

The main observation made during the present investigation is that dichlorvos, injected in chick eggs before incubation, even at low concentrations, produced embryotoxicity and teratogenicity. The developmental anomalies were found at all developmental stages, including 3rd, 7th and 15th day of incubation. The developmental defects observed on day 3, small, undifferentiated and unfolded brain parts, patent neurocoel, incomplete somite formation, twisting of spinal cord, ectopia cardis position and under-developed eyes; on day 7 increased embryo lethality, reduction in CR length, microcephaly, non-distinct brain parts, microphthalmia, short beak, twisting of spinal

cord, micromelia, ectopia cardis defects and short and thick neck and on day 15, dwarfism, monopia, micromelia, amelia, turned beak and ectopia cardis were increased with the increase of dose concentration.

These results are more or less in conformity with earlier reports that organophosphorus insecticides are toxic to embryonic and fetal tissues and can induce teratogenicity in chick. Miscioni *et al.* (1977) have categorized a whole set of abnormalities encountered in chick embryos following malathion treatment. Abnormalities such as micromelia, dwarfism, parrot beak and abnormal feathering were commonly observed and were classified as type I abnormalities. Another set of abnormalities, designated as type II, included defects such as short neck, tibiotarsal arthrogryposis and muscular hypoplasia of the legs. Many other studies have also shown embryotoxic and teratogenic effects of different organophosphorus insecticides in chick embryos (Marliac, 1964; Khera and Bedok, 1967; Greenberg and LaHam, 1969; Meiniel and Autissier-Navarro, 1980; Kitos *et al.*, 1981; Misawa *et al.*, 1981).

The teratogenic effects of diazinon with regard to skeletal development, particularly extremities and vertebrae were examined in chick (Misawa *et al.*, 1982). Inhibited growth of femur, tibia, metatarsi and digits were observed on day 15 following injection of insecticide (0.1 mg diazinon) on day 3 after incubation. In another related study, phosphamidon induced severe dwarfism as well as neural defects including complete agenesis of the eye in the chick embryo (Mufti and Dad, 1977) and abnormalities in the developing heart and kidneys (Mufti and Nasim, 1987).

Garrison and Wyttenbach (1985) showed that white leghorn chicken eggs treated with dicrotophos in a dose ranging from 250 μg – 2 mg/egg administered on day 1, 2, 3 and 4 of incubation, resulted into the production of malformed embryos showing a variety of abnormalities. These abnormalities included general growth retardation, abnormal cranial sense organs, notochordal folding with deformities of the spinal cord, deformities in the neural epiphysis and distention of major blood vessels. Jena and Bhunya (1992) studied the genotoxicity of as organophosphate, monocrotophos, upon chronic exposure in chick *in vivo* test system employing micronucleus bioassay. The induced frequency of micronuclei in the erythrocytes of both bone marrow and peripheral blood was significantly higher than the respective control values, which revealed the genotoxic potential of monocrotophos. Rao *et al.* (1992) suggested that RPR-V, an organophosphate, has teratogenic effects on chick embryos when injected on day 4 of incubation. As the dose was increased, the hatchability decreased and the incidence of deformities increased. Ghosh *et al.* (1998) observed that the survival rate, growth rate and size and the cholinesterase activity significantly declined, while mortality rate and the frequency of abnormalities increased in the Methyl parathion intoxication in chick embryos.

Some investigations on harmful effects of these insecticides on other developing animals have also been done. In most of these studies, it has been shown that very small

quantities of OP insecticides caused severe abnormalities or embryonic malformations. malathion, malaoxon, parathion and paraoxon caused dose dependent development, notochordal defects and reduced growth in African clawed frog (Snawder and Chambers, 1990).

Methamidophos is embryotoxic to mice and like other OPs produces different undesirable side effects including death (Murphy, 1980). Mufti and Nazir (1988) studied the effect of Malathion on mice and found that dose of 1 mg and 3 mg/g BW proved to be lethal for embryos and the viable ones were highly malformed.

Dichlorphos inhibits AChE in the embryos of Japanese quail. This enzyme inhibition leads to retardation of the development, to reduce accumulation of glucose and amino acids in the sub-embryonic liquid and finally to death of the embryo, suggesting that the developmental retardation is due to the restricted supply of glucose and amino acids (Kaltner *et al.*, 1993).

The two principal determinants of anticholinesterase activity for various organophosphorus insecticides are steric hindrance and the electrophilic strength of phosphorus atom. Structure-activity correlation revealed that whereas steric hindrance is the principal factor governing inhibitory potency for rats and hens, the electrophilicity of the phosphorus atom is the principal determinant of anticholinesterase activity in trout (Kemp and Wallace, 1990).

In birds, the mechanisms of teratogenic action appears to be related to diminished embryonic nicotin-amide adenine dinucleotide levels as a result of Kynurenine formamidase inhibition (Seifert and Casida, 1978; Eto *et al.*, 1980) and/or altered levels of available acetylcholine causing neuromuscular blocking effects during development (Landauer, 1975).

All studies, including present one, indicate that in spite of being non-cumulative and biodegradable, these OP insecticides are potentially dangerous to developing embryos even when given at comparatively low concentrations.

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INSECTICIDAL ACTIVITY OF SOME *BACILLUS THURINGIENSIS* STRAINS AGAINST *TRIBOLIUM CASTANEUM* (HERBST) (COLEOPTERA : TENEBRIONIDAE)

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Abstract: The biotoxicity analysis of crystal protein of some *Bacillus thuringiensis* strains has been carried out against the larvae of red flour beetle, *Tribolium castaneum* (Herbst). Seven isolates found highly active against *T. castaneum*. The most toxic isolate SG 31.11 has calculated LC_{50} value of 0.2 µg/mg of artificial diet.

Key words: *Bacillus thuringiensis*, stored grain pest, *Tribolium castaneum*, biotoxicity, insecticidal activity.

INTRODUCTION

Tribolium castaneum is a serious pest of stored grains throughout the world. It not only affects the quantity but also the quality of stored grains. The quantitative estimation of the loss incurred by red flour beetle is difficult because this insect is found in flour mills, godowns, and warehouses with other associated stored grain pest complex. To control the infestation of this insect, many synthetic pesticides have been used for several years now. However, these pesticides produce several adverse effects, which include accumulation of lethal chemicals in food chain and environment, lack of selectivity towards beneficial insects and evolution of resistance. These factors have directed the attention of scientists from traditional chemical pesticides to biopesticides.

Microbial control of insect pest of crops using entomopathogens is an ecologically sound pest management strategy. Although insect viruses and fungal pathogens are used as microbial control agents, but *Bacillus thuringiensis* Berliner (Bt) appears to have the greatest potential for this purpose. This gram-positive, spore forming crystalliferous bacterium synthesizes a proteinaceous parasporal crystalline inclusion (δ-endotoxin) during the sporulation phase. These crystalline proteins are highly specific against different insect orders, and non-target organisms like parasitoids, predators and vertebrates are not affected by their use (Aronson *et al*., 1986; Whiteley and Schnepf, 1986). A promising variety of crystal proteins (cry proteins) have been recognized in different Bt strains. Of these crystal proteins CryIII are reported to be toxic against coleoptera (Herrnstadt *et al*., 1986). This study presents our initial efforts to assess the potential of Bt

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strains, isolated from different environmental samples, as a biological control agent of *T. castaneum*.

MATERIALS AND METHODS

Bacterial culture and isolation of crystal proteins

The strains of *B. thuringiensis* used in the present study, were very kindly supplied by the Culture Collection Laboratory, Centre of Excellence in Molecular Biology, Punjab University, Lahore. These samples were collected from different areas of Pakistan. Most of the strains selected for the study, were isolated from wheat grain, wheat dust, pulse dust, soil and dead insects *E. coli* clone of CryIII was obtained from the Donald Dean Lab., and HD1, from ATCC.

B. thuringiensis cells were grown on petri plates of T3 medium, it contained 3g of tryptone, 2g of Tryptose, 1.5g of yeast extract, 0.005g of $MnCl_2.2H_2O$ 2.5 ml of 1M potassium phosphate (pH 6.9) and 15g of agar/liter.

Cultures were streaked on petri plates and incubated at 30±1°C for 3 to 5 days until sporulation took place. Cells were harvested by washing twice with autoclaved distilled water centrifugation at 7,000 rpm for 10 min., at 4°C. The pellet was resuspended in 50 mM sodium carbonate, pH 9.5 containing 10 mM dithiothreitol at 37°C for overnight. After centrifugation at 7,000 rpm for 10 min., at 4°C, the supernatant was collected and the concentration of soluble crystal proteins (protoxin) was quantified by the microassay method of Biorad using bovine serum albumen (Sigma) as a standard. The solubilized protoxin was activated by treating its 20 µg with 1 µg of trypsin. Solutions were mixed well and incubated at 37°C for 3 hours.

Insect rearing and toxicity assay

Tribolium castaneum were reared on a diet containing semolina and 10% yeast extract. We maintained 20 individual matting pairs in glass jar containing 250g of diet and covered it with muslin cloth. The rearing jar was placed in the insectory set at 30±1°C, a photoperiod of 16.8 (L:D) and a relative humidity of 50±5.

Larvicidal test was carried out with third instar of *T. castaneum* by incorporating suspension containing two-fold serial dilutions of activated proteins into the artificial diet and maintained according to the rearing conditions. Control bioassays were performed with solubilization buffer. Thirty larvae were used for each experiment and each experiment was duplicated. Mortality was counted after five days. LC_{50} and its 95% confidence limits were calculated with probit analysis (Raymond, 1985).

RESULTS AND DISCUSSION

During the present study, different strains of Bt were checked for larvicidal activity against the third instar larvae of *T. castaneum*. Most of the strains were collected from the province of Punjab, some from NWFP and few from Sindh. The main objective to collect the strains from different areas of country is to cover the heterogeneity exist among the populations of Bt. The sources of isolation for most of the strains selected for study were stored grain, and their dust (Table I). It was assumed that we could find novel and highly active strains by screening the host material available to *T. castaneum* in the natural environment.

Table I: Larvicidal activity of *Bacillus thuringiensis* samples collected from different sources and localities.

Isolate	Collection place	Source	LC_{50}*	LC_{50} 95% Confidence Limits	
				Lowest	Highest
CryIIIA	-	*E. coli* clone	0.65	0.40	2.42
SG 31.11	Shakargarh	Wheat grain	0.32	0.21	0.50
Hfz 24.8	Hafizabad	Wheat dust	0.60	0.34	0.79
GU 29.2	Gujranwala	Wheat dust	0.41	0.26	0.63
MR 1.7	Murid-K	Wheat dust	0.33	0.26	0.52
JR 6.3	Chitral	Soil	0.35	0.20	0.72
Hfz 26.8	Hafizabad	Wheat dust	0.30	0.21	0.47
Hfz 2.1	Hafizabad	Wheat dust	0.27	0.16	0.43
Gu 9.1	Gujranwala	Pulse dust	0.38	0.26	0.80
Gu 9.2	Gujranwala	Pulse dust	0.47	0.31	1.02
HM 10.4	Gujrat	Soil	0.32	0.20	0.59

*LC_{50}, concentration at which 50% of larvae were killed.

Of the total samples 1 isolate showed varying level of toxicity against *T. castaneum* larvae in the screening tests. CryIII A protein, obtained from *E. coli* cloned with CryIII gene, showed LC_{50} value as 0.65 µg/mg of diet.

Isolates SG 31.11, Hfz 24.8, HM 10.4, MR 1.7, JR 6.3, Hfz 26.8 and Hfz 2.1 were most toxic with LC_{50} values of approximately 0.3 µg/mg of diet; LC_{50} value of other strains ranged from 0.4 to 0.7 µg/mg of diet (Table I).

The active protein of SG 31.11 was sequenced and data showed that it resemble with a novel CryIII proteins reported by Sato *et al.* (1994). The toxic proteins of other strains were not characterized, so it is difficult to tell whether they are novel proteins or have any homology with existing Cry proteins.

The present study had shown that potential candidate Bt to use in the *T. castaneum* control program could be isolated from a variety of source materials. However, a qualitative survey of the most active isolates is necessary for further evaluation.

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TERATOGENIC EFFECTS OF SUBLETHAL DOSES OF METHAMIDOPHOS IN MICE

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Abstract: An organophosphorous insecticide, methamidophos was tested for its embryotoxicity and teratogenecity in mice. Different sublethal doses of the insecticide i.e., 12.5, 25 and 50% of LD_{50} comprising 1.125, 2.25 and 4.5 µg.g BW were prepared by dissolving it in water in such a way that each 0.1 ml of the solution contains desired concentration. These doses were administered orally to the pregnant mice on different days of gestation. The fetuses were recovered on day 15 of gestation. Fetal body weight and crown rump length decreased in higher doses and longer exposure groups. Morphometric studies of these fetuses were also done which were comprised of the measurements of size of brain, eye, ear, snout, lower limbs and upper limbs. Sizes of these organs decreased significantly (P 0.001) in higher dose groups. Some morphological abnormalities including brain defects, dermal haemorrhagic patches, microphthalmia, limb defects and a high rate of resorptions were found in all dose groups. The present study indicates that concentrations of this insecticide used are highly dangerous for mouse development which may be equally harmful for human too.

Key words: Organophosphates, methamidophos, mouse, sublethal doses, developmental anomalies.

INTRODUCTION

Pollution is the major problem of modern age. It has created various hazards which are severely damaging the living conditions of almost all the organisms. Amongst many other factors, causing pollution, the insecticides contribute a lot. The insecticides, of course, have increased the agri-production possibilities but the environmental and health side effects of their use have rendered differences for living creatures (Zilberman and Siebert, 1990). Worldwide production of these insecticides continue to rise with a 10 fold increase in production between 1955 and 1985 (Rosenstock, 1987).

Organophosphorus insecticides have almost completely replaced chlorinated hydrocarbons due to their biodegradable and non-accumulative nature but now they are found to be relatively toxic to central nervous system of non-target organisms while inhibiting acetylcholinesterase which results in an accumulation of free acetylcholine in nervous tissue (Kobayashi *et al*., 1980).

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In a case report of suicidal attempts with organophosphates, the course of intoxication was such that OPS were absorbed into the blood and finally transferred to body tissue where they inhibited AchE at both nicotinic and muscarinic synapses (Braeckmann *et al.*, 1993).

Almost all the organophosphates are involved in the inhibition of AchE in birds and mammals. Rat, mouse and human's acetylcholinesterase exhibited a 90-100% inhibition after the exposure of organophosphates (Dauberschmidt *et al.*, 1997). In short we can assess that organophosphates are extremely toxic for almost all the vertebrates like fishes, aves and mammals. So there is further need of extensive studies on the effect of these chemicals on non-target organisms especially with reference to their embryotoxic and teratogenic effects. The present study was designed to investigate harmful effects of Methamidophos on the development of mice so as to extrapolate these results in man.

MATERIALS AND METHODS

Albino mice (*Mus musculus*) were used in the experiment. Breeding stock was kept in controlled condition i.e., 12 hour light/dark cycle at 28±1°C. Estrus females were caged with males for overnight mating. Females having vaginal flug on sperm in vaginal tract were separated and that day was designated as day '0' of gestation.

LD_{50} values (9 µg/g BW) of insecticide (Methamidophos) for pregnant mothers were used in this experiment (Tayyaba, 1999). Three sublethal concentrations i.e., 1.125, 2.25 and 4.5 µg/g BW were used as 12.5, 25 and 50% of LD_{50} values. The doses were prepared by dissolving the insecticide in water in such a way that each 0.1 ml contains the desired concentration. The dose was administered orally with the help of syringe having rubber tubing. In this way dose was not wasted. All the treatments were performed on gestation days 6 and 6, 9, 12 and 6-12 as acute, subchronic and chronic doses.

On day 15 the pregnant mothers were weighed and anaesthetized and uteri bearing the fetuses were dissected out. The fetuses were then taken out from these gravid uteri and placed in Bouin's fixative for 48 hours. Afterwards they were washed in 70% alcohol and preserved in 80% alcohol for morphological and morphometric studies of different body organs.

RESULTS

A higher fetal mortality was noted in all treated groups as compared to the controls (Table I). There was no fetal recovery in case of 4.5 µg chronic dose group (Table I). There was a significant ($P<0.001$) reduction in body weight and crown rump length of fetuses of treated groups as compared to control with the exception of 1.125 µg/g BW group in which only chronic group showed significant decrease ($P<0.05$; Table I).

Table I: Developmental defects induced by Methamidosphos in mice.

Parameters	Control	Doses (µg/g BW)								
		1.125			2.25			4.5		
		(s)	(d)	(c)	(s)	(d)	(c)	(s)	(d)	(c)
No. of fetuses	145	16.00	30.00	24.00	48.00	13.00	9.00	82.00	15.00	♣
Resorbed fetuses (%)	11.72	18.75	23.33	25.00	16.07	23.08	22.22	14.63	26.67	♣
Brain defects (%)	5	0.00	0.00	25.00	47.90	0.00	22.00	10.90	6.60	♣
Microphthalmia (%)	10	12.50	6.30	54.00	27.00	15.40	66.60	1.20	13.30	♣
Short pinnae (%)	0.00	0.00	6.60	0.00	2.00	23.00	66.60	0.00	40.00	♣
Limb defects (%)	0.00	12.50	3.30	0.00	20.80	23.00	33.00	12.20	13.30	♣
Skin haemorrhage (%)	8.00	81.00	9.30	66.60	89.60	53.00	0.00	93.90	86.00	♣
Short tail (%)	0.00	0.00	0.00	0.00	0.00	0.00	33.00	0.00	0.00	♣
Snout defects (%)	0.00	62.50	0.00	0.00	18.70	7.70	33.00	19.70	0.00	♣
CR length (mm±S.E)	14.02 ±1.50	14.00 ±0.18	13.75 ±0.11	13.44 ±0.07	12.23 ±0.96	12.70 ±0.51	12.09 ±0.16	12.60 ±0.16	12.40 ±0.14	♣
Body wt. (mg±S.E)	431.76 ±88.16	430.60 ±30.01	421.63 ±15.44	400.62 ±10.48	300.85 ±46.25	319.72 ±31.67	323.33 ±66.43	284.24 ±13.29	315.71 ±17.17	♣

♣ = No fetal recovery.

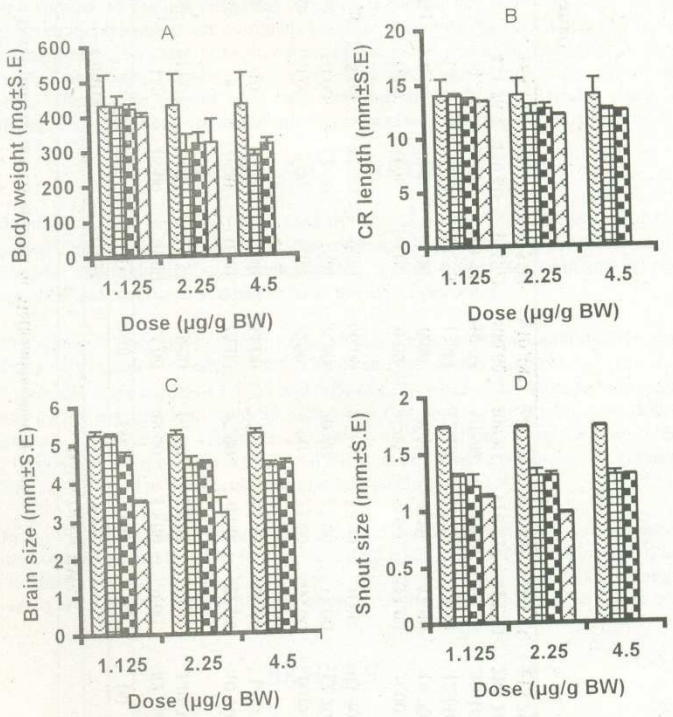

Fig. 1: Histograms showing the effects of different sublethal doses of methamidophos on: A, body weight; B, CR length; C, brain size and D, snout size of 15 days old fetuses. Asterisks show significant difference against controls (*=P<0.05; **=P<0.01; ***=P<0.001).

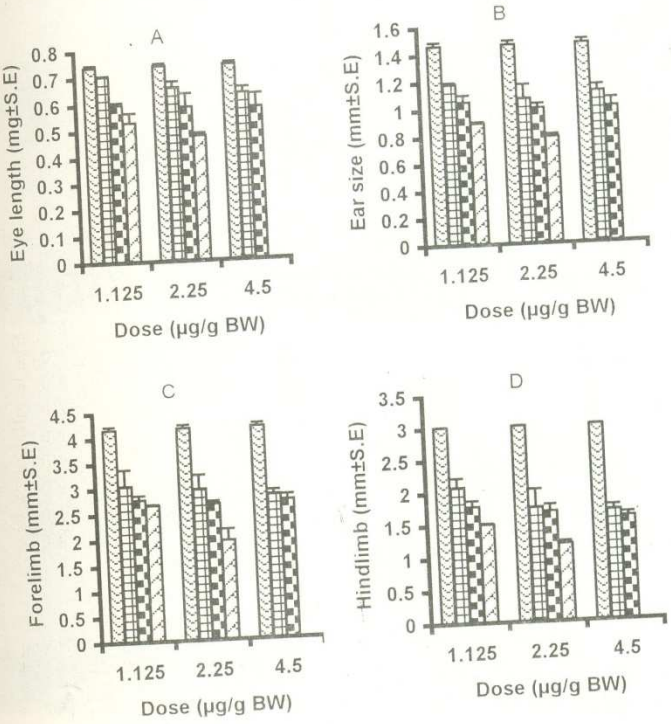

Fig. 2: Histograms showing the effects of different sublethal doses of methamidophos on: A, eye length; B, Pinnae size; C, fore limb and D, hind limb size of 15 days old fetuses. Asterisks show significant difference against controls (*=P<0.05; **=P<0.01; ***=P<0.001).

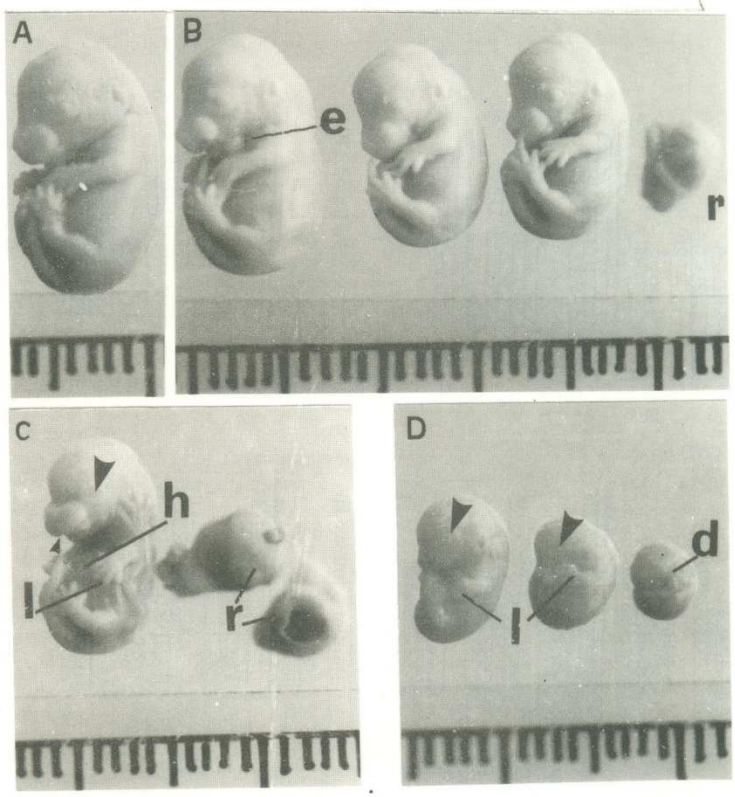

Fig. 3: Macrophotographs of 15 days old fetuses recovered from mothers exposed to different concentrations of methamidophos. A, control fetus with almost normal development; B, members of 1.125 µg/g BW dose group; C, fetuses from 2.25 µg/g BW dose group; D, fetuses from 4.5 µg/g BW dose group. *Note*: Developmental anomalies including ectopia cardis (e), a dead fetus (d), haemorrhagic spots (h), peddle like fore limbs (i), some resorbed fetuses ®, microphthalmia (large arrow head) and cleft lip (small arrow head).

During morphometric observations of different body parts like eye, brain, snout, ear and fore and hind limbs, there was also significant decrease ($P<0.001$) in the size of these organs as compared to control with the exception of brain in which 1-125 µg/g BW single dose group does not show significant decrease (Figs.1 and 2)

Different developmental defects like microphthalmia, short pinnae, skin haemorrhage, limb defects, brain defects (Table I) and cleft lip (Fig.3) were also observed.

DISCUSSION

The present investigation has clearly shown that incidences of teratogenecity and embryotoxicity were increased by the use of sublethal concentrations of Methamidophos.

Fetal mortality was 100% at 4.5 µg/g BW chronic dose level (Table I). The foetal body weight and crown rump length were significantly reduced as compared to controls. Similarly sizes of brain, eye, limbs, ear and snout decreased significantly as compared to control. Different developmental defects like microphthalmia, short pinnae, skin haemorrhage, limb defects, brain defects and cleft lip were also noted.

There are reports in the literature which clearly show that if these organophosphates are used in high concentration and over a long period of time they can found to be embryotoxic and potentially teratogenic in mammals. Vogin (1969) showed in rabbits that when dose level of 3, 12 and 24 mg/kg/day of dichlorvos were administered on day 6 of gestation. No litter was produced at 24 mg/kg dose level. Similarly Mufti and Asmatullah (1997) observed 85% fetal mortality at 50% of LD_{50} dose level in case of Diazinon while it was 61% at 50% of LD_{50} dose level in case of Malathion.

In many previous studies it has been shown that fetal body weight and CR length decreased with increase in dose concentration of Diazinon (Mufti and Asmatullah, 1991; Asmatullah, 2000). Karlow and Martin (1961) also found that a continuous administration of Malathion for 10 week to rats before and during pregnancy also resulted into dwarfism. In another study, dose of 18.6 mg/kg BW of cyclophosphomide showed significant decrease in fetal body weight and increased incidence of eye defects and cleft palate (Ujhazy *et al.*, 1993).

Dobbin (1967) and Lechner and Abdel-Rehman (1984) found decreased fetal weight and increased incidence in external haemorrhagic spots on the fetuses of rats following the administration of Malathion. In another study incidences of Acrania, microphthalmia and microcardia were observed after the administration of 100 mg/kg of Malathion in rabbits (Machine and McBride, 1989). Similarly Azodrin another organophosphate showed almost same results as it caused 100% malformations in the avian embryos at low dose levels (Abbott, 1972).

Ishikawa *et al.* (1975) reported that acetylcholine inhibitors induced cardiac anomalies in nine of 23 chick embryos at a dose level of 20 mg. The anomalies induced were interventricular septal defects, atrial septal defects and double aortic arch. The present results are also supported by the study of Wytterboch and Thompson (1985) that cardiac defects like enlargement and thinning of atrium and dorsal aorta were produced in mice exposed to Malathion.

It is thus quite apparent that the insecticide tested, if ingested by pregnant mothers can cause serious abnormalities and can even prove to be embryolethal so there is need to have extensive research work to know the dangers being caused by these insecticides in developing non-target organisms.

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EFFECT OF *BACILLUS THURINGIENSIS* ON THE SURVIVAL OF *LUCILIA CUPRINA* (WIED) (CALLIPHORIDAE : DIPTERA)

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Abstract: The effect of *Bacillus thuringiensis* on the survival of *Lucilia cuprina* were observed. LD_{50} was found to be $14x10^6$ cells/ml. The treatment resulted in vacuolization of plasma and destruction of haemocytes. Morphological studies of treated flies revealed that bacterial attack caused no apparent decrease in the size of haemocytes as compared to control ones. Differential haemocytes counting (DHC) of the treated flies showed a significant quantitative differences in the haemocyte number as compared to the control flies.

Key words: *Lucilia cuprina, Bacillus thuringiensis*, haemocyte morphology.

INTRODUCTION

Insect pests are major cause of damage as they cause diseases in animals and humans. Current strategies are aimed at reducing crop loses by using chemical pesticides. But these are becoming ineffective against insects, as insects have developed resistance against them because of their extensive use. The death of target as well as non-target species is a major drawback of these chemical insecticides. These are also expensive and cause health hazards (Estruch *et al.*, 1999). Due to above mentioned disadvantages scientists are now moving towards the biological control, which broadly means any method of control that utilizes living organisms or their natural products (Price, 1975).

Over the past few decades many members of different groups have been evaluated as vector control agents. Out of all the bacteria tested for this purpose the only one considered an operational success is the bacterium *Bacillus thuringiensis* (Federici, 1999). *Bacillus thuringiensis* is a rod shaped, gram-positive, catalase positive, spore forming bacterium that is used for special control of some Lepidopteran and Coleopteran insect pests as well as Dipteran vectors of infectious diseases. Its insecticidal activity is connected with parasporal crystalline proteins delta endotoxins produced during sporulation (Knowles, 1994). *Bacillus thuringiensis* insecticidal proteins have been used commercially for over forty years and now represent 98% of all biopesticides. These compounds give fast, drastic but short-lived results. They attack against the target species, and are harmless to mammals. The first known strains of *B. thuringiensis* produced

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proteins toxic to Lepidopteran, but there are now many strains that affect Dipterans, Coleoptrans, Orthopterans and Hymenopterans (Malla, 1997).

Dipteran pests are serious nuisance as well as vectors of many diseases in humans and animals such as malaria, onchocerciasis, equire encephalitis and dog heat worm. Dipteran pests are also a major problem in poultry and cattle industries. They also infest plants, *e.g.*,. Hesian fly, Medfly and Mexfly for which a *B. thuringiensis* product would be valuable. Pests are normally killed by *B. thuringiensis* in larval stages (Beegle, 1978).

The aim of present work was to find the susceptibility of *Lucilia cuprina* to *Bacillus thuringiensis*. *Lucilia cuprina* chosen for the present study belongs to order Diptera and family Calliphoridae. It is also known as sheep blow fly, and is indigenous to Africa but now has spread to other continents like Asia and Australia. Its medical importance was mainly associated with myiasis. It breeds in meat or carrion and may cause facultative myiasis in man by infecting festering sore and wounds (Service, 1980). Now it is also a well known vector of Anthrax affecting sheep, goats, cattle and even humans, causing death if left untreated. Their practice of sitting on dog faces, decaying matter and then on human food means that they can easily transmit undesirable organisms and can readily spread diseases through a community.

Our present work is concerned with the microbial control of *L. cuprina* through a Dipteran specific strain of *B. thuringiensis*.

MATERIALS AND METHODS

Collection and maintenance of insects

The Australian blow flies, *Lucilia cuprina* used for the present work were collected from the meat shops from different localities around Quaid-e-Azam Campus, Punjab University, Lahore. These adult flies were fed on fresh minced beef and pieces of banana peels. The eggs obtained from these flies were kept in sterilized glass jars covered with muslin cloth. The 3rd instar larvae obtained settled down and this marked the onset of the puparial life. The adults emerged nearly five days after the start of puparial life. The larvae were reared on beef throughout their life. The colonies of flies were maintained at 30°C, 12 hours photoperiod and relative humidity ranging from 65% to 75%.

The bacteria used were *Bacillus thuringiensis* Kurstaki (Abbott : Diptera strain). These bacteria were reared on nutrient agar medium at 37°C. Nutrient broth was used as the inoculation medium.

Experimental procedures

Following steps were taken in regard to the entire experimental set-up:

Preparation of bacterial inoculum

The overnight cultures of *B. thuringiensis* were prepared in nutrient broth. These were then used for inoculation studies.

Biotoxicity assays

Milk sugar (1 ml) solution was poured in each sterilized jar having cotton pad and filter paper at the bottom. Then 7 ml of bacterial inoculum was added in each jar. Jars used as control contained 7 ml of autoclaved nutrient broth. Five flies were placed in each jar. Three replicates were set up for the experimental purpose.

Blood film formation and fixation

The flies were given the fumes of glacial acetic acid. A fine sterilized needle was used to puncture the abdomen of the flies, which was gently pressed to squeeze out the blood. The blood thus obtained was evenly spread out into a film by sliding the edge of another glass slide at an angle of 45°C. After air drying, it was fixed in methanol for 5 minutes, and then stained in Giemsa's stain for one minute. After differentiation and dehydration, mounting was carried out in Canada Balsam.

Experimental studies

The sizes of different type of blood cells were measured by using an ocular micrometer. Differential haemocyte count (DHC) was done by marking a spot in a film randomly. All the cells in the marked spot were counted and categorized. Approximately 160 cells per experimental stage were counted and classified.

Estimation of LD_{50}

LD_{50} was determined by computerized probit analysis. Three concentrations ($12x10^6$ cells/ml, $14x10^6$ cells/ml and $16x10^6$ cells/ml) were selected for calculating LD_{50}.

RESULTS

The blood or haemolymph of *Lucilia cuprina* is contained in the general body cavity, as in case of all the insects and has two components, the plasma, which is the liquid part and the haemocytes or the blood cells (Fig.1D-F). Three types of blood cells were distinguished on the basis of light microscopy, which were as follows:

1. Prohaemocytes.
2. Plasmatocytes.
3. Granular cells.

Prohaemocytes are small to medium sized cells. These are round or ellipsoidal and occasionally fusiform. The nucleus is central and occupies almost all the cell body so that cytoplasm forms only a narrow rim around it. These cells are deeply basophilic but nucleus is eosinopilic. These are germ or stem cells (Ratcliffe and Rowley, 1981) (Fig.1B).

Plasmatocytes are highly polymorphic haemocytes and large in size as compared to prohaemocytes. These cells have basophilic cytoplasm but their nuclei are eosinophilic with granular chromatin. Plasmatocytes have more cytoplasm surrounding their nuclei as compared to prohaemocytes. Very few plasmatocytes were seen dividing (Fig.1A).

Granular cells are compact cells of variable size, usually round or disk shaped with a relatively small nucleus enveloped in a large volume of cytoplasm, which characteristically contains many prominent granules. Their nuclei are eosinophilic and smaller in size as compared to those of plasmatocytes (Fig.1C).

When *L. cuprina* was fed on different concentrations of *B thuringiensis* LD_{50} was found to be $14x10^6$ cells/ml of *Bt* liquid culture after 24 hours.

The blood films of control as well as treated flies were studied at 8 hours, 16 hours and 24 hours of intervals. After 8 hours of treatment, the direct microscopic observation of the blood film showed that many bacterial cells were scattered in the vicinity of the haemocytes (Fig.2A). After 16 hours of treatment, many bacteria approached the haemocytes and ultimately they were found attached to the cell membranes of the haemocytes (Fig.2B). After 24 hours of treatment, many bacteria were seen to be residing inside the haemocytes (Fig.2C).

Morphological study and DHC

The size of prohaemocytes in control flies ranged from 7.5 to 8.5 μ and the size of their nuclei ranged from 7.0 to 8.0 μ, whereas in *Bt* treated flies blood, the size of prohaemocytes ranged from 5.5 to 6.5 μ and that of their nuclei ranged from 5.0 to 6.0 μ. It showed that there is 2% decrease in the size of prohaemocytes in treated flies as compared to control ones.

The size of plasmatocytes in control flies of blood ranged from 11.0 to 16.0 μ and that of their nuclei ranged from 7.0 to 7.6 μ, whereas in *Bt* treated flies the size of plasmatocytes ranged from 10.3 to 12.6 μ and that of their nuclei ranged from 6.3 to 7.0 μ. It showed that there was 3% decrease in the size of the whole cell and 0.6% decrease in the size of the nuclei of plasmatocytes.

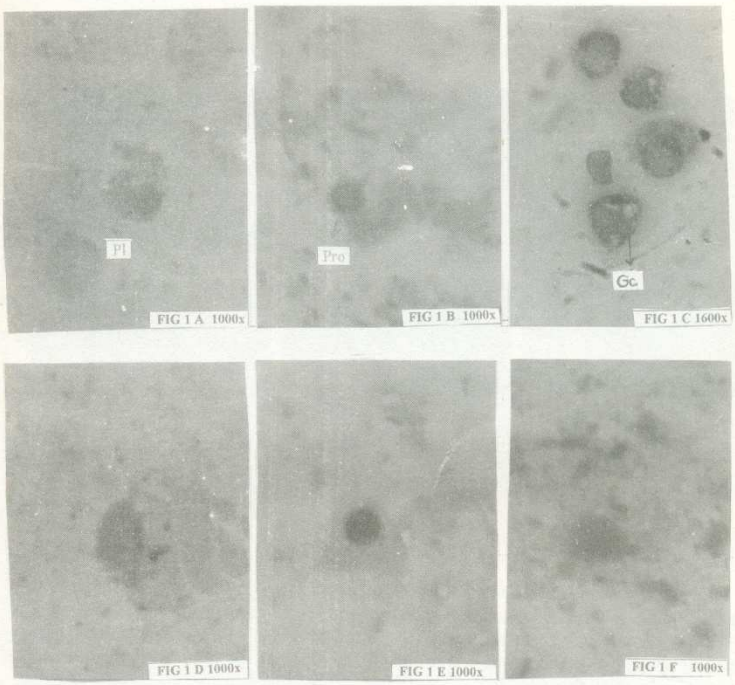

Fig. 1: Control (A-C), A: Pl; Plasmatocytes; B: Pro; Prohaemocytes; C: Gc; Granular cells. General blood picture of control (D-F), D: after 8 hr; E: after 16 hr; F: after 24 hr.

The size of granular cells ranged from 12.6 to 16.0 μ and that of their nuclei ranged from 7.0 to 8.6 μ in control flies, whereas in *Bt* treated flies the size of granular cells ranged from 8.6 to 11.0 μ and that of their nuclei ranged from 5.3 to 6.3 μ. It showed that there was 4% decrease in the size of the whole cell and 2% decrease in the size of the nuclei of granular cells.

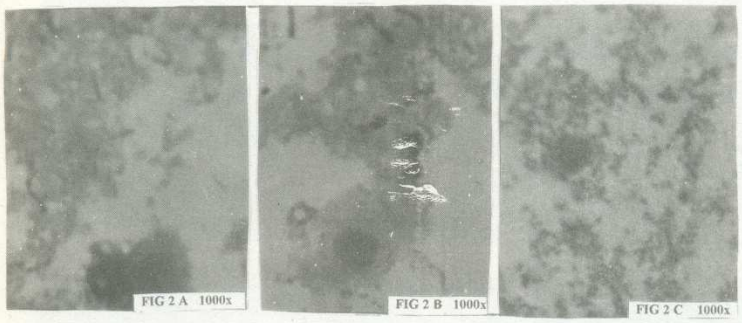

Fig. 2: ***B. thuringiensis*** **(A-C), A: after 8 hr; B: after 16 hr; C: after 24 hr.**

DHC showed that in the control flies prohaemocytes were found to be 15% after 8, 6 and 24 hours, whereas prohaemocytes were found to be 7.5% after 8 hours of treatment, 9% after 16 hours of treatment and 10% after 24 hours of treatment. The statistical analysis showed that there was a significant difference in the prohaemocytes number of control and treated flies blood.

Plasmatocytes were found to be 55% after 8, 16 and 24 hours in control flies blood, whereas in *Bt* treated flies blood, plasmatocytes were found to be 45% after 8 hours, 48% after 16 hours and 52.5% after 24 hours. Statistical analysis showed that there was a significant difference in the plasmatocyte number of control and *Bt* treated flies.

Granular cells were found to be 10% in control flies blood after 8, 16 and 24 hours of treatment, whereas in *Bt* treated flies they were found to be 4% after 8 hours, 6% after 16 hours and 7.5% after 24 hours. Statistical analysis showed that there was a significant difference in granular cells number of control and *Bt* treated flies.

Table I: Showing the effect of *Bt* treatment on the sizes of different types of haemocytes and their nuclei (at 100x).

(30 cells of each type were measured)

Type of cell	Control		*B.t.* Treated	
	Size (μ)	S.D.	Size (μ)	S.D.
Prohaemocytes	7.5-8.5	7.5±0.58	5.5-6.5	5.8±0.46
Nuclei of prohaemocytes	7.0-8.0	7.5±0.44	5.0-6.0	5.5±0.44
Plasmatocytes	11.0-16.0	13.5±1.87	10.3-12.6	11.4±0.92
Nuclei of plasmatocytes	7.0-7.6	7.2±0.25	6.3-7.0	6.6±0.24
Granular cells	12.6-16.0	14.5±1.27	8.6-11.0	9.7±0.97
Nuclei of granular cells	7.0-8.6	7.7±0.70	5.3-6.3	5.8±0.49

Table II: Showing percentages of different types of haemocytes after *Bt* treatment.

Duration of treatment	Cell Types					
	Prohaemocytes		Plasmatocytes		Granular cells	
	Control		Control		Control	
	Total	%	Total	%	Total	%
	30	15	110	55	20	10
	Treated		Treated		Treated	
	Total	%	Total	%	Total	%
8 hours	15	7.5	90	45	8	4
16 hours	18	9	96	48	12	6
24 hours	20	10	105	52.5	15	7.5

Table III: Showing the results of effects of *Bt* treatment on haemocytes size at different durations.

ANALYSIS OF VARIANCE					
Source	DF	SS	MS	F	P
Factor	1	12.32	12.32	1.27	0.293
Error	8	77.84	9.73	-	-
Total	9	90.16	-	-	-

Table value of F = 5.32

There is no significant difference in the size of haemocytes of control and *Bt* treated flies.

Table IV: Showing the results of effects of *Bt* treatment on prohaemocyte number at different durations.

ANALYSIS OF VARIANCE					
Source	**DF**	**SS**	**MS**	**F**	**P**
Factor	1	228.17	228.17	72.05	0.001
Error	4	12.67	3.17	-	-
Total	5	240.83	-	-	-

Table value of F = 7.71

There is a significant difference in the plasmatocyte number of control and *Bt* treated flies.

Table V: Showing the results of effects of *Bt* treatment on plasmatocyte number at different durations.

ANALYSIS OF VARIANCE					
Source	**DF**	**SS**	**MS**	**F**	**P**
Factor	1	228.17	228.17	72.05	0.001
Error	4	12.67	3.17	-	-
Total	5	240.83	-	-	-

Table value of F = 7.71

There is a significant difference in the plasmatocyte number of control and *Bt* treated flies.

Table VI: Showing the results of effects of *Bt* treatment on granular cell number at different durations.

ANALYSIS OF VARIANCE					
Source	**DF**	**SS**	**MS**	**F**	**P**
Factor	1	104.17	104.17	16.89	0.015
Error	4	24.67	6.17	-	-
Total	5	128.83	-	-	-

Table value of F = 7.71

There is a significant difference in the granular cell number of control and *Bt* treated flies.

DISCUSSION

Microbiological control of insects is an important aspect of biological control. Nearly all entomopathogen bacteria are from class "Schizomycetes".

In the present study treatment of *L. cuprina* with *B. thuringiensis* Kurstaki resulted in certain abnormalities *e.g.*.. disruption of haemocytes and ultimate death of flies after 24 hours of treatment.

The blood film studies revealed that haemolymph was affected both in its plasma and the cellular contents. The plasma became coagulated due to scattering of cytoplasmic contents and the bacteria became entangled in this thickened plasma. This seems to be the first defense against the invasion of these foreign particles. Phagocytosis is the most important function of haemocytes. The different steps in this process could be seen clearly such as attachment of *B. thuringiensis* to the cell membranes of various haemocytes and their ingestion by these cells. Detoxification of poisons is done by haemocyte phagocytosis, encapsulation of entomopathogenic microorganisms and storing the antibacterial enzyme, Lysozyme (Hoffman and Frodsham, 1993). Most affected cells were plasmatocytes, but granular cells were also affected to a lesser extent. The prolonged treatment resulted in disruption of cells (Salt, 1970).

DHC of the control and the infected flies was done in order to correlate the resultant quantitative changes. During the present study, DHC revealed that nearly 20% of the haemocytes burst and their contents became scattered around them in infected haemolymph. Upto 90% cell lysis has been reported by other workers in some other insects depending upon the pathogenicity of the bioagents (Pearson and Ward, 1988). The control *L. cuprina* blood showed only 1% distorted haemocytes, which is a natural phenomenon in all the insects. The haemocytes decreased in number in response of treatment.

The bacteria belonging to *B. thuringiensis* Kurstaki produce entomocidal crystal proteins (ICP). The protein is proteolytically cleaved in alkaline circumstances of the larval gut juices into smaller fragments. Thus a proteolytically resistant core has toxicity against insect larvae. Characterization of the core is very important in the elucidation of the entomocidal mechanism of crystal proteins (Ogiwara *et al*., 1971).

Toxic effects of *B. thuringiensis* have been found in insects other than crop pests. *e.g*... in 1997, Akhurst found that the larval of *L. cuprina* were susceptible to some strains of *B. thuringiensis*.

Statistical analysis revealed that there existed a significant difference between the haemocytes number of control and *Bt* treated insects. But a non-significant difference was found in size of haemocytes of the control and *Bt* treated insects.

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EFFECT OF SEASONAL VARIATIONS ON BIOLOGICAL PARAMETERS OF AN AQUA FARM, AGRICON, MULTAN

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Abstract: Density and diversity of plankton was used as a measure of water quality. Photoplankton were abundant as compared to zooplankton. Forty three phytoplankton genera were recorded. Among these nine were of *Cyanophyta*, seventeen of *Chlorophyta*, seven of *Euglenbphyta* and ten of *Chrysophyta*. *Chlorophyta* were abundant in summer. *Chrysophyta* showed an inverse correlation with temperature. *Euglenophyta* was rarely observed. Sixteen genera of zooplankton were observed including twelve of protozoans and four of rotifers. Diversity index ranged from 3.16 to 4.13 which indicates unpolluted water.

Key words: Seasonal variations, water quality, Biological parameters, Fish farm.

INTRODUCTION

In recent years, aquaculture is being projected as a possible solution to the food problems faced by the masses. It gives higher productivity per unit as compared to agriculture and animal husbandry. Water quality studies are important and have been taken up because these play a key role in aquaculture (Pillay, 1990). The water quality determines the survival and growth of the cultured animals and plants (Dehadrai, 1992). The actual harvest of the fish from a piece of water is the ultimate terminal link between a chain of successive biological events within the watermass (Mishra and Saksena, 1992). The quality and quantity of phytoplankton is a good indicator of water quality. The high relative abundance of chlorophyta is indicative of productive water. Blue green algal blooms secrete toxic substances and cause phytoplankton die-off (Shepherd and Bromage, 1992). Diversity indices are used to measure stress in the environment and describe how the individuals are distributed among the species. Unpolluted environments are characterized by a large number of species occurring in relatively low numbers in a community thus having a maximum diversity (Mason, 1988). The present study deals with the effect of seasonal variations on biological parameters of a commercial fish farm.

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MATERIALS AND METHODS

The details of the location and the experimental protocols have been described elsewhere (Ali *et al.*, 1994). The water samples for the qualitative and quantitative study of plankton were preserved by using 4% formaline solution (Battish, 1992) and examined under a compound microscope (OSK 9715-HB-I), using 10X ocular and 10X and 40X objectives.

The identification of zooplankton and phytoplankton up to generic level was carried out by using following literature:

Fritsch (1979), Tonapi (1980), Huet (1986), APHA (1989), Battish (1992).

The relative abundance of different phyla was also calculated.

Diversity index of phytoplankton during each month was calculated by using the following formula:

Diversity index (H) = S-1/lnN (Boyd, 1981)

where

S = No. of phytoplankton genera
N = No. of total phytoplankton.
ln = Natural logarithm.

RESULTS

The monthly distribution of phytoplankton and zooplankton is given in Table I and Figure 1.

Table I: Monthly distribution and relative abundance (%) of phytoplankton and zooplankton.

	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.
No. of phyto-plankton	244	261	275	327	194	168	289	294
No. of zoo-plankton.	17	21	29	25	23	12	11	17
Total No. of organisms observed.	261	282	304	352	217	180	300	311
Relative abun-dance of phytoplankton	93.48	92.55	90.46	92.89	89.40	93.33	96.33	94.53

Forty three genera of phytoplankton were observed. They belong to *Cyanophyta* (9 genera), *Chlorophyta* (17 genera), *Euglenophyta* (7 genera) and *Chrysophyta* (10 genera). Sixteen genera of zooplankton were observed. Twelve were protozoans and four were rotifers.

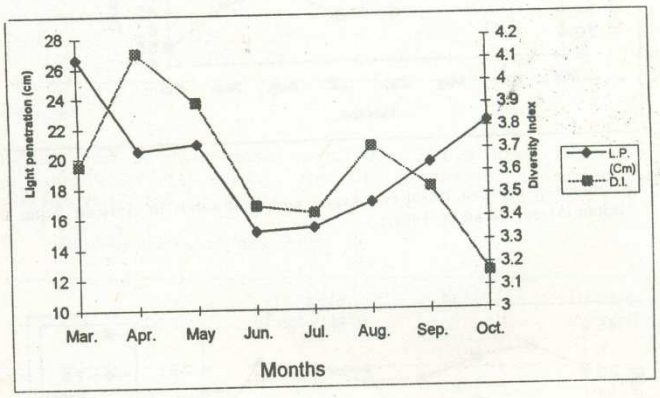

Fig. 1: Relationship between light penetration and diversity index in Agricon Aqua Farm, Multan (March-October, 1993).

Relative abundance

Phytoplankton were abundant as compared to zooplankton throughout the study period (Table I). *Chlorophyta* was relatively abundant when considered during the whole study period (Table II).

During March, *Chlorophyta* and *Chrysophyta* both were relatively abundant followed by *Cyanophyta* and *Euglenophyta*. During April, *Cyanophyta* was relatively abundant followed by *Chlorophyta*, *Chrysophyta* and *Euglenophyta*. From May to September, *Chlorophyta* was most abundant followed by *Cyanophyta, Chrysophyta* and *Euglenophyta*. During October, *Chlorophyta* was relatively less abundant followed by *Cyanophyta*, *Chrysophyta* and *Euglenophyta* (Table II).

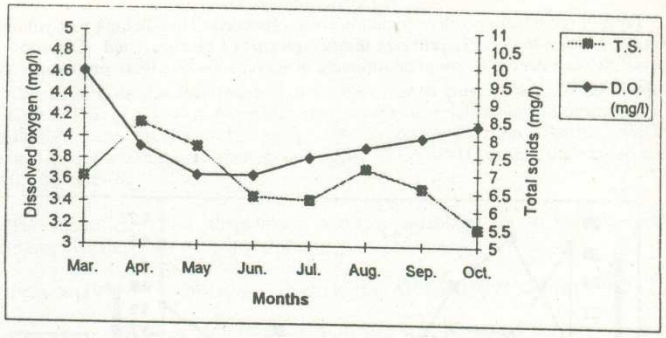

Fig. 2: Relationship between dissolved oxygen and total solids in Agricon Aqua Farm, Multan (March-October, 1993).

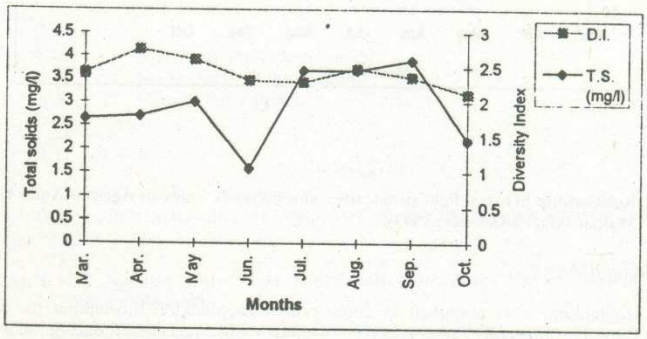

Fig. 3: Relationship between total solids and diversity index in Agricon Aqua Farm, Multan (March-October, 1993).

Cyanophyta showed irregular fluctuations. *Chlorophyta* showed increasing trend during study period with a peak in September. *Chrysophyta* showed a decreasing trend from March to June and then increasing trend from June to October. *Euglenophyta* was rarely observed. It showed irregular behaviour.

Table II: **Relative abundance (%) of phyla during study period.**

	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Mean
Cyanophyta	23.75	40.42	23.68	33.80	20.27	23.30	18.33	26.36	26.73
Chlorophyta	32.56	34.02	60.19	57.67	64.51	63.88	65.00	49.51	53.05
Chrysophyta	32.56	17.02	5.59	1.13	3.68	4.44	7.66	12.86	10.55
Euglenophyta	4.59	1.06	0.98	0.28	0.92	1.66	5.33	5.78	2.62
Protozoa	3.83	4.96	5.26	2.27	4.14	2.77	2.02	4.50	3.48
Rotifera	2.68	2.48	4.27	4.82	6.45	3.88	1.66	0.96	3.30

Diversity index

Diversity index of phytoplankton ranged from 3.16 to 4.13 (Table III). The relationship of diversity index with light penetration, dissolved oxygen and total solids is shown in figures 1, 2 and 3, respectively.

Table III: **Diversity index of phytoplankton.**

Month	No. of genera(s)	Total No. of individuals (N)	ln N	Diversity index (H)
March	21	244	5.497	3.63
April	24	261	5.564	4.13
May	23	275	5.616	3.91
June	21	327	5.789	3.45
July	19	194	5.267	3.42
August	20	168	5.123	3.71
September	21	289	5.666	3.53
October	19	294	5.583	3.16

DISCUSSION

Chlorophyta was relatively abundant in March to April and highly abundant from May to September. Salam and Parveen (1997) reported similar trend in relative abundance of *Chlorophyta* from February to July indicating the productivity of water. In the present study, *Cyanophyta* was rarely present except in April. This observation was in contrast to the study by Salam and Parveen (1997) who reported that *Cyanophyta* was highly abundant during August and September indicating water pollution in that body of water which was mainly fed by seepage water while in the present study, water was regularly replenished. These results also show interlocking fluctuations of *Chlorophyta* and *Cyanophyta*. When *Chlorophyta* population is at its peak, *Cyanophyta* are at minimum level. When *Cyanophyta* population is at its peak, *Chlorophyta* are at minimum level. Boyd (1981), Shepherd and Bromage (1992) reported that *Cyanophyta* secrete toxic substances and results in massive phytoplankton die-offs, favouring blue green algae.

Our results indicated a negative correlation between *Chrysophyta* and temperature. Similarly Munawar *et al.* (1991) also showed a negative correlation between *Chrysophyta* and temperature. *Englenophyta* were rarely observed throughout the study period which followed irregular distribution pattern.

In the present study, diversity index remained above 3. Diversity index greater than 3 is an indicator of clean water while values in the range of 1-3 are characteristic of moderately polluted conditions and values less than 1 characterize heavy pollution (Mason, 1988). Therefore, in the present study, it can be concluded that water was productive and unpolluted as indicated by diversity index.

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QUALITATIVE ANALYSIS OF DRINKING WATER OF LAHORE FOR THE PRESENCE OF COLIFORM BACTERIA

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Abstract: Water samples, from direct supplies and overhead tanks of different localities in Lahore were collected and analyzed for the presence of coliform bacteria by using lactose broth and Endo agar media. It was observed that almost all of the samples harboured coliform bacteria indicating some forms of fecal pollution. The results show that either the water-storage or water pipe lines are not being managed properly.

Key words: Drinking water, pollution, coliforms, lactose fermenting bacteria, *E. coli*.

INTRODUCTION

The quality of water, for both drinking and recreation purposes is now a matter of national and international concern (Collins *et al*., 1995). Every impact which changes the quality of our surface and subsoil waters to such a degree that turns it unsuitable either for human consumption or for survival of fauna and flora is considered water pollution (Chow, 1964; Ramalho, 1977; Eckenfelder, 1989; Chhatwal *et al*., 1989; Shukla and Shrivastiva, 1992; Hussain, 1998). Odours from polluted waters and trash in the water system affect quality of life, tourism and economic development. Water pollution harms aquatic life and ecological balance. During and after rain, runoff carries pollutants including fecal coliform bacteria, pesticides, fertilizers, chemicals, oil and grease etc., into water ways. Contamination also occurs from sanitary sewer systems and waste water treatment plants (Edmonds, 1978; Dufour, 1984; Sterrit and Lester, 1988).

Water that contains large numbers of bacteria may be perfectly safe to drink. The important consideration, from a microbiological standpoint, is the kinds of microorganisms that are present. Water from streams and lakes, which contains multitudes of autotrophs and saprophytic heterotrops is potable as long as pathogens for human are lacking. The intestinal pathogens such as those that cause typhoid fever, cholera and bacillary dysentery are of prime concern. The fact that human fecal material is carried away by water in sewage systems that often empty into rivers and lakes presents a colossal sanitary problem, making constant testing of municipal water supplies for the presence of fecal microorganisms as essential procedure for the maintenance of water purity (Benson, 1994). Bacterial contamination cannot be detected by sight, smell or taste. Therefore, a water supply has to be tested through microbiological methods. Coliform bacteria have been used to assess the quality of water and presence of pathogens. Although several of

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the coliform bacteria are not usually pathogenic themselves but they serve as an indicator of potential bacterial pathogens contamination. The simpler, quicker and safer nature of the method for analyzing these microorganisms as compared to the efforts required to verify the presence of individual pathogens, has made it a popular routine water assay protocol (Gaudy and Gaudy, 1980; USEPA, 1986; Francy *et al.*, 1993).

There are a number of methods and media available for the detection and enumeration of indicator microorganisms, such as multiple-tube most probable number fermentation technique (MPN), membrane filter method, presence/absence test and employing defined substrate (AWWA, APHA and WEF, 1992; Benson, 1994; Collins *et al.*, 1995). The present study was intended to process drinking water from direct supplies and over-head tanks of different areas of Lahore, for the presence of coliform bacteria. The informations are relevant to public-health authorities.

MATERIALS AND METHODS

Samples of drinking water were collected from different areas of Lahore (Table I). Direct supply lines and as well as over-head tank of houses, were sampled by taking about 100 ml of water in sterile glass bottles.

Qualitative analysis of the samples for coliform bacteria

Water samples were analyzed by multiple-tube most probable number (MPN) fermentation technique (Benson, 1994; Collins *et al.*, 1995). The procedure was completed in three steps named, presumptive, confirmed and completed tests.

Presumptive test

Double strength and single strength lactose broth were prepared according to Merck (1996/97). Single strength lactose broth (SSLB) contained (% W/V) 0.5 each of peptone and lactose, and 0.3 meat extract. The double strength lactose broth (DSLB) contained double amounts of all the ingredients. For a given sample nine test tubes were fitted with Durham's tubes and divided into three sets. 10 ml of DSLB was dispensed into each of the three test tubes of one set, and SSLB into other two sets. The tubes were cotton plugged and sterilized by autoclaving. On cooling down the DSLB-test tubes were inoculated with 10 ml, one set of SSLB with 1 ml and the other with 0.1 ml of a water sample. The inoculated tubes were incubated at 37°C for 24-48 hours, and then observed for gas production. The most probable number (MPN) of coliform bacteria was determined from MPN table (Benson, 1994).

Confirmed test

Endo agar medium was prepared by mixing 3.6 gm of Endo agar (Oxide) and 0.4 ml of 10% (W/V) alcoholic basic fuchsin in 100 ml of distilled water. The medium was autoclaved and allowed to cool down to about 50°C before pouring in pre-sterile petri plates. Following solidification the petri plates were streaked on from the cultures of lactose broth. The plates were incubated for 24 hours at 37°C and observed for the presence of coliforms' colonies.

Completed test

Bacterial colonies from the Endo agar plates were subcultured into lactose broth fermentation tubes and on nutrient agar slopes. Both were incubated at 37°C for 24 hours. Gas production was noted in the test tubes, while growth on nutrient agar slants was processed for Gram's staining.

Table I: Drinking water sampling sites and dates.

Sample No.	Sampling locality	Collection date
1.	Mian Meer Colony	15.09.2000
2.	Gulshan-e-Ravi	21.09.2000
3.	Zoology Department, Punjab University, Lahore	21.09.2000
4.	Thokar Niaz Baig	11.10.2000
5.	Ghari Shahoo	11.10.2000
6.	Faisal Town	12.10.2000
7.	Allama Iqbal Town	14.10.2000
8.	Rehman Pura	16.10.2000
9.	Dharam Pura	20.10.2000
10.	Jain Mandar	20.10.2000
11.	Davis Road	26.10.2000
12.	Sanda	28.10.2000
13.	Ichhra	01.11.2000
14.	Karim Park	03.11.2000
15.	Samanabad	04.11.2000
16.	Sabzazar	20.11.2000
17.	Shadbagh	21.11.2000

RESULTS

Presumptive test

Sparing the three samples for which stored water was not available about 36% of the samples showed gas production both in the direct supplies as well as in the stored water. About 29% of the samples yielded gas in stored water only. 7% of the samples indicated gas production only in direct supplies. 29% samples did not give the positive reaction at all (Fig.1).

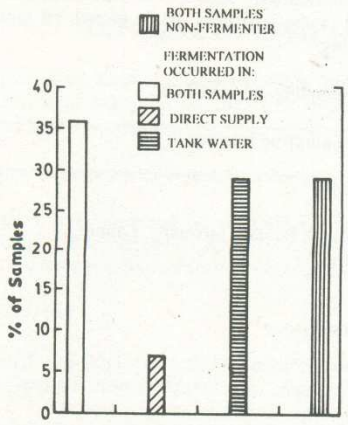

Fig. 1: Incidence of lactose fermentation by water samples.

Results of the presumptive test showed that water of both direct supplies as well as over-head tanks of sample number 8, 10, 11, 12 and 14 produced gas in lactose broth. Samples number 6, 9, 15 and 16 were found negative for the gas production at all. Four samples *i.e.*, 1, 2, 3 and 7 indicated gas production only in overhead tank water. For sample number 13, the direct supply gave positive reaction, while the overhead tank water did not produce gas. In case of samples, 4, 5 and 17 only direct supplies were sampled and they all showed positive results. Most probable number of lactose fermenter bacteria of these samples, are shown in Table II and Fig.2

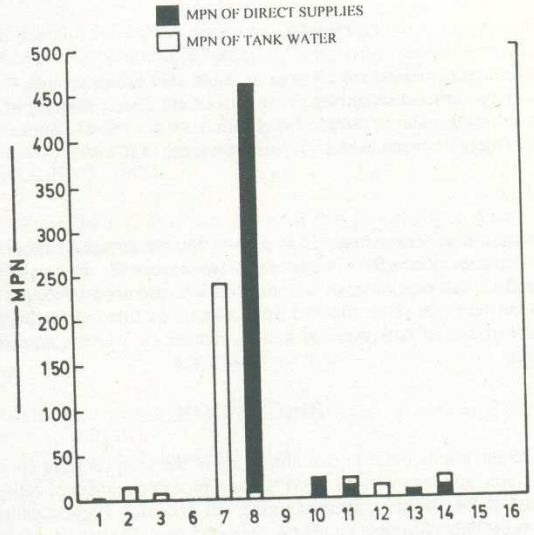

Fig. 2: Graph showing most probable number (MPN) of water samples.

Table II: Indication of coliform bacteria in water samples of direct supplies from pipelines (D.S.) and household overhead tanks (T.W.) of different localities of Lahore.

Sample	[a]MPN/ 100 ml of sample		Confirmed Test on Endo agar				Completed Test			
			Pink/red colonies		Metallic sheen of colonies		Gas production		Grams' staining	
	D.S.	T.W.	D.S.	T.W.	D.S.	T.W.	D.S.	T.W.	D.S.	T.W
1[c]	-	4	+	+	-	-	-	-	g-ve rods	b-ve
2	-	15	+	+	-	-	-	+	g-ve rods	b-ve
3	-	7	+	+	-	-	-	+	g-ve rods	b-ve
4	1100+	•	+	•	+	•	+	•	g-ve rods	•
5	4	•	+	•	-	•	-	•	g-ve rods	•
6	-	-	+	+	-	-	-	-	g-ve rods	b-ve
7	-	240	+	+	-	+	-	+	g-ve rods	b-ve
8	460	9	+	+	-	-	+	-	g-ve rods	b-ve
9	-	-	+	+	-	-	-	-	g-ve rods	b-ve
10	23	4	+	+	-	-	+	-	g-ve rods	b-ve
11	21	23	+	+	+	+	+	+	g-ve rods	b-ve
12	15	15	+	+	-	-	+	+	g-ve rods	b-ve
13	9	-	+	+	-	-	-	-	g-ve rods	b-ve
14	15	23	+	+	-	-	+	+	g-ve rods	b-ve
15	-	-	+	+	-	-	-	-	g-ve rods	b-ve
16	-	-	-	-	-	-	-	-	g-ve rods	b+ve cocci
17	240	•	+	•	+	•	+	•	g-ve rods	•

[a]: most probable number of coliforms; [b]: amount (ml) of sample; b-: gram-ve bacilli; c+: gram+ve cocci; •: sample was not available; -: negative for fermentation; +: positive for fermentation.

Confirmed test

All the samples yielded red colonies on Endo agar except sample 16, irrespective to their status of gas production during presumptive tests. Direct supplies of sample number 4 and 17, while tank water of sample 7 and both types of water of sample 11, gave rise red colonies with metallic sheen, which indicated presence of *E. coli*.

Completed test

Inoculation of colonies from Endo agar to lactose fermenter tubes showed results similar to the ones obtained in presumptive test except for direct supplies of sample number 5 and 13, and tank water of 1, 8 and 10, which did not produce gas at this step. All cultures on nutrient agar slant, streaked from colonies on Endo agar represented Gram-ve bacilli except in case of tank water of sample number 16, which comprised of Gram+ve staphylococci.

DISCUSSION

The present results indicate that almost all of the samples were contaminated with coliform bacteria, and regarding their MPN (most probable number of coliforms/100 ml of sample) most of the samples appeared unsafe for drinking. Highest contamination was noted in direct supply of sample number 4, collected from Thokar Niaz Baig as envisaged by a figure of more than one thousand for MPN. All the colonies of this sample on Endo agar possessed metallic sheen, which indicated the presence of *E. coli* in the water. Similarly, direct supplies of sample numbers 8 and 17 and overhead tank water of 7 were also highly contaminated with MPN values of 460, 240 and 240, respectively. Colonies of sample numbers 7, 11 and 17 also expressed metallic sheen. Remaining samples indicated 4 to 23 MPN (Table II, Fig.2). These all values are higher than the established safe level for drinking water, which is 3 MPN (Baker and Breach, 1980).

It was noticed that all the samples except No. 16 irrespective to their lactose fermenter/ non-fermenter nature formed pink to red colonies on Endo agar. Although the Endo agar medium has been considered selective for lactose fermenters (Benson, 1994), but it is also reported that the medium is slightly selective one and some enteric pathogens such as *Salmonella* may form faint pink colonies, while *Shigella* may develop slightly pinker colonies (Rohde, 1973). *Shigella* as well as *Salmonella* are non-fermenters of lactose and Gram-negative bacilli (Benson, 1994; Collins *et al.*, 1995). Emergence of pink colonies on Endo agar for the samples No. 6, 9 and 15, which appeared non-lactose fermenter during the presumptive as well as completed tests indicate the presence of such enteric pathogens. Similarly, for sample numbers 1-3 and 7, the water taken from the direct supplies did not produce gas during the presumptive and the completed tests but pink to red bacterial colonies appeared on the Endo agar medium. A similar situation was observed for tank water of the sample No.13. These results strongly suggest that while

analyzing water samples for the prevalence of coliform bacteria, the sample, which yield no gas production at the presumptive test stage, must be processed further on selective media such as Endo agar and EMB etc., to monitor the presence of enteric non-fermenter pathogens. It is important to note at this level that many workers in their protocols for the analyses of water samples for coliforms have mentioned that if no gas is produced by a sample at presumptive test stage, the samples need no further processing (Benson, 1994; Collins *et al*., 1995; Black, 1996).

From the results obtained it is recommended that almost all the water samples are unsafe for drinking purpose and many human gastrointestinal diseases might have been prevailing in these areas. These information, however, suffice to open the eyes of public health authorities to feel their responsibilities in relation to the provision of safe drinking water for the urban population.

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DEVELOPMENTAL PATHWAYS IN A FUNGUS GROWING TERMITE, *ODONTOTERMES OBESUS* (RAMBUR) (TERMITIDAE: MACROTERMITINAE)*

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Abstract: Developmental pathways of *O. obesus* based on the collection brought from the nest, were studied. It was found that workers in the colony were of two types (major workers and minor workers) differing only in minor details unlike those of the genus *Macrotermes*. However, after second instar larva they can be easily differentiated, as they have different mandibular size and shape (not pattern). Detailed studies on caste differentiation of a field colony of *O. obesus* revealed that major and minor workers develop from third instar larva. Both major and minor workers after their differentiation from third instar larva pass through three successive instars to become an adult sclerotized major or minor worker. There were many qualitative and quantitative changes from third instar larva to the adult workers. Biometrically all the instars of major workers and minor workers were significantly different in all the characters studied. However, for the total body length third instar major workers were not significantly different from adult workers. The origin of presoldier takes place from third instar minor worker larva. The presoldier after five significantly different instars develops into a soldier. It is in the last moult that rectangularly oval head characteristic of the species appears. The origin of alate line takes place from second instar larva. The nymph after five instars developed into alate. Biometrically all the instars were significantly different.

Key words: Developmental pathways, fungus, termite.

INTRODUCTION

Since the first publication by Grassi and Sandias nearly hundred years ago (1893-94) on caste determining mechanism in termites, this fascinating subject has attracted the attention of a large number of workers. Among the more important publications on the subject of caste differentiation are those of Miller (1942), Light (1944), Light and Weesner (1951), Luscher (1952, 1960, 1962, 1972, 1975, 1977), Buchli (1958), Lebrun (1967a,b), Springhetti (1970, 1971, 1973), Nagin (1972), Symthe and Mauldin (1972), Sewell and Watson (1981), Watson and Sewell (1981) and Noirot (1985a) on lower termites and those of Weesner (1953), Noirot (1955, 1956), Williams (1959a,b), Sands (1965a), Luscher (1976), Okot-Kotber (1981a,b, 1985) and Noirot (1985b) on higher termites.

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In Pakistan, a detailed study on developmental pathways has been carried out by Afzal (1981) on the lower termite, *Bifiditermes beesoni* (Gardner). Some observations on caste differentiation in the fungus growing termite, *O. gurdaspurensis* have been made by Akhtar and Rana (1988).

The present study deals with the developmental lines of workers, soldiers and imagoes of *O. obesus* based on field colonies.

MATERIALS AND METHODS

The material used in the present study consisted of populations of subterranean nests of *O. obesus*. The population of the nest was separated by suspending the collection in water and was preserved in 80% alcohol. Measurements of different instars of worker, soldier and alate lines were taken with the calibrated ocular micrometer. Photographs of the various developmental stages were taken using an Olympus photomicroscope.

Taxonomic terms and measurements used in the present study are as explained by Emerson (1945) and Ahmad (1950).

To study the origin of major and minor worker developmental lines, following characters were measured.

Total body length.
Length of head to side base of mandibles.
Maximum width of head.
Length of hind tibia.

For the study of the developmental pathways of the soldier line, different larval instars were measured for the following characters:

Total body length.
Length of head to side base of mandibles.
Maximum width of head.
Length of left mandible.
Length of hind tibia.
Length of pronotum.
Width of pronotum.

For the study of the developmental pathways of the alate line following characters were studied:

Total body length.
Length of head to side base of mandibles.
Maximum width of head.
Length of hind tibia.
Length of wing pads.

Numerical data of various characters were analysed for mean ($\bar{x}$), standard deviation (S.D.) and co-efficient of variability (C.V.) according to Sokal and Rohlf (1969). For comparison of different instars, data were analysed by student "t" test.

RESULTS

Developmental line of major worker

On the basis of the statistical analysis of the measurements of the immature stages in the samples of field population of *O. obesus* it is inferred that the major worker passes through three larval instars followed by three successive instars of major workers (Fig.1).

The larval instar can be easily distinguished from the major worker instar as the larva is fragile and white due to absence of sclerotization (including the mandibles) and the gut appears empty and colourless. However, the workers contain gut content and appear coloured due to some pigmentation.

First instar larva

Head and body whitish, unpigmented, head nearly round; whitish brain mass visible through cuticle; head width 0.335-0.490 mm; brain occupying nearly the whole of head capsule. Mandibles unpigmented, whitish; left mandible with apical and first marginal tooth minutely indicated; right mandible with a slight indication of first marginal and apical tooth (Plate 1A). Antennae 12-segmented, transparent; first and second segments distinct, nearly equal. Antennal portion near third and fourth segment not clearly segmented; segmentation distinct beyond fourth segment. Abdomen with a pair of styli. Tarsi indistinctly 4-segmented (Table I).

Table I: Biometric analysis of different characters of first instar larva of *O. obesus* (measurements are in mm)

Character	Range (n-25)	Mean±S.D.	C.V.
Total body length	0.8450-1.729	1.346±0.201	14.911
Length of head to side base of mandibles	0.258-0.335	0.294±0.021	7.065
Width of head	0.335-0.490	0.429±0.031	7.321
Length of hind tibia	0.181-0.232	0.192±0.014	7.392

Second instar larva

Head and body whitish; unpigmented. Head nearly round; brain area visible through cuticle, large occupying 75% of the whole head capsule. Mandibles

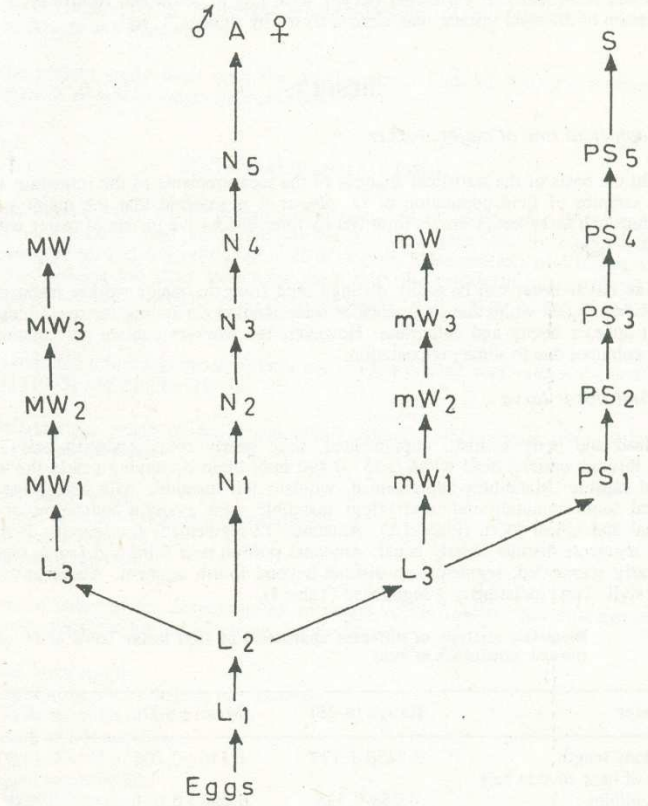

Fig. 1: **A scheme of post-embryonic development in a mature field colony of *O. obesus*. L_1-L_3, larval instars 1-3; MW_1-MW_3, major worker instars 1-3; MW, major worker; mW_1-mW_3, minor worker instars 1-3; mW, minor worker; PS_1-PS_5, pre-soldier instars 1-5; S, soldier; N_1-N_5, nymphal instars 1-5; A, alate.**

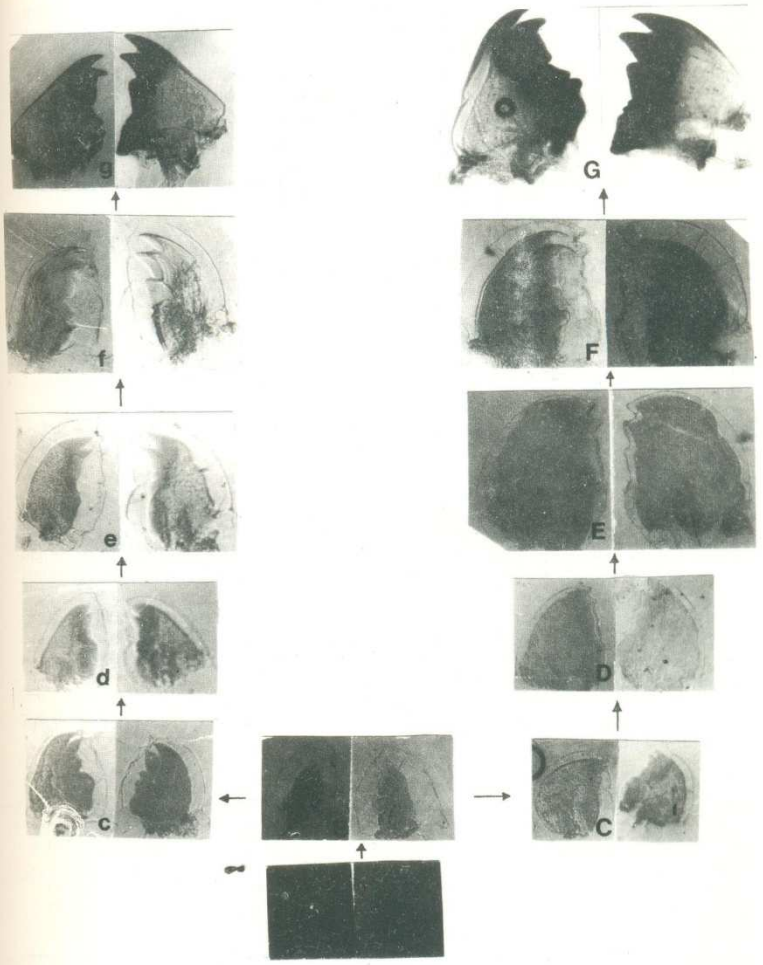

Plate 1: Stages of mandibular development of major and minor workers of O. obesus. A, first stage larva; B, second stage larva; C, third stage larva of major workers; c, third stage larva of minor workers; D,E,F, major worker instars 1-3; d,e,f, minor worker instars 1-3; G, major worker; g, minor worker. Magnification; A,B,c = X200; C-G and d-g = X100.

unpigmented; inner mandibular side dirty white; rest of the area transparent. Pigmentation not started yet; right mandible with apical and first marginal tooth slightly more developed than first instar larvae; notch between first and second marginal tooth slightly indicated; left mandible with first marginal and apical tooth distinct; notch between first and second marginal in initial stage of development; slightly indicated (Plate 1B). Antennae with 13 articles. Abdomen with a pair of styli. Tarsi 4-segmented (Table II).

Table II: Biometric analysis of different characters of second instar larva of *O. obesus* (measurements are in mm)

Character	Range (n=25)	Mean±S.D.	C.V.
Total body length	1.73-2.296	1.986±0.154	7.744
Length of head to side base of mandibles	0.361-0.387	0.382±0.009	2.380
Width of head	0.542-0.619	0.577±0.0004	3.328
Length of hind tibia	0.335-0.387	0.336±0.015	4.211

Third instar larva

Head and body dirty white, unpigmented. Head nearly round; brain not visible through cuticle. Mandibles more differentiated than second instar larvae; all the teeth have developed and visible under the old cuticle; apical and first marginal with a slight brownish tinge; right mandible with a distinct notch between first and second marginal tooth; second notch between posterior margin of second marginal tooth and molar plate slightly indicated. Left mandible also with a distinct notch between first marginal and second marginal tooth; second notch between second marginal and molar plate also slightly indicated, unpigmented (Plate 1C). Antennae with 14 articles. Abdomen with a pair of styli. Tarsi 4-segmented (Table III).

Table III: Biometric analysis of different characters of third instar larva of *O. obesus* (measurements are in mm)

Character	Range (n=25)	Mean±S.D.	C.V.
Total body length	2.477-3.122	2.847±0.156	5.494
Length of head to side base of mandibles	0.490-0.568	0.521±0.024	4.547
Width of head	0.645-0.748	0.708±0.029	4.954
Length of hind tibia	0.439-0.490	0.450±0.015	3.267

First instar major worker

The first instar major worker develops from third instar larva. Head and body slightly brownish yellow; brain area visible through cuticle; head brain index 0.6; mandibles more differentiated than third instar larva; teeth slightly pigmented in some

specimens; left mandible with outer cuticle provided with distinct apical and first plus second marginal; posterior end of the first and second marginal tooth convex; developing mandible within the cuticle with distinct apical, first plus second marginal and with an indication of a notch between first plus second and third marginals (Plate 1D). Antennae with 15 articles. Abdomen with a pair of styli. Tarsi 4-segmented (Table IV).

Table IV: Biometric analysis of different characters of first instar major worker (measurements are in mm).

Character	Range (n=25)	Mean±S.D.	C.V.
Total body length	2.709-3.612	3.288±0.232	7.056
Length of head to side base of mandibles	0.542-0.619	0.569±0.023	3.991
Width of head	0.722-0.851	0.771±0.033	4.234
Length of hind tibia	0.593-0.722	0.660±0.030	4.549

Second instar major worker

Head and thorax slightly darker than the abdomen; head oval narrowing posteriorly; brain clearly visible through cuticle; brain smaller in proportion to head than in earlier stages; mandibles at tips more pigmented than first instar major worker; left mandible almost like that of first instar major worker; right mandible with old cuticle indicating first and second marginals; notch between third marginal and molar plate present; developing mandible within old cuticle with distinct apical, first and second marginals but with regressed third marginal tooth; notch also not distinct (Plate 1E). Antennae with 16 articles; dirty white. Abdomen with a pair of styli. Tarsi 4-segmented (Table V).

Table V: Biometric analysis of different characters of second instar major worker (measurements are in mm).

Character	Range (n=25)	Mean±S.D.	C.V.
Total body length	3.612-4.076	3.885±0.021	3.686
Length of head to side base of mandibles	0.774-0.851	0.820±0.026	3.160
Width of head	1.032-1.187	1.125±0.039	3.494
Length of hind tibia	0.774-0.877	0.825±0.024	2.898

Third instar major worker

Head yellowish brown, much darker, oval. Brain clearly visible through cuticle. Mandibles sclerotized, brownish red; left mandible with distinct apical, first and second marginal teeth; posterior margin of first and second marginal teeth and anterior margin of third marginal making a notch, which at this stage is well indicated, deep (Plate 1F).

Antennae with 17 articles; distal articles darker than proximal; distal articles yellowish brown; first antennal article as long as third and fourth combined. Abdomen with a pair of styli. Tarsi 4-segmented (Table VI).

Table VI: Biometric analysis of different characters of third instar major worker (measurements are in mm).

Character	Range (n=10)	Mean±S.D.	C.V.
Total body length	4.386-4.902	4.670±0.155	3.328
Length of head to side base of mandibles	0.851-0.929	0.880±0.029	3.251
Width of head	1.161-1.238	1.215±0.026	2.098
Length of hind tibia	0.851-0.903	0.872±0.024	2.741

Major worker

Like third instar major worker except that head much brownish and darker; brain much smaller as compared to head size. Mandibles strongly sclerotized (Plate 1G; Table VII).

Table VII: Biometric analysis of different characters of major worker of *O. obesus* (measurements are in mm).

Character	Range (n=25)	Mean±S.D.	C.V.
Total body length	4.386-5.031	4.736±0.162	3.429
Length of head to side base of mandibles	1.032-1.445	1.297±0.095	7.339
Width of head	1.393-1.625	1.529±0.069	4.525
Length of hind tibia	0.980-1.238	1.138±0.075	6.626

Comparison of different instars of major workers of O. obesus

Total body length

The range, mean and standard deviation of total body length of different instars of major worker line are given in Table VIII. Frequency distribution of total body length of different instars is illustrated in Fig.2. The co-efficient of variability was maximum for first instar larva, whereas it was minimum for third instar major worker indicating that the growth has completed and the specimens are therefore relatively less variable. Comparison of different instars of major worker line of *O. obesus* was carried out by student "t" test, which revealed significant differences ($P<0.05$) between all the

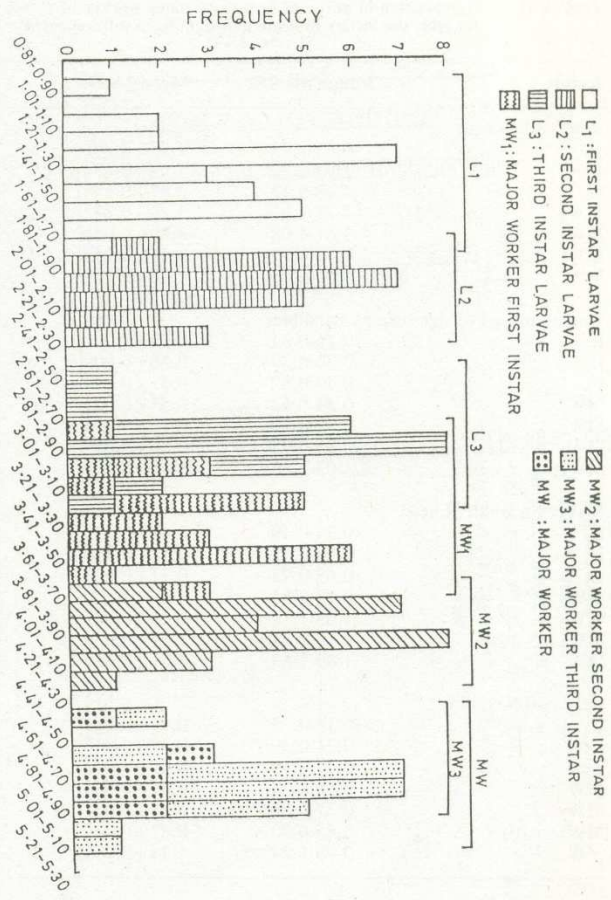

Fig. 2: Frequency distribution of total body length in different instars of major worker line of *O. obesus*.

instars. However, third instar major worker and adult major worker were not significantly different (P>0.05) for total body length (Table VIII).

Table VIII: Age variation in different instars of major worker of *O. obesus* (vertical lines alongside the instars indicate non-significant differences; P>0.05).

Instar	Range (n=25)	Mean±S.D.	C.V.
Total body length			
L_1	0.85-1.73	1.35±0.201	14.91
L_2	1.70-2.30	1.99±0.154	7.74
L_3	2.48-3.12	2.85±0.156	5.49
MW_1	2.71-3.61	3.29±0.232	7.07
MW_2	3.61-4.08	3.89±0 021	3.69
MW_3	4.39-4.90	4.67±0 155	3.33
MW	4.39-5.03	4.74±0.162	3.43
Length of head to side base of mandibles			
L_1	0.26-0.34	0.29±0.021	7.07
L_2	0.36-0.39	0.38±0.009	2.38
L_3	0.49-0.57	0.52±0.024	4.55
MW_1	0.54-0.62	0.57±0.023	3.99
MW_2	0.77-0.85	0.82±0.026	3.16
MW_3 (n=10)	0.85-0.93	0.88±0.029	3.25
MW	1.03-1.45	1.30±0.095	7.34
Maximum width of head			
L_1	0.34-0.49	0.43±0.031	7.32
L_2	0.54-0.62	0.58±0.004	3.33
L_3	0.65-0.75	0.71±0.029	4.05
MW_1	0.72-0.85	0.77±0.33	4.23
MW_2	1.03-1.19	1.13±0.039	3.49
MW_3 (n=10)	1.16-1.24	1.22±0.026	2.10
MW	1.39-1.63	1.53±0.069	4.53
Length of hind tibia			
L_1	0.18-0.23	0.19±0.014	7.39
L_2	0.34-0.39	0.37±0.015	4.21
L_3	0.44-0.49	0.45±0.015	3.27
MW_1	0.59-0.72	0.66±0.030	4.55
MW_2	0.77-0.88	0.83±0.024	2.90
MW_3 (n-10)	0.85-0.90	0.87±0.024	2.74
MW	0.98-1.24	1.14±0.075	6.63

Length of head to side base of mandibles

The range, mean and standard deviation of length of head to side base of mandibles for different instars of major worker line are given in Table VIII. Frequency distribution of various instars of major workers of *O. obesus* for length of head to side base of mandibles is illustrated in Fig.3.

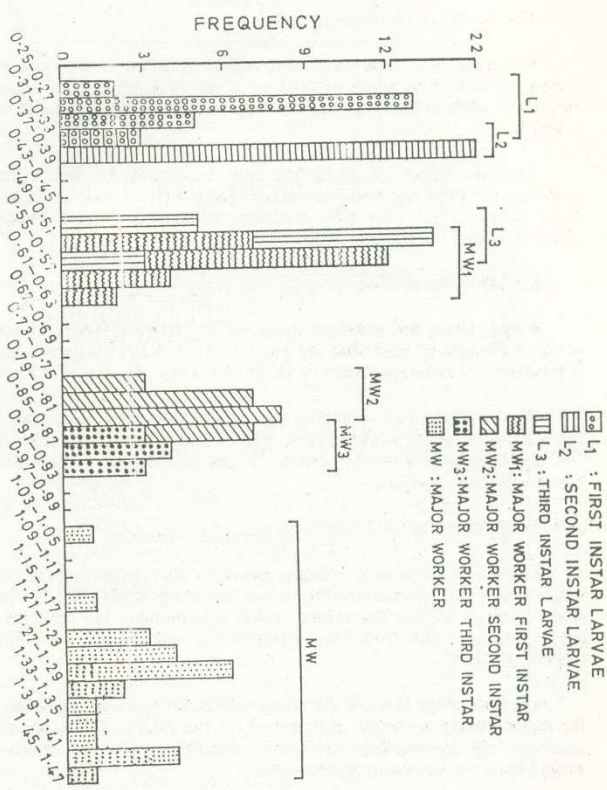

Fig. 3: Frequency distribution of length of head to side base of mandible in different instars of major worker line of *O. obesus*.

The co-efficient of variability was maximum for adult major workers, whereas, it was minimum for second instar larva of *O. obesus* (Table VIII). Comparison of different instars of major workers of *O. obesus* for maximum width of head revealed significant differences ($P<0.05$) in all the instars studied.

Maximum width of head

The range, mean and standard deviation of different instars of major workers of *O. obesus* for maximum width of head are given in Table VIII. Frequency distribution of maximum width of head of different instars of major workers of *O. obesus* is illustrated in Fig.4.

The co-efficient of variability was maximum for first instar larva, whereas, minimum for third instar major worker (Table VIII). Comparison of different instars of major workers of *O. obesus* for maximum width of head revealed significant differences ($P<0.05$) in all the instars studied.

Length of hind tibia

Range, mean and standard deviation of different instars of major workers of *O. obesus* for length of hind tibia are given in Table VIII. Frequency distribution of length of hind tibia of different instars of *O. obesus* is also illustrated in Fig.5.

The co-efficient of variability was maximum for first instar larva and was minimum for third instar major worker (Table VIII). Comparison of different instars of *O. obesus* (for length of hind tibia) by student "t" test revealed significant differences ($P<0.05$) between different instars.

Developmental line of minor worker

Like the major worker, the minor worker also passes through three larval and three successive minor worker instars before becoming adult (Fig.1). The first two larval instar of major worker and minor worker are common, but the third larval instar of the minor worker differ from the corresponding stage in the development of the major worker (Plate 1C).

The third stage larva of the minor worker differ from that of the third stage larva of the major worker in overall structure of the mandibles. The minor worker larva possess relatively anteroposteriorly elongated mandibles, whereas in major worker larva, mandibles are more robust and broader.

Third instar larva

Head and body whitish; unpigmented, head round. Mandibles unpigmented, left mandible with indications of apical and first marginal teeth; right mandible also with indication of apical and first marginal teeth (Plate 1C). Antennae with 13-14 articles, whitish, first and second article larger than others, second article nearly twice as long as third. Abdomen unpigmented and have a pair of styli. Tarsi 4-segmented (Table IX).

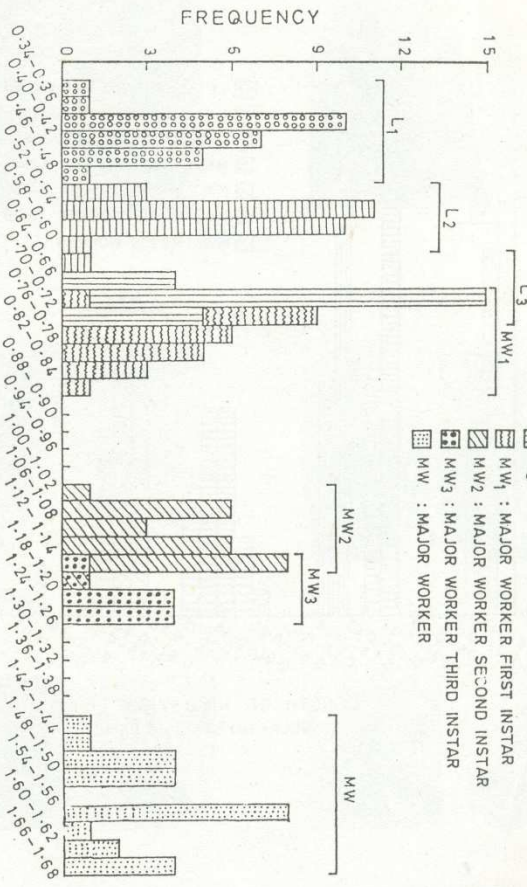

Fig. 4: Frequency distribution of maximum width of head in different instars of major worker line of *O. obesus*.

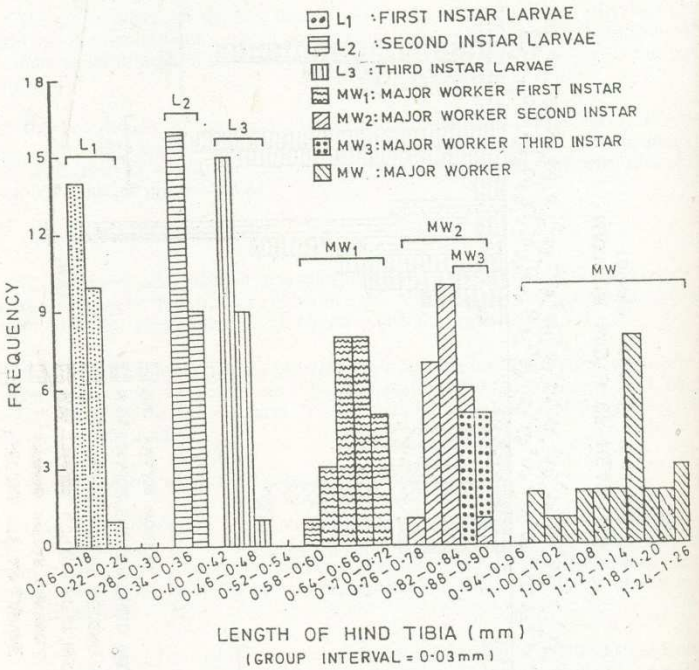

Fig. 5: **Frequency distribution of length of hind tibia in different instars of major worker line of *O. obesus*.**

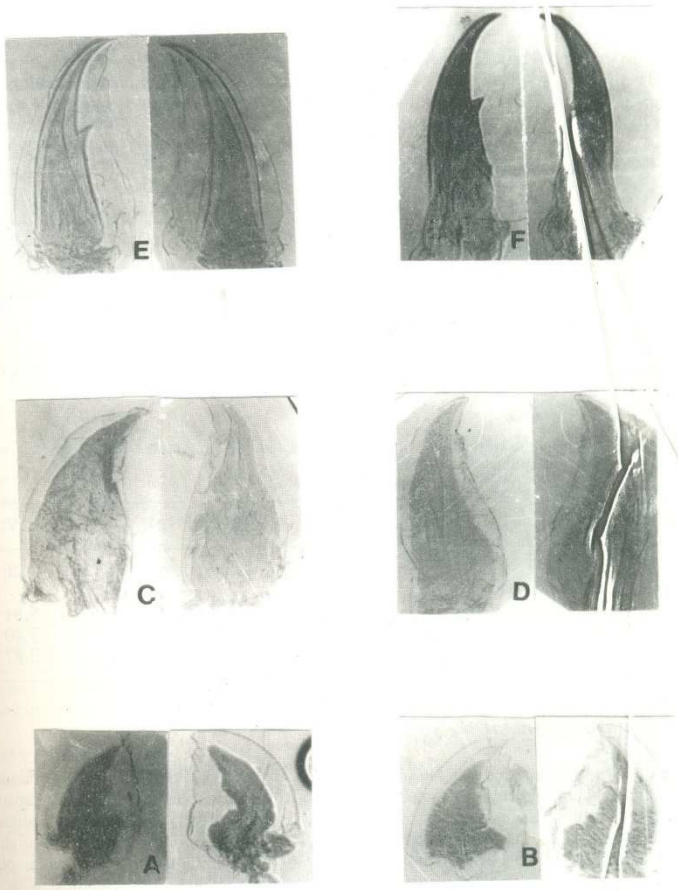

Plate II: Stages of mandibular development during soldier differentiation. A, first instar pre-soldier; B, second instar pre-soldier; C, third instar pre-soldier; D, fourth instar pre-soldier; E, fifth instar pre-soldier; F, mature soldier. Magnification: X100.

First instar minor worker

Head slightly darker than abdomen; head round; brain not clearly visible through cuticle. Mandibles whitish; unpigmented; right mandible with apical and first marginal more developed than third instar larva; left mandible first marginal and second marginal teeth differentiating within old cuticle (Plate 1D). Antennae with 14 articles. Abdomen with a pair of styli. Tarsi 4-segmented (Table X).

Table IX: **Biometric analysis of different characters of third instar larva of *O. obesus* (measurements are in mm).**

Character	Range (n=10)	Mean±S.D.	C.V.
Total body length	2.19-2.65	2.37±0.14	6.02
Length of head to side base of mandibles	0.39-0.44	0.41±0.018	4.32
Width of head	0.57-0.67	0.62±0.67	4.60
Length of hind tibia	0.36-0.41	0.38±0.015	3.85

Table X: **Biometric analysis of different characters of first instar minor worker of *O. obesus* (measurements are in mm).**

Character	Range (n=10)	Mean±S.D.	C.V.
Total body length	2.84-3.10	2.96±0.097	3.26
Length of head to side base of mandibles	0.46-0.57	0.52±0.030	5.81
Width of head	0.72-0.80	0.74±0.025	3.40
Length of hind tibia	0.44-0.46	0.45±0.013	2.87

Second instar minor worker

Head slightly darker than the abdomen; weakly sclerotized. Head nearly rounded; brain area visible through cuticle, nearly occupying whole of the head capsule. Mandibles more differentiated than the first instar minor worker; teeth slightly pigmented; left mandible with notch between first and second marginal teeth slightly indicated, second notch between posterior margin of second marginal and molar plate indistinctly indicated; tips of teeth slightly pigmented (Plate 1E). Antennae with 15 articles. Abdomen with a pair of styli. Tarsi 4-segmented (Table XI).

Third instar minor worker

Head and thorax more darker than the second instar minor worker; weakly sclerotized. Head round; brain clearly visible through cuticle, occupies 50% area of the head capsule. Mandibles more darkly pigmented than second instar minor worker; teeth completely differentiated (Plate 1F). Antennae with 16 articles. Abdomen weakly sclerotized, a pair of styli present. Tarsi 4-segmented (Table XII).

Table XI: **Biometric analysis of different characters of first second minor worker of *O. obesus* (measurements are in mm).**

Character	Range (n=10)	Mean±S.D.	C.V.
Total body length	3.15-3.61	3.41±0.130	3.83
Length of head to side base of mandibles	0.57-0.62	0.58±0.018	3.04
Width of head	0.77-0.83	0.79±0.018	2.30
Length of hind tibia	0.67-0.70	0.68±0.014	2.00

Table XII: **Biometric analysis of different characters of third instar minor worker of *O. obesus* (measurements are in mm).**

Character	Range (n=10)	Mean±S.D.	C.V.
Total body length	3.61-3.82	3.70±0.066	1.80
Length of head to side base of mandibles	0.62-0.67	0.64±0.018	2.75
Width of head	0.85-0.93	0.89±0.022	2.47
Length of hind tibia	0.70-0.75	0.72±0.021	2.88

Minor worker

Head brownish, much darker than third instar minor worker. Head oval; brain clearly visible, appearing smaller in comparison to size of the head capsule. Mandibles much sclerotized with well differentiated notches and fully developed teeth (Plate 1G). Antennae with 17 articles, first and second antennal articles much larger than others. Abdomen with a pair of styli. Tarsi 4-segmented (Table XIII).

Table XIII: **Biometric analysis of different characters of minor worker of *O. obesus* (measurements are in mm).**

Character	Range (n=10)	Mean±S.D.	C.V.
Total body length	3.74-4.00	3.87±0.081	2.09
Length of head to side base of mandibles	0.77-0.85	0.81±0.022	2.68
Width of head	0.96-1.08	1.01±0.041	4.03
Length of hind tibia	0.93-0.96	0.94±0.013	1.35

Comparison of different instars of minor worker of O. obesus

Total body length

Range, mean and standard deviation of different instars of minor worker of *O. obesus* for total body length are given in Table XIV.

Table XIV: Age variation in different parameters in measurement of different instars of minor worker of *O. obesus* (vertical lines alongside the instars indicate non-significant differences; P>0.05).

Instar	Range (n=10)	Mean±S.D.	C.V.
Total body length			
L_1	2.19-2.63	2.37±0.143	6.02
mW_1	2.84-3.10	2.96±0.097	3.26
mW_2	3.15-3.61	3.41±0.130	3.83
mW_3	3.61-3.82	3.70±0.066	1.80
mW	3.74-4.00	3.87±0.081	2.09
Length of head to side base of mandibles			
L_3	0.39-0.44	0.41±0.018	4.32
mW_1	0.46-0.57	0.52±0.030	5.81
mW_2	0.57-0.62	0.58±0.018	3.04
mW_3	0.62-0.67	0.64±0.018	2.75
mW	0.77-0.85	0.81±0.022	2.68
Maximum width of head			
L_3	0.57-0.67	0.62±0.028	4.60
mW_1	0.72-0.80	0.74±0.025	3.40
mW_2	0.77-0.83	0.79±0.018	2.30
mW_3	0.85-0.93	0.89±0.022	2.47
mW	0.96-1.08	1.01±0.041	4.03
Length of hind tibia			
L_3	0.36-0.41	0.38±0.015	3.85
mW_1	0.44-0.46	0.45±0.013	2.87
mW_2	0.67-0.70	0.68±0.014	2.00
mW_3	0.70-0.75	0.72±0.021	2.88
mW	0.93-0.96	0.94±0.013	1.35

Frequency distribution of different instars of minor worker of *O. obesus* for total body length is illustrated in Fig.6.

The co-efficient of variability was 6.02, 3.26, 3.83, 1.80 and 2.09 for third instar larva, first instar, second instar, third instar minor workers and adult minor workers, respectively (Table XIV).

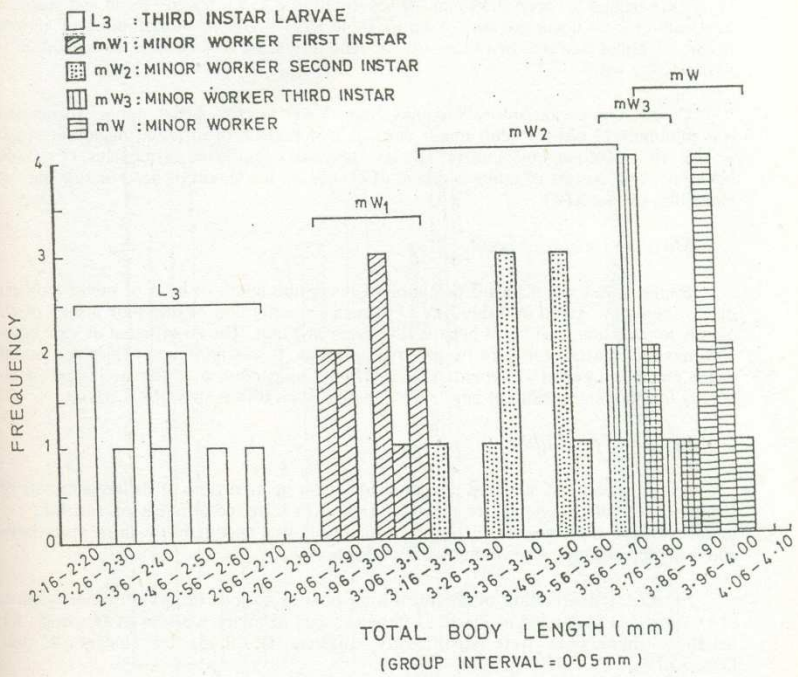

Fig. 6: Frequency distribution of total body length in different instars of minor worker line of *O. obesus*.

Comparison of different instars by student "t" test revealed significant differences ($P<0.05$) between all the instars of *O. obesus* for total body length.

Length of head to side base of mandibles

For length of head to side base of mandibles, different instars of minor worker of *O. obesus* ranged between 0.39 mm - 0.85 mm (Table XIV). Range, mean and standard deviation of each instar are also given in Table XIV. Frequency distribution of various instars of minor workers of *O. obesus* for length of head to side base of mandibles is illustrated in Fig.7.

Co-efficient of variability was maximum (5.81) for first instar minor worker and was minimum (2.68) for adult minor worker. Comparison of different instars of minor worker of *O. obesus* by student "t" test revealed significant differences ($P<0.05$) between all the instars of minor workers of *O. obesus* for length of head to side base of mandibles (Table XIV).

Maximum width of head

Range, mean and standard deviation of maximum width of head of minor workers of *O. obesus* are given in Table XIV. Frequency distribution of different instars of *O. obesus* for maximum width of head is illustrated in Fig.8. The co-efficient of variability was maximum (4.60) for third instar larva, whereas, it was minimum (2.30) for second instar minor worker of *O. obesus* (Table XIV). Comparison of different instars of *O. obesus* for maximum width of head revealed significant differences ($P<0.05$).

Length of hind tibia

Range, mean and standard deviation of length of hind tibia of different instars of minor worker of *O. obesus* are given in Table XIV. The co-efficient of variability of minor workers of *O. obesus* for length of hind tibia was maximum for third instar larva and was minimum for adult minor worker (Table XIV).

Frequency distribution of length of hind tibia of various instars of minor workers of *O. obesus* is illustrated in Fig.9. Different instars of minor workers of *O. obesus* for length of hind tibia were significantly different ($P<0.05$) by student "t" test (Table XIV).

Developmental line of soldier

The statistical data pertaining to immature stages in a field colony of *O. obesus* reveal that the third stage larva from which arises the developmental line of minor worker, also give rise to soldier line. The pre-soldier arising from the third instar larva

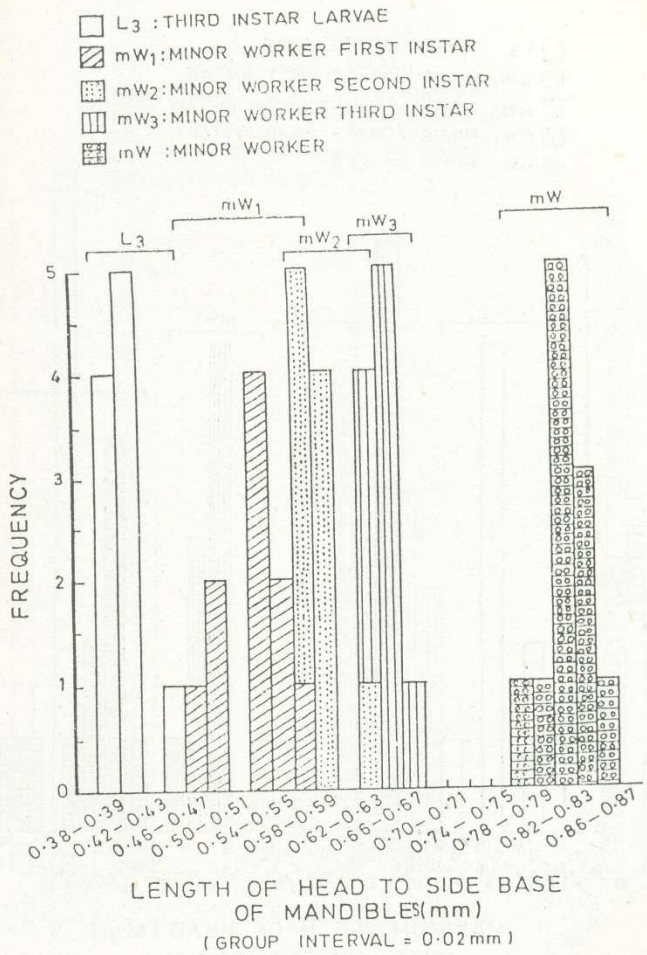

Fig. 7: **Frequency distribution of length of head to sidebase of mandibles in different instars of minor workers line of *O. obesus*.**

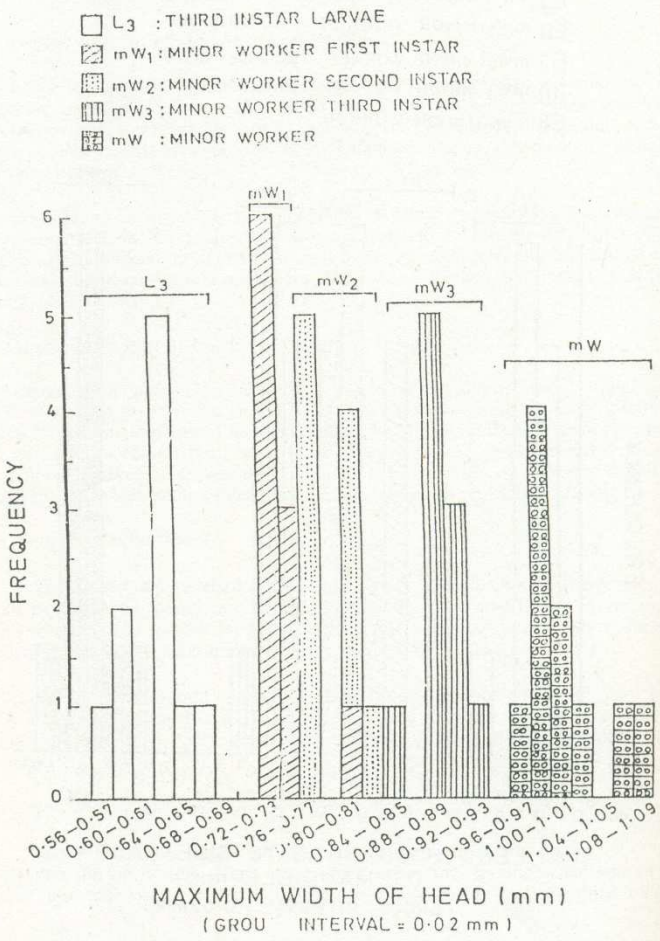

Fig. 8: **Frequency distribution of maximum width of head in instars of minor worker line of *O. obesus*.**

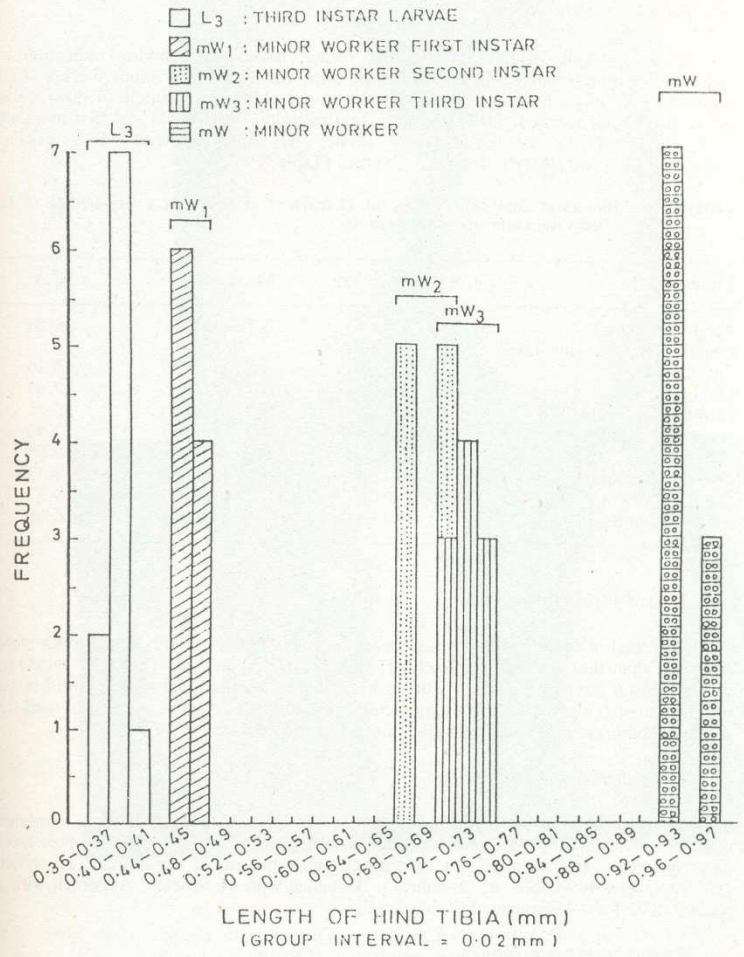

Fig. 9: **Frequency distribution of length of hind tibia different in instars of minor worker of *O. obesus*.**

moults for five times before becoming adult (Fig.1). The various stages of development of the soldier are described below in detail.

First instar pre-soldier

Head and body whitish, head nearly round. Mandibles showing indication of soldier development within the mandibles of third instar larva of minor worker. Left mandible with apical tooth clearly visible within the older intact cuticle of third instar larva, mandibular tooth slightly indicated; right mandible with apical and first marginal tooth developed like the left mandible (Plate 2A). Antennae with 14-15 articles, abdomen with a pair of styli. Tarsi 4-segmented (Table XV).

Table XV: Biometric analysis of different characters of first instar pre-soldier of *O. obesus* (measurements are in mm).

Character	Range (n=25)	Mean±S.D.	C.V.
Total body length	3.15-3.61	3.37±0.136	4.04
Length of head to side base of mandibles	0.54-0.64	0.60±0.031	5.10
Maximum width of head	0.75-0.83	0.78±0.021	2.67
Length of left mandible	0.36-0.44	0.41±0.024	5.74
Length of hind tibia	0.67-0.73	0.70±0.018	2.56
Length of pronotum	0.21-0.31	0.26±0.025	9.65
Width of pronotum	0.46-0.54	0.50±0.024	4.73
Length of brain (n=20)	0.26-0.36	0.32±0.030	9.37
Width of brain (n=20)	0.49-0.65	0.56±0.039	6.98

Second instar pre-soldier

Head and abdomen whitish; head nearly round. Mandibles with apical tooth more elongated than the first instar pre-soldier, tips slightly pigmented. The older cuticle of the third instar larva of the minor worker still persist; first marginal tooth of third instar larva of minor worker still not completely absorbed (Plate 2B). Antennae with 15 articles. Abdomen with a pair of styli. Tarsi 4-segmented (Table XVI).

Third instar pre-soldier

Head and abdomen whitish, head nearly oval. Mandibles whitish, more elongated than second instar pre-soldier; first marginal tooth of third instar minor worker larva much absorbed; sclerotization not started yet, inner margin of mandibles wavy (Plate 2C). Brain clearly visible, much reduced. Antennae with 16 articles. Abdomen with a pair of styli. Tarsi 4-segmented (Table XVII).

Fourth instar pre-soldier

Head oval, slightly pigmented, dirty white; mandibles brownish yellow, well differentiated; left mandible with distinct tooth, right mandible with slight indication of tooth (Plate 2D). Brain visible, much reduced as compare to head capsule. Abdomen not

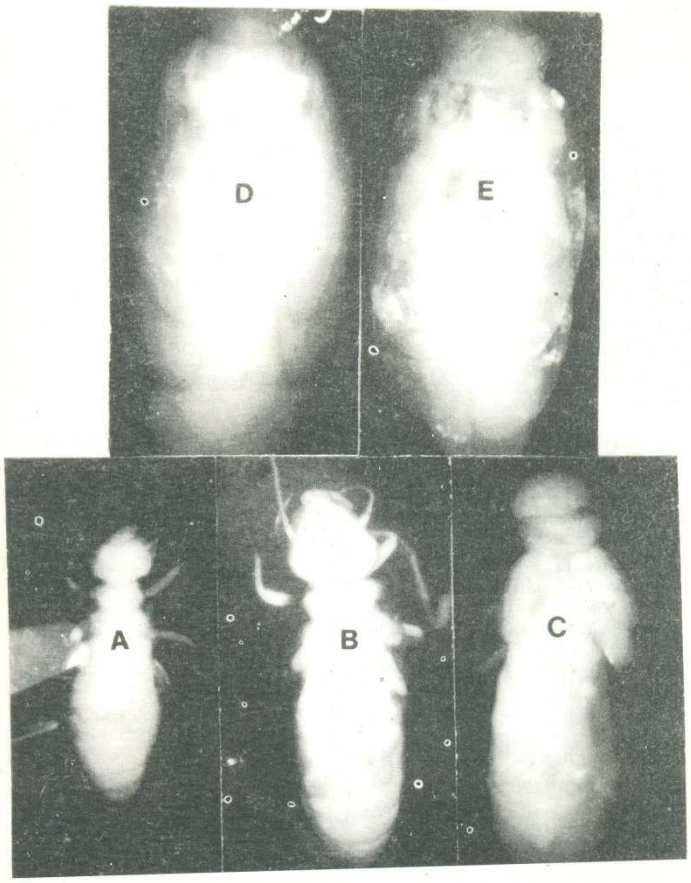

Plate III: Stages of development of wing pads during alate differentiation. A, first instar nymph; B, second instar nymph; C, third instar nymph; D, fourth instar nymph; E, fifth instar nymph.

completely pigmented. Antennae with 16 articles. Abdomen with a pair of styli. Tarsi 4-segmented (Table XVIII).

Table XVI. Biometric analysis of different characters of second instar pre-soldier of *O. obesus* (measurements are in mm).

Character	Range (n=10)	Mean±S.D.	C.V.
Total body length	3.48-3.74	3.63±0.077	2.13
Length of head to side base of mandibles	0.59-0.72	0.66±0.044	6.65
Maximum width of head	0.83-0.90	0.88±0.027	3.10
Length of left mandible	0.44-0.52	0.48±0.022	4.59
Length of hind tibia	0.72-0.77	0.75±0.015	1.99
Length of pronotum	0.23-0.31	0.28±0.028	9.75
Width of pronotum	0.46-0.59	0.52±0.050	9.60
Length of brain	0.28-0.34	0.31±0.091	6.16
Width of brain	0.52-0.59	0.56±0.025	4.48

Table XVII: Biometric analysis of different characters of third instar pre-soldier of *O. obesus* (measurements are in mm).

Character	Range (n=25)	Mean±S.D.	C.V.
Total body length	3.74-4.52	4.15±0.218	5.25
Length of head to side base of mandibles	0.88-1.01	0.95±0.030	3.16
Maximum width of head	0.98-1.08	1.04±0.033	3.14
Length of left mandible	0.88-0.93	0.91±0.014	1.51
Length of hind tibia	0.88-0.096	0.93±0.024	2.55
Length of pronotum	0.31-0.39	0.33±0.023	6.92
Width of pronotum	0.72-0.81	0.76±0.024	3.15
Length of brain (n=24)	0.31-0.41	0.34±0.028	8.27
Width of brain (n=24)	0.52-0.65	0.59±0.031	5.19

Fifth instar pre-soldier

Head rectangularly oval; head and abdomen much sclerotized than fourth instar pre-soldier. Mandibles darkly pigmented, more pointed (Plate 2E). Brain clearly visible, appearing smaller in comparison to the size of the head capsule. Antennae with 16 articles, dirty white throughout its length. Abdomen with a pair of styli. Tarsi 4-segmented (Table XIX).

Table XVIII: Biometric analysis of different characters of fourth instar pre-soldier of *O. obesus* (measurements are in mm).

Character	Range (n=10)	Mean±S.D.	C.V.
Total body length	4.52-4.90	4.69±0.121	2.57
Length of head to side base of mandibles	0.96-1.03	0.98±0.024	2.47
Maximum width of head	1.03-1.11	1.08±0.027	2.54
Length of left mandible	0.90-0.93	0.92±0.013	1.46
Length of hind tibia	0.93-0.98	0.96±0.017	1.78
Length of pronotum	0.34-0.41	0.38±0.021	5.65
Width of pronotum	0.75-0.88	0.79±0.047	5.98
Length of brain	0.31-0.41	0.35±0.040	11.41
Width of brain	0.54-0.62	0.60±0.027	4.53

Table XIX: Biometric analysis of different characters of fifth instar pre-soldier of *O. obesus* (measurements are in mm).

Character	Range (n=10)	Mean±S.D.	C.V.
Total body length	5.31-5.55	5.31±0.200	3.76
Length of head to side base of mandibles	1.34-1.50	1.42±0.053	3.77
Maximum width of head	1.29-1.37	1.31±0.024	1.87
Length of left mandible	0.96-1.06	1.01±0.028	2.82
Length of hind tibia	1.03-1.16	1.10±0.044	3.98
Length of pronotum	0.49-0.57	0.54±0.027	5.06
Width of pronotum	0.96-1.03	0.99±0.024	2.47
Length of brain	0.28-0.34	0.32±0.017	5.39
Width of brain	0.54-0.62	0.59±0.024	4.13

Mature soldier

Head rectangularly oval; head and abdomen fully sclerotized. Mandibles darkly pigmented, more pointed (Plate 2F). Brain visible in some specimens. Antennae with 16-17 articles, light brown throughout its length. Abdomen with a pair of styli. Tarsi 4-segmented (Table XX).

Comparison of different instars of soldiers of O. obesus

Total body length

Range, mean and standard deviation of different instars of soldiers for total body length are given in Table XXI. Frequency distribution of different instars of soldiers of *O. obesus* for total body length is illustrated in Fig.10.

Table XX: **Biometric analysis of different characters of soldiers of *O. obesus* (measurements are in mm).**

Character	Range (n=10)	Mean±S.D.	C.V.
Total body length	5.88-6.19	6.05±0.124	2.04
Length of head to side base of mandibles	1.42-1.50	1.45±0.034	2.33
Maximum width of head	1.32-1.39	1.35±0.025	1.84
Length of left mandible	1.01-1.06	1.04±0.018	1.69
Length of hind tibia	1.11-1.16	1.14±0.021	1.88
Length of pronotum	0.52-0.57	0.54±0.023	4.22
Width of pronotum	0.96-1.01	0.98±0.019	1.92
Length of brain	0.34-0.38(n=5)	0.36±0.022	6.04
Width of brain	0.57-0.62(n=5)	0.60±0.021	3.50

The co-efficient of variability was maximum (5.25) for third instar pre-soldier and was minimum for adult soldiers (Table XXI). Comparison of different instars of soldiers by student "t" test revealed significant differences ($P<0.05$).

Length of head to side base of mandibles

Range, mean and standard diviation of various instars of soldiers of *O. obesus* for length of head to side base of mandibles are given in Table XXI. Frequency distribution of different instars of soldiers is illustrated in Fig.11. The co-efficient of variability was maximum (6.65) for second instar pre-soldier, whereas it was minimum for adult soldiers. Comparison of different instars based on student "t" test revealed significant differences ($P<0.05$) between different instars. However, non-significant differences ($P>0.05$) were noted between fifth instar pre-soldier and adult soldier (Table XXI).

Maximum width of head

Range, mean and standard deviation of different instars of soldiers of *O. obesus* for maximum width of head are given in Table XXI. Frequency distribution of maximum width of head of different instars of soldiers of *O. obesus* is illustrated in Fig.12.

The co-efficient of variability is also indicated in Table XXI. As is indicated in Table maximum C.V. value was noted for third instar pre-soldiers, whereas, for adult soldier C.V. value was minimum.

Comparison of different instars of soldiers by student "t" test revealed significant differences ($P<0.05$) between all the instars (Table XXI).

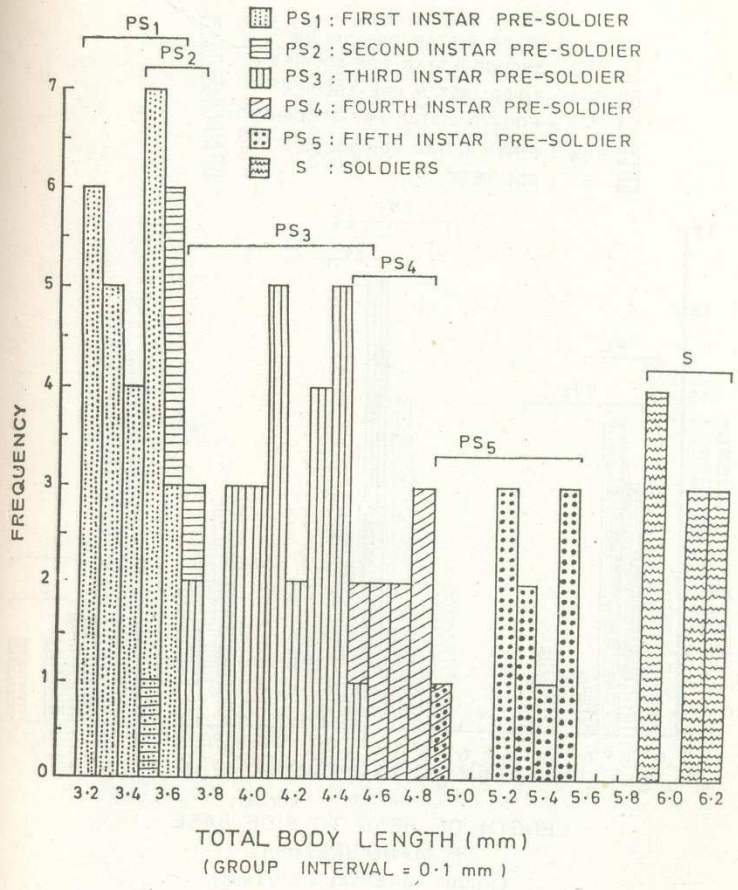

Fig. 10: **Frequency distribution of total body length in different instars of soldier line of *O. obesus*.**

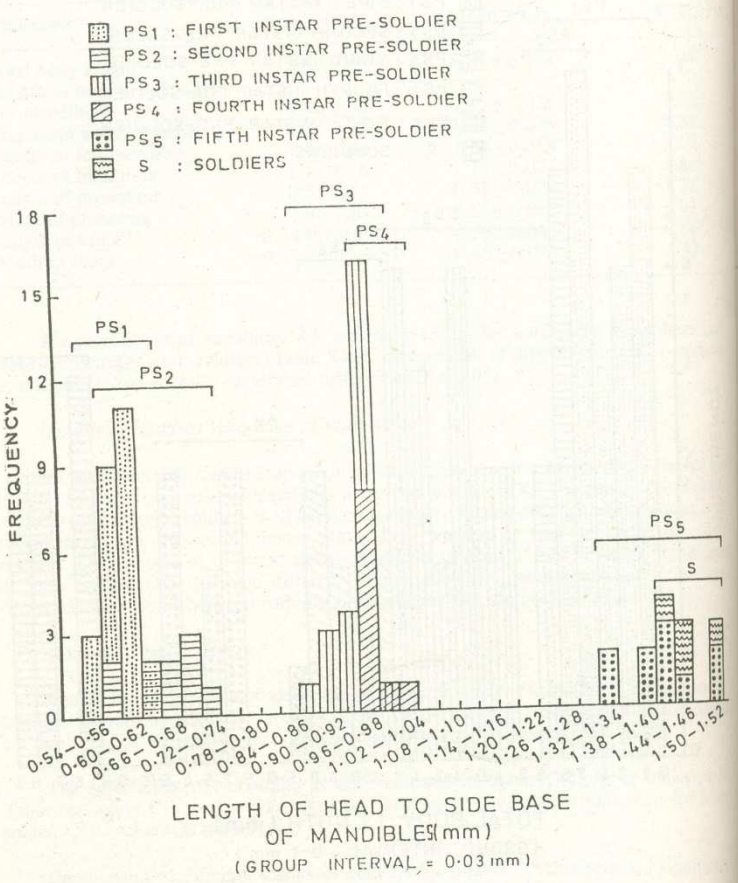

Fig. 11: **Frequency distribution of length of head to side base of mandibles in differe instars of soldier line of *O. obesus*.**

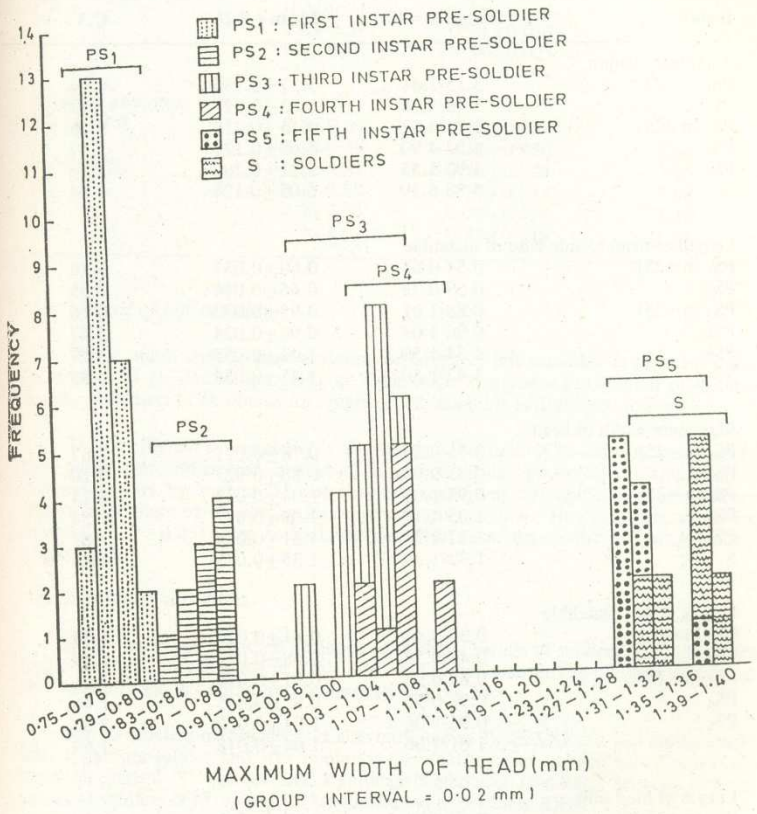

Fig. 12: **Frequency distribution of maximum width of head in different instars of soldier line of *O. obesus*.**

Table XXI: **Age variation in different parameters in measurement of different parameters of soldier instars of *O. obesus* (vertical lines alongside the instars indicate non-significant differences; P>0.05).**

Instar	Range (n=10)	Mean±S.D.	C.V.
Total body length			
PS_1 (n=25)	3.15-3.61	3.37±0.136	4.04
PS_2	3.48-3.74	3.63±0.077	2.13
PS_3 (n=25)	3.74-4.52	4.15±0.218	5.25
PS_4	4.52-4.90	4.69±0.121	2.57
PS_5	4.90-5.55	5.31±0.200	3.76
S	5.88-6.19	6.05±0.124	2.04
Length of head to side base of mandible			
PS_1 (n=25)	0.54-0.64	0.60±0.031	5.10
PS_2	0.59-0.72	0.66±0.044	6.65
PS_3 (n=25)	0.88-1.01	0.95±0.030	3.16
PS_4	0.96-1.03	0.98±0.024	2.47
PS_5	1.34-1.50	1.42±0.053	3.77
S	1.42-1.50	1.45±0.034	2.33
Maximum width of head			
PS_1 (n=25)	0.75-0.83	0.78±0.021	2.67
PS_2	0.83-0.90	0.88±0.027	3.10
PS_3 (n=25)	0.98-1.08	1.04±0.033	3.14
PS_4	1.03-1.11	1.08±0.027	2.54
PS_5	1.27-1.37	1.31±0.024	1.87
S	1.32-1.39	1.35±0.025	1.84
Length of left mandible			
PS_1 (n=25)	0.36-0.44	0.41±0.024	5.74
PS_2	0.44-0.52	0.48±0.022	4.59
PS_3 (n=25)	0.88-0.93	0.91±0.014	1.51
PS_4	0.90-0.93	0.92±0.013	1.46
PS_5	0.96-1.06	1.01±0.028	2.82
S	1.01-1.06	1.04±0.018	1.69
Length of hind tibia			
PS_1 (n=25)	0.67-0.72	0.70±0.018	2.56
PS_2	0.72-0.77	0.75±0.015	1.99
PS_3 (n=25)	0.88-0.96	0.93±0.024	2.55
PS_4	0.93-0.98	0.96±0.017	1.78
PS_5	1.03-1.16	1.10±0.044	3.98
S	1.11-1.16	1.14±0.021	1.88

Cont'd,..

Length of pronotum			
PS_1 (n=25)	0.21-0.31	0.26±0.025	9.65
PS_2	0.23-0.31	0.28±0.028	9.75
PS_3 (n=25)	0.31-0.39	0.33±0.023	6.92
PS_4	0.34-0.41	0.38±0.021	5.65
PS_5	0.49-0.57	0.54±0.027	5.06
S	0.52-0.57	0.54±0.023	4.22
Width of pronotum			
PS_1 (n=25)	0.46-0.54	0.50±0.024	4.73
PS_2	0.46-0.59	0.52±0.050	9.60
PS_3 (n=25)	0.72-0.81	0.76±0.024	3.15
PS_4	*0.75-0.88*	*0.79±0.047*	*5.98*
PS_5	0.96-1.03	0.99±0.024	2.47
S	0.96-1.01	0.98±0.019	1.92

Length of left mandible

Range, mean and standard deviation of length of left mandible in different instars of soldiers of *O. obesus* are given in Table XXI. Frequency distribution of different instars of soldiers of *O. obesus* for length of left mandible is illustrated in Fig.13.

The co-efficient of variability is also indicated in Table XXI and was maximum for first instar pre-soldier and minimum for fourth instar pre-soldier. Comparison of different instars by student "t" test revealed significant differences between different instars of soldiers of *O. obesus* for length of left mandible. However, non-significant differences ($P>0.05$) were noticed between fifth instar pre-soldier and adult soldiers (Table XXI).

Length of hind tibia

Range, mean and standard deviation of different instars of soldiers of *O. obesus* for length of hind tibia are given in Table XXI. Frequency distribution of various instars of *O. obesus* for length of hind tibia is illustrated in Fig.14.

The co-efficient of variability is also indicated in Table XXI and was maximum for fifth instar pre-soldier and was minimum for fourth instar pre-soldiers. Comparison based on student "t" test revealed significant differences ($P<0.05$) between different instars of soldiers of *O. obesus* for length of hind tibia (Table XXI).

Length of pronotum

Range, mean and standard deviation of different instars of soldiers of *O. obesus* for length of pronotum are given in Table XXI. Frequency distribution of different instars of soldiers of *O. obesus* for length of pronotum is illustrated in Fig.15.

Length of pronotum			
PS_1 (n=25)	0.21-0.31	0.26±0.025	9.65
PS_2	0.23-0.31	0.28±0.028	9.75
PS_3 (n=25)	0.31-0.39	0.33±0.023	6.92
PS_4	0.34-0.41	0.38±0.021	5.65
PS_5	0.49-0.57	0.54±0.027	5.06
S	0.52-0.57	0.54±0.023	4.22
Width of pronotum			
PS_1 (n=25)	0.46-0.54	0.50±0.024	4.73
PS_2	0.46-0.59	0.52±0.050	9.60
PS_3 (n=25)	0.72-0.81	0.76±0.024	3.15
PS_4	0.75-0.88	0.79±0.047	5.98
PS_5	0.96-1.03	0.99±0.024	2.47
S	0.96-1.01	0.98±0.019	1.92

Length of left mandible

Range, mean and standard deviation of length of left mandible in different instars of soldiers of *O. obesus* are given in Table XXI. Frequency distribution of different instars of soldiers of *O. obesus* for length of left mandible is illustrated in Fig.13.

The co-efficient of variability is also indicated in Table XXI and was maximum for first instar pre-soldier and minimum for fourth instar pre-soldier. Comparison of different instars by student "t" test revealed significant differences between different instars of soldiers of *O. obesus* for length of left mandible. However, non-significant differences ($P>0.05$) were noticed between fifth instar pre-soldier and adult soldiers (Table XXI).

Length of hind tibia

Range, mean and standard deviation of different instars of soldiers of *O. obesus* for length of hind tibia are given in Table XXI. Frequency distribution of various instars of *O. obesus* for length of hind tibia is illustrated in Fig.14.

The co-efficient of variability is also indicated in Table XXI and was maximum for fifth instar pre-soldier and was minimum for fourth instar pre-soldiers. Comparison based on student "t" test revealed significant differences ($P<0.05$) between different instars of soldiers of *O. obesus* for length of hind tibia (Table XXI).

Length of pronotum

Range, mean and standard deviation of different instars of soldiers of *O. obesus* for length of pronotum are given in Table XXI. Frequency distribution of different instars of soldiers of *O. obesus* for length of pronotum is illustrated in Fig.15.

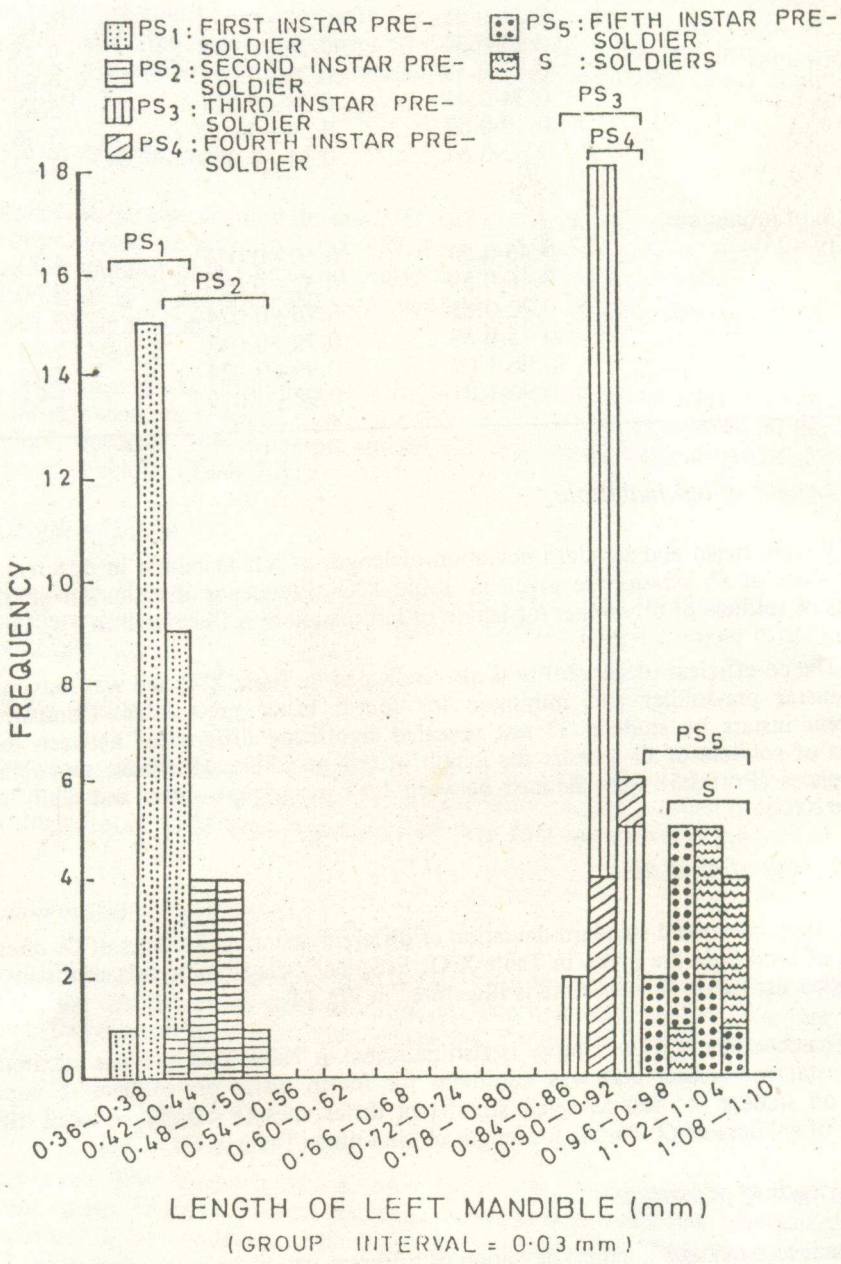

Fig. 13: **Frequency distribution of length of left mandible in different instars of soldier line of *O. obesus*.**

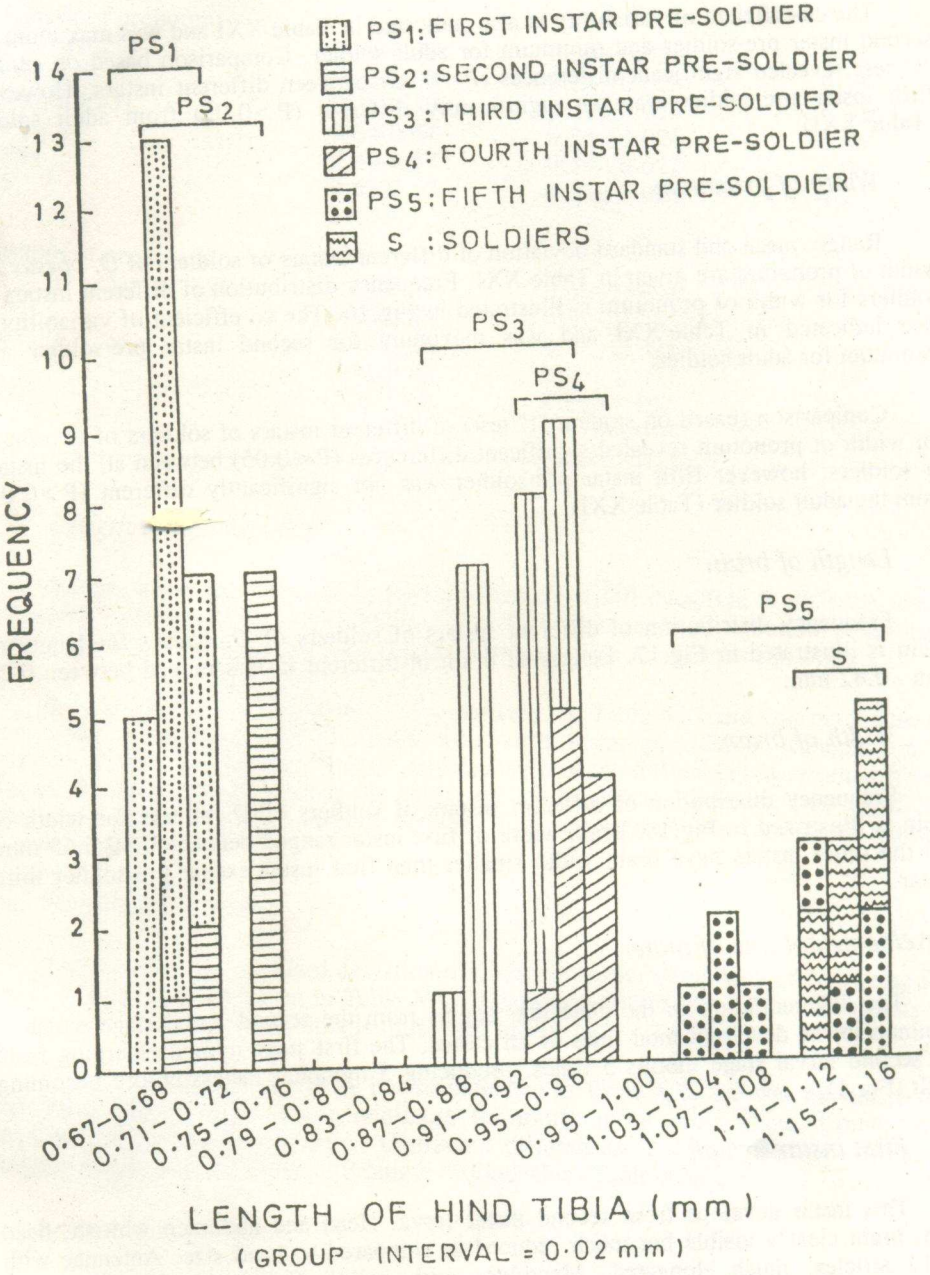

Fig. 14: **Frequency distribution of length of hind tibia in different instars of soldier line of *O. obesus*.**

The co-efficient of variability is also indicated in Table XXI and was maximum for second instar pre-soldier and minimum for adult soldier. Comparison based on student "t" test revealed significant differences ($P<0.05$) between different instars. However, fifth instar pre-soldier was not significantly different ($P>0.05$) from adult soldier (Table XXI).

Width of pronotum

Range, mean and standard deviation of different instars of soldiers of *O. obesus* for width of pronotum are given in Table XXI. Frequency distribution of different instars of soldiers for width of pronotum is illustrated in Fig.16. The co-efficient of variability is also indicated in Table XXI and was maximum for second instar pre-soldier and minimum for adult soldier.

Comparison (based on student "t" test) of different instars of soldiers of *O. obesus* for width of pronotum revealed significant differences ($P<0.05$) between all the instars of soldiers, however fifth instar pre-soldier was not significantly different ($P>0.05$) from the adult soldier (Table XXI).

Length of brain

Frequency distribution of different instars of soldiers of *O. obesus* for length of brain is illustrated in Fig.17. Length of brain in different instars ranged between 0.26 mm - 0.42 mm.

Width of brain

Frequency distribution of different instars of soldiers of *O. obesus* for width of brain is illustrated in Fig.18. Brain width of first instar ranged between 0.49-0.65 mm. All the other instars have brain width smaller than first instar except pre-soldier third instar.

Developmental line of alate

The differentiation of the alate line begins from the second larval stage which is common to the developmental lines of all castes. The first stage nymph emerging from the second larval stage moults 5 times, producing 5 nymphal instars before becoming adult (Fig.1).

First instar nymph

This instar develops from second instar larva. Head and abdomen whitish, head oval; brain clearly visible but much reduced as compared to head size. Antennae with 16-17 articles, much elongated. Mandibles with apical, first marginal and second marginal teeth slightly indicated. Thorax broader than second larval instar, mesothorax and metathorax with slight indication of wing pads (Plate 3A), abdomen with a pair of cerci and a pair of styli, Tarsi 4-segmented (Table XXII).

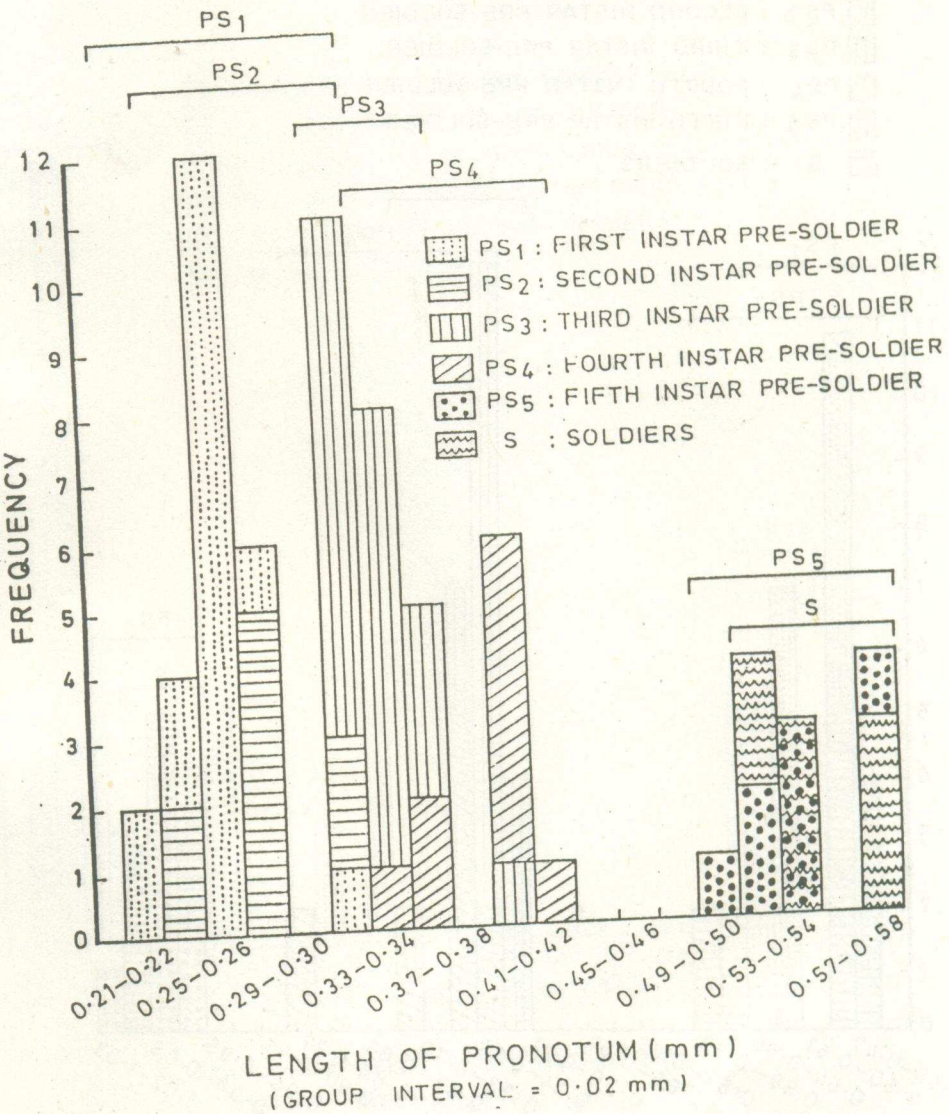

Fig. 15: **Frequency distribution of length of pronotum in different instars of soldier line of *O. obesus*.**

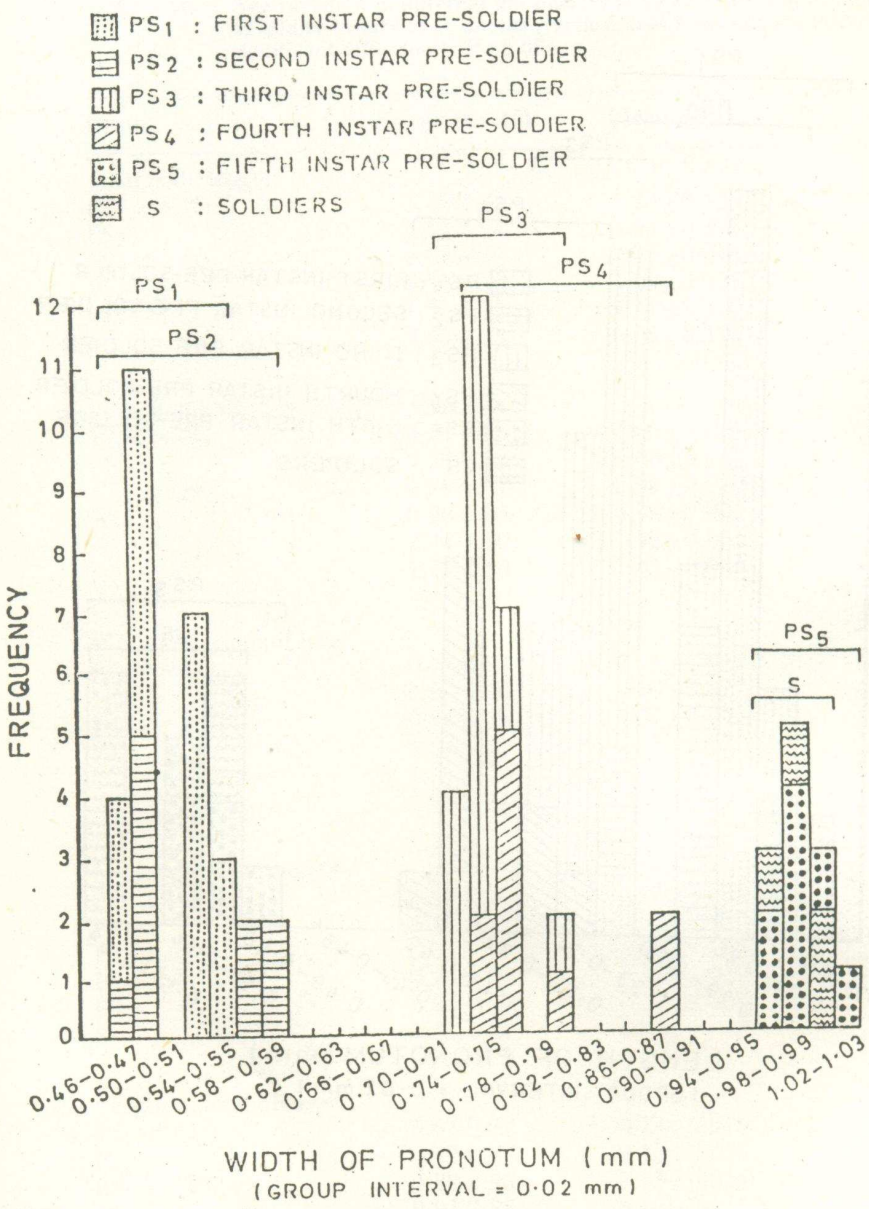

Fig. 16: **Frequency distribution of width of pronotum in different instars of soldier line of *O. obesus*.**

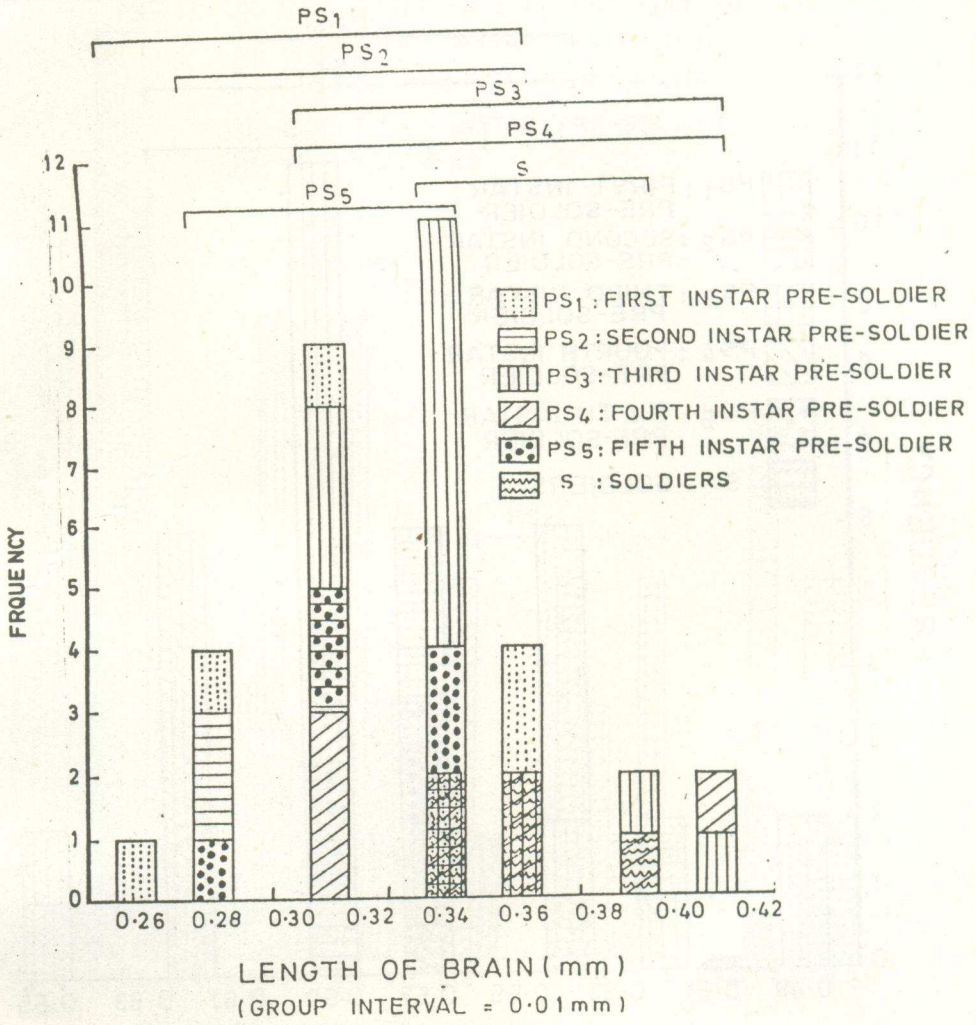

Fig. 17: **Frequency distribution of length of brain in different instars of soldier line of *O. obesus*.**

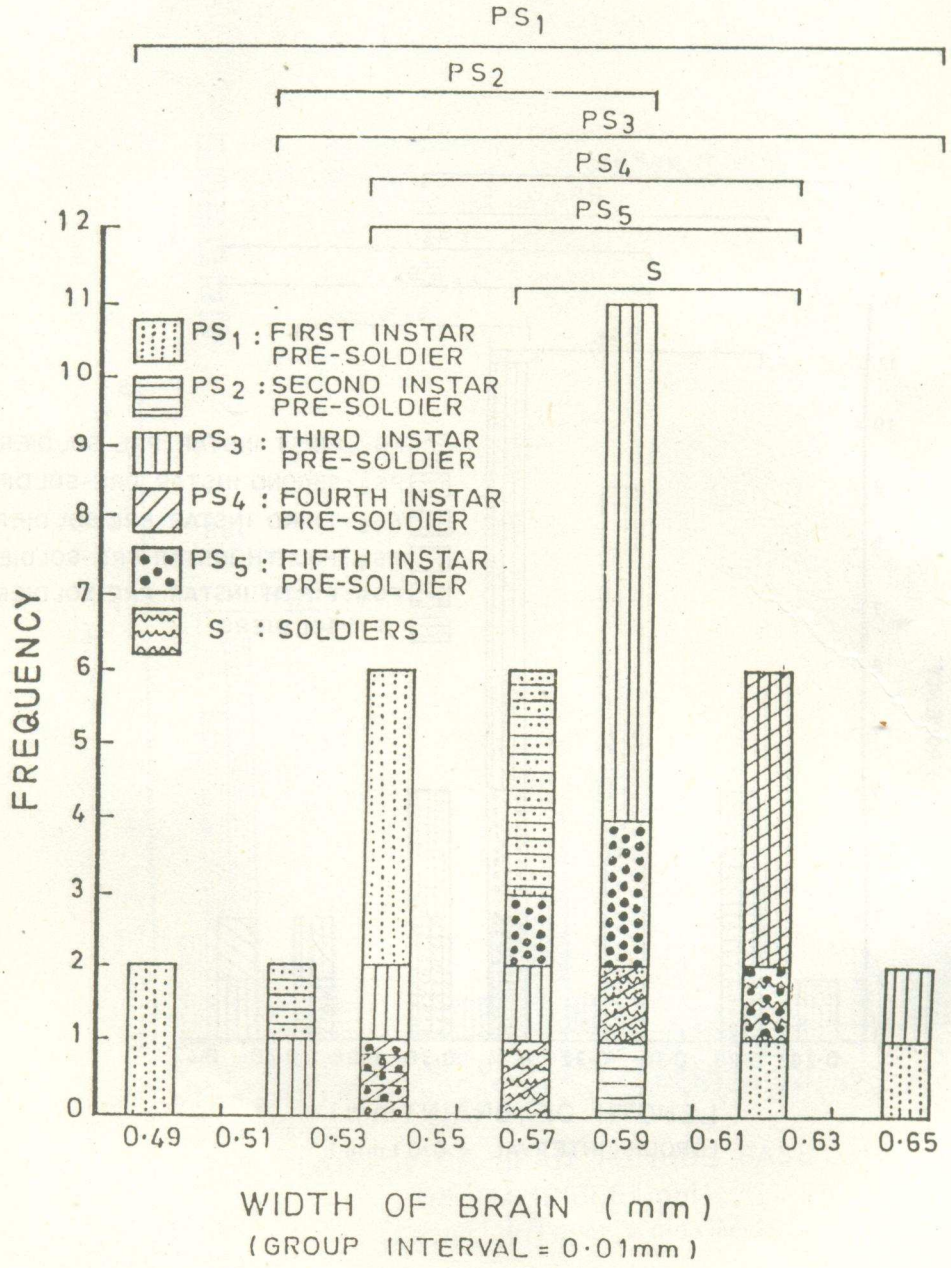

Fig. 18: **Frequency distribution of width of brain in different instars of soldier line of *O. obesus*.**

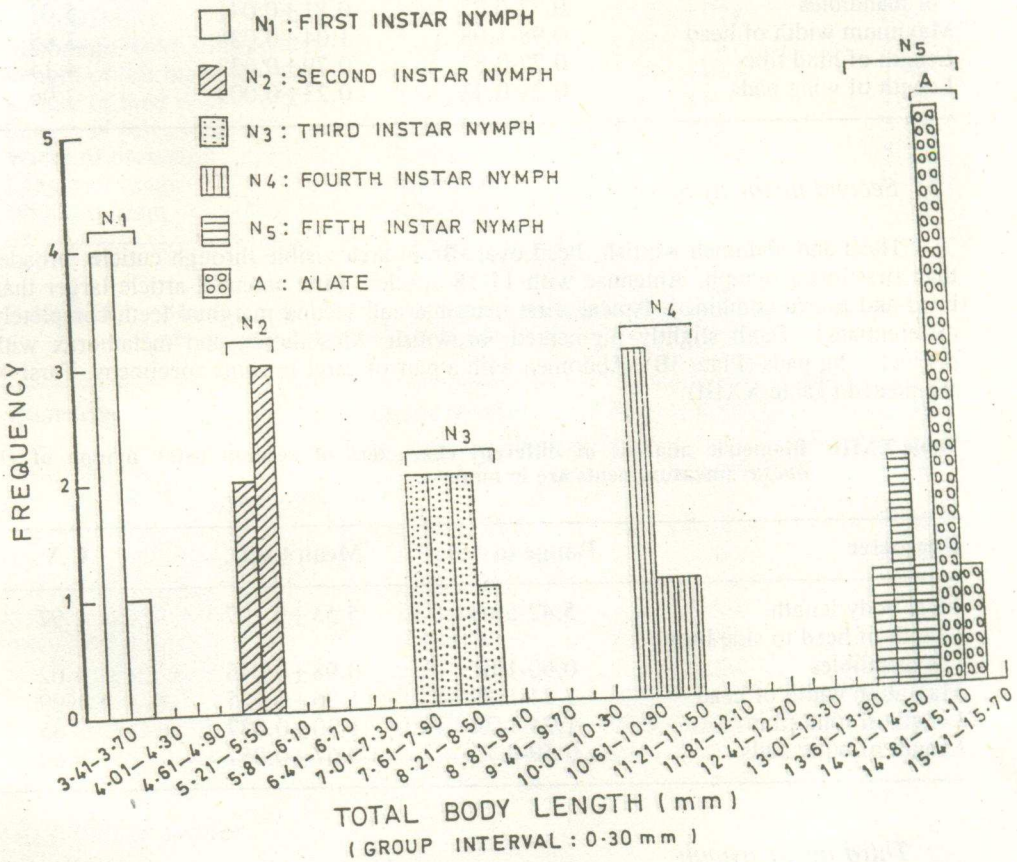

Fig. 19: **Frequency distribution of total body length in different instars of alate of *O. obesus*.**

Table XXII: Biometric analysis of different characters of first instar nymph of *O. obesus* (measurements are in mm).

Character	Range (n=5)	Mean±S.D.	C.V.
Total body length	3.48-3.77	3.63±0.112	3.10
Length of head to side base of mandibles	0.77-0.85	0.81±0.041	5.03
Maximum width of head	0.98-1.08	1.04±0.038	3.65
Length of hind tibia	0.77-0.83	0.79±0.033	4.14
Length of wing pads	0.21-0.23	0.23±0.009	3.96

Second instar nymph

Head and abdomen whitish, head oval. Brain area visible through cuticle, broader than first instar nymph. Antennae with 17-18 articles. First antennal article larger than third and fourth combined. Apical, first marginal and second marginal teeth completely differentiated. Teeth slightly pigmented, brownish. Mesothorax and metathorax with distinct wing pads (Plate 3B). Abdomen with a pair of cerci in some specimens. Tarsi 4-segmented (Table XXIII).

Table XXIII: Biometric analysis of different characters of second instar nymph of *O. obesus* (measurements are in mm).

Character	Range (n=5)	Mean±S.D.	C.V.
Total body length	5.42-5.68	5.55±0.107	1.92
Length of head to side base of mandibles	0.90-1.01	0.98±0.045	4.62
Maximum width of head	1.24-1.29	1.26±0.025	1.99
Length of hind tibia	1.24-1.34	1.30±0.037	2.85
Length of wing pads	0.59-0.72	0.67±0.057	8.60

Third instar nymph

Head and abdomen whitish. Head nearly round. Brain larger than second instar nymph. Antennae with 19 antennal articles. Differentiation of teeth more pronounced, slightly more pigmented than second instar nymph. Eyes slightly indicated. Thorax with wing pads longer than second instar nymph, extending up to 1/4 of abdomen (Plate 3C). Styli present in some specimens. Tarsi 4-segmented (Table XXIV).

Table XXIV: Biometric analysis of different characters of third instar nymph of *O. obesus* (measurements are in mm).

Character	Range (n=7)	Mean±S.D.	C.V.
Total body length	7.74-8.51	8.11±0.267	3.29
Length of head to side base of mandibles	1.08-1.24	1.15±0.067	5.81
Maximum width of head	1.60-1.75	1.68±0.059	3.53
Length of hind tibia	1.70-1.81	1.74±0.038	2.16
Length of wing pads	1.68-1.86	1.77±0.060	3.40

Fourth instar nymph

Head and abdomen dirty white, head nearly round with minute bristles and hairs. Labrum whitish. Mandibles more pigmented than third instar nymph. Eyes clearly indicated, much darker. Antennae with 19 articles. Thorax with wing pads larger than third instar nymph covering nearly 7/8 part of abdomen (Plate 3D). Styli present in some specimens. Tarsi 4-segmented (Table XXV).

Table XXV: Biometric analysis of different characters of fourth instar nymph of *O. obesus* (measurements are in mm).

Character	Range (n=5)	Mean±S.D.	C.V.
Total body length	10.53-10.96	10.67±0.174	1.63
Length of head to side base of mandibles	1.50-1.57	1.54±0.028	1.80
Maximum width of head	2.09-2.27	2.16±0.067	3.09
Length of hind tibia	2.06-2.32	2.20±0.096	4.36
Length of wing pads	4.95-5.21	5.09±0.103	2.03

Fifth instar nymph

Head light brown, brain not visible through cuticle of head. Eyes large, well developed, separated from the lower margin of head by nearly one fifth of its long diameter. Ocellus slightly indicated. Mandibles more pigmented than fourth instar nymph. Teeth of mandibles dark brown with blackish tinge. Antennae with 19 articles, slightly brownish. Wing pads light brown (Plate 3E). Abdomen slightly pigmented, clear indication of abdominal segments. Tarsi 4-segmented (Table XXVI).

Table XXVI: Biometric analysis of different characters of fifth instar nymph of *O. obesus* (measurements are in mm).

Character	Range (n=10)	Mean±S.D.	C.V.
Total body length	14.19-15.22	14.77±0.332	2.25
Length of head to side base of mandibles	1.81-1.93	1.86±0.036	1.93
Maximum width of head	2.27-2.40	2.32±0.043	1.86
Length of hind tibia	2.42-2.58	2.49±0.043	1.73
Length of wing pads	5.42-5.78	5.61±0.121	2.15

Alate

Head slightly reddish brown, brain not visible through cuticle. Eyes fully developed. Ocellus large, oval, clearly indicated. Antennae with 19 antennal articles, reddish brown. Postclypeus strongly arched. Wings fully developed, reddish brown. Labrum larger than wide. Tarsi 4-segmented (Table XXVII).

Table XXVII: Biometric analysis of different characters of alates of *O. obesus* (measurements are in mm).

Character	Range (n=5)	Mean±S.D.	C.V.
Total body length	14.83-15.22	15.04±0.148	0.99
Length of head to side base of mandibles	1.86-1.93	1.88±0.029	1.52
Maximum width of head	2.58-2.68	2.61±0.042	1.61
Length of hind tibia	2.68-2.83	2.75±0.121	2.12

Comparison of different instars of alate line of O. obesus

Total body length

The range, mean and standard deviation of total body length of different instars of alate line of *O. obesus* are given in Table XXVIII. Frequency distribution of total body length of different instars is illustrated in Fig.19.

The co-efficient of variability (Table XXVIII) was maximum for third instar nymph, whereas, it was minimum for alates, indicating that the growth has completed and the specimens are therefore relatively less variable. Comparison of different instars of alate line of *O. obesus* for total body length was carried out by student "t" test, which revealed significant differences between all the instars except in the case of fifth instar nymph which was not significantly different ($P>0.05$) from alates.

Table XXVIII: **Age variation in different instars of alate line of *O. obesus* (vertical lines alongside the instars indicate non-significant differences; P > 0.05).**

Instar	Range (n=5)	Mean±S.D.	C.V.
Total body length			
N_1	3.48-3.77	3.63±0.112	3.10
N_2	5.42-5.68	5.55±0.107	1.92
N_3 (n=7)	7.74-8.51	8.11±0.267	3.29
N_4	10.53-10.96	10.67±0.174	1.63
N_5 (n=10)	14.19-15.22	14.77±0.332	2.25
A	14.83-15.22	15.04±0.148	0.99
Length of head to side base of mandibles			
N_1	0.77-0.85	0.81±0.041	5.03
N_2	0.90-1.01	0.98±0.045	4.62
N_3 (n=7)	1.08-1.24	1.15±0.067	5.81
N_4	1.50-1.57	1.54±0.028	1.80
N_5 (n=10)	1.81-1.93	1.86±0.036	1.93
A	1.86-1.93	1.88±0.029	1.52
Maximum width of head			
N_1	0.98-1.08	1.04±0.038	3.65
N_2	1.24-1.29	1.26±0.025	1.99
N_3 (n=7)	1.60-1.75	1.68±0.059	3.53
N_4	2.09-2.27	2.16±0.067	3.09
N_5 (n=10)	2.27-2.40	2.32±0.043	1.86
A	2.58-2.68	2.61±0.042	1.61
Length of hind tibia			
N_1	0.77-0.83	0.79±0.033	4.14
N_2	1.24-1.34	1.30±0.037	2.85
N_3 (n=7)	1.70-1.81	1.74±0.038	2.16
N_4	2.05-2.32	2.20±0.096	4.36
N_5 (n=10)	2.42-2.58	2.49±0.043	1.73
A	2.68-2.83	2.75±0.121	2.12
Length of wing pads			
N_1	0.21-0.23	0.23±0.009	3.96
N_2	0.59-0.72	0.67±0.057	8.60
N_3 (n=7)	1.68-1.86	1.77±0.060	3.40
N_4	4.95-5.21	5.09±0.103	2.03
N_5 (n=10)	5.42-5.78	5.61±0.121	2.15

Length of head to side base of mandibles

The range, mean and standard deviation of length of head to side base of mandibles for different instars of alate line of *O. obesus* are given in Table XXVIII. Frequency distribution of length of head to side base of mandibles of different instars is illustrated in Fig.20.

The co-efficient of variability was maximum for third instar nymph and was minimum for alates of *O. obesus* (Table XXVIII). Comparison of different instars of alate line of *O. obesus* was carried out by student "t" test. There were significant differences ($P<0.05$) between different instars of alate of *O. obesus*. However, fifth instar nymph was not significantly different from alates.

Maximum width of head

The range, mean and standard deviation of different instars of alate line of *O. obesus* for maximum width of head are given in Table XXVIII. Frequency distribution of maximum width of head of alate of *O. obesus* is illustrated in Fig.21.

The co-efficient of variability was maximum (3.65) for first instar nymph, whereas it was minimum (1.61) for alates. Comparison of different instars of alate line of *O. obesus* for maximum width of head was carried out by student "t" test, which revealed significant differences ($P<0.05$) between all the instars.

Length of hind tibia

The range, mean and standard deviation of different instars of alate line of *O. obesus* for length of hind tibia are given in Table XXVIII. Frequency distribution of different instars is illustrated in Fig.22.

The co-efficient of variability was maximum for fourth instar nymph, whereas it was minimum for fifth instar nymph (Table XXVIII). Comparison of different instars of alate line for length of hind tibia of *O. obesus* revealed singificant differences ($P<0.05$).

Length of wing pads

The range, mean and standard deviation of length of wing pads for different instars of alate line of *O. obesus* are given in Table XXVIII. Frequency distribution of different instars for length of wing pags is illustrated in Fig.23.

The co-efficient of variability was maximum for second instar nymph and was minimum for fourth instar nymph (Table XXVIII).

Comparison of different instars of alate line of *O. obesus* for length of wing pads was carried out by student "t" test, which revealed singificant differences ($P<0.05$) between all the instars.

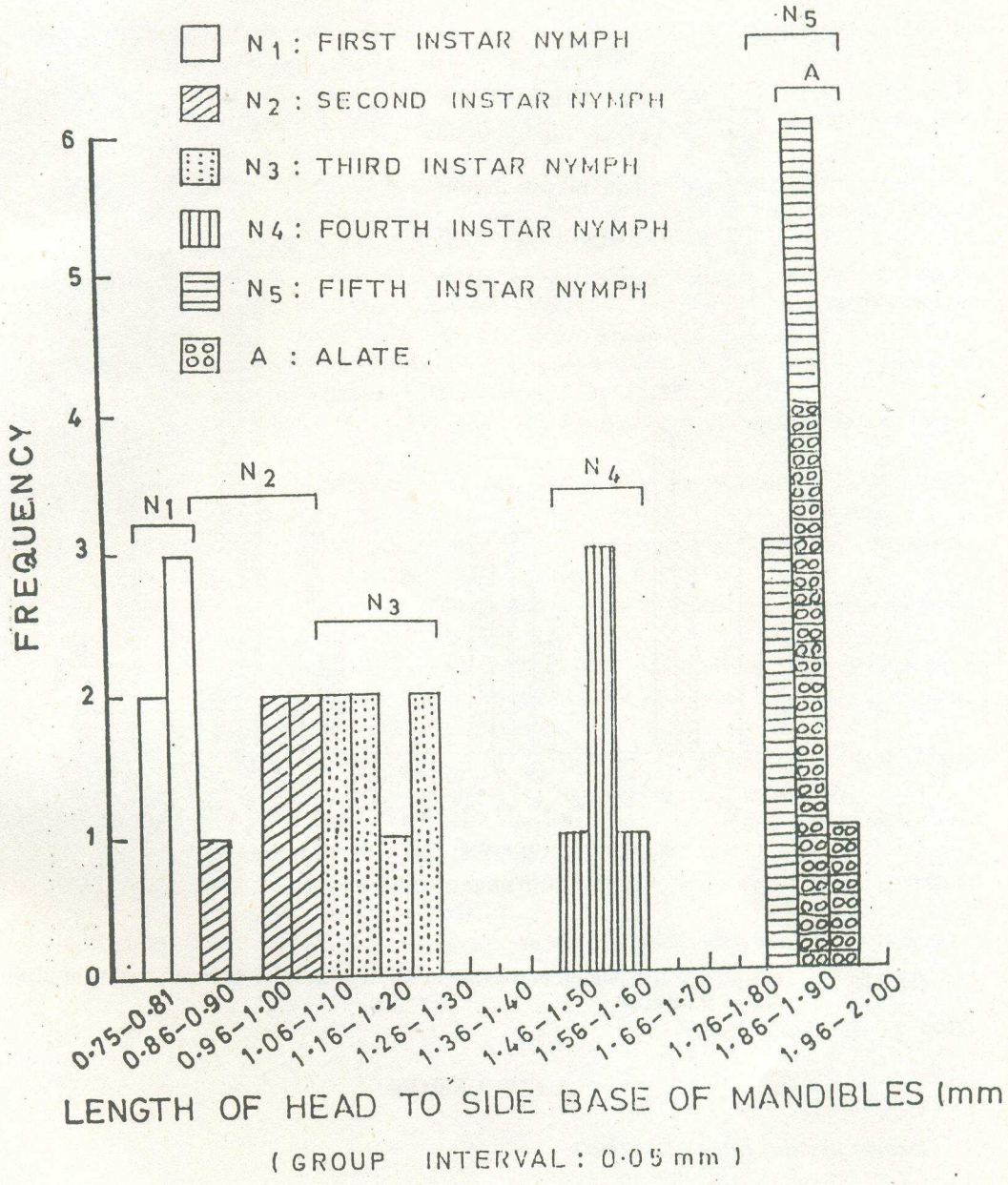

Fig. 20: **Frequency distribution of length of head to side base of mandibles in different instars of alate of *O. obesus*.**

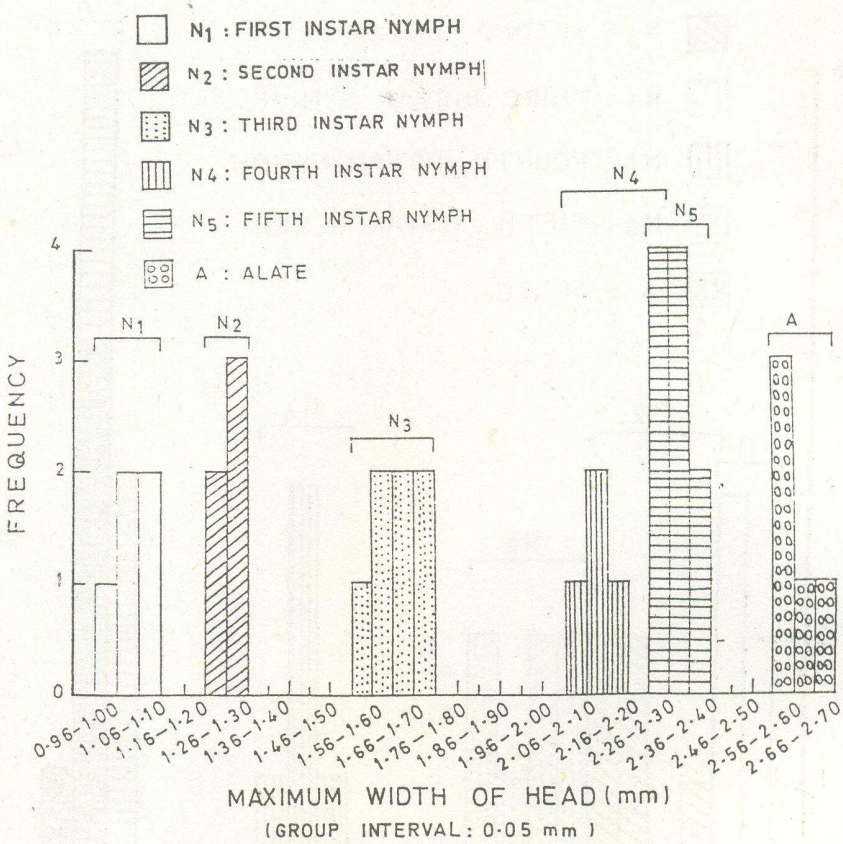

Fig. 21: **Frequency distribution of maximum width of head of different instars of alate line of *O. obesus*.**

DISCUSSION

Developmental lines of workers

In the field collections of *O. obesus*, two types of workers, major and minor, were detected. The major and minor workers are as distinct as in the fungus growing termites belonging to the genus *Macrotermes*. Nevertheless, they can be distinguished by

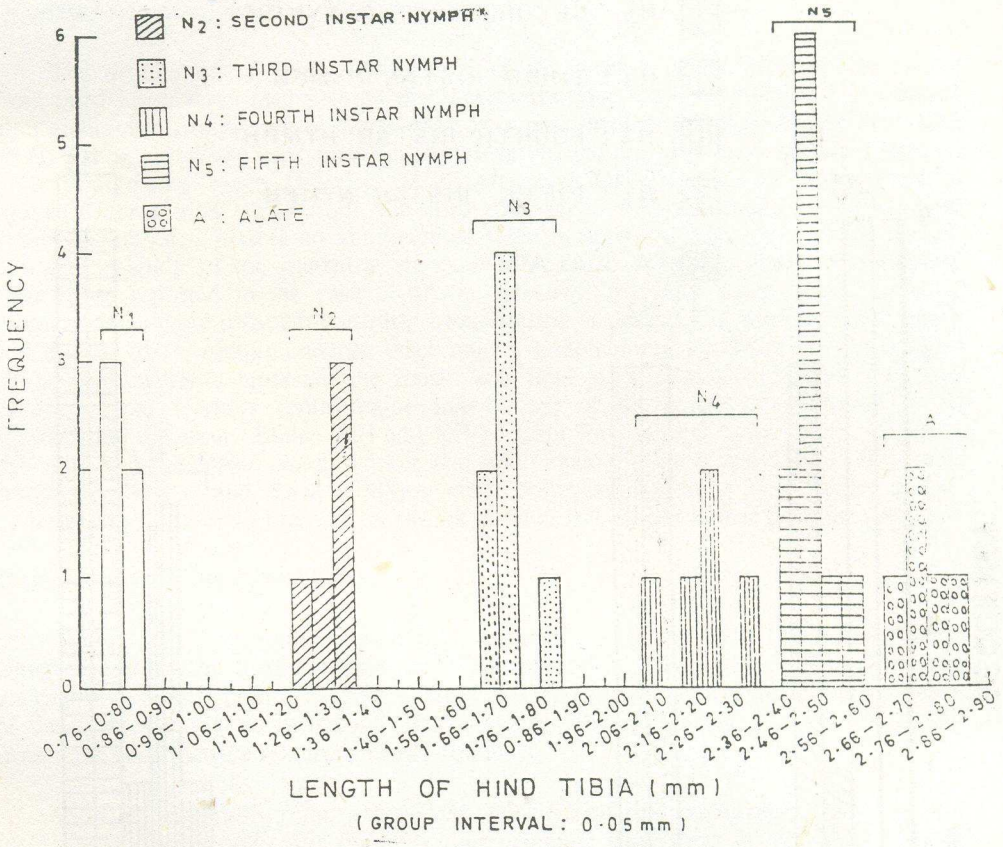

Fig. 22: **Frequency distribution of length of hind tibia in different instars of alate line of *O. obesus*.**

differences in their mandibular shape (not pattern) and also by the differences in the measurements (Table VIII and XIV). Okot-Kotber (1985) has reported that besides other morphological differences, sex differences also exist in the major and minor workers of *M. michaelseni*. Major workers develop from third instar male larvae, whereas the minor workers develop from female third instar larvae.

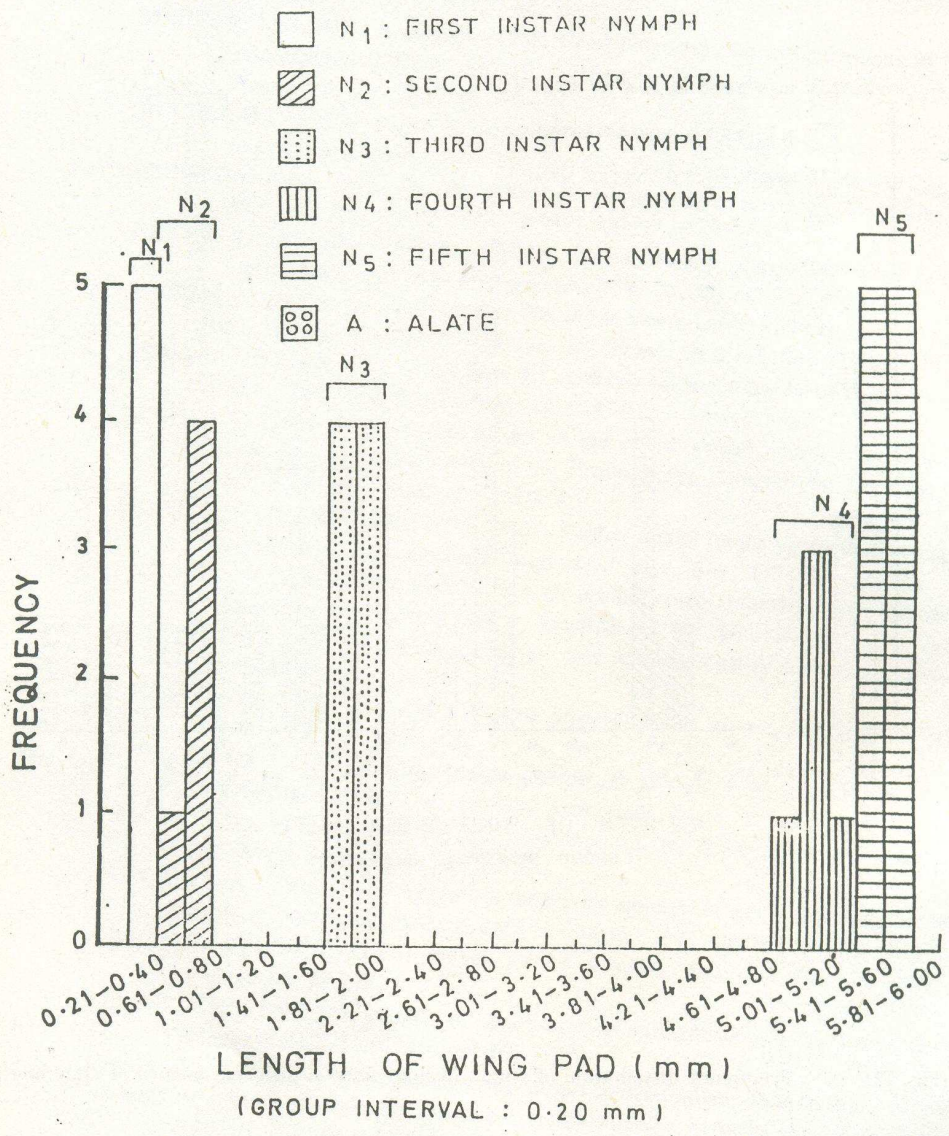

Fig. 23: **Frequency distribution of length of wind pads of different instars of alate line of *O. obesus*.**

Noirot (1985b) has reported that the workers are preceded by two larval stages in all the Termitidae except the fungus growing Macrotermitinae in which the workers are preceded by three larval instars. The present study supports Noirot in that the workers of *O. obesus* also develops from third instar larva.

The developmental stages through which the worker passes after differentiation from third instar larva may vary in the Macrotermitinae (Noirot, 1985b). The workers after emergence from the third larval instar may pass through one stage before becoming adult. The intermediate stage between the last larval and the adult stage has been referred to as "unpigmented stage" (Luscher, 1976). However, Okot-Kotber (1985) report that in case of *Macrotermes michaelseni*, the third stage larva moults into adult workers, both major and minor. There is no unpigmented stage in between. The result of the present biometrical study of the immature stages of *O. obesus*, however, differ significantly from those reported in the case of *M. michaelseni*, the only other fungus growing termite whose caste differentiation has been studied in detail. The workers (both major and minor) of *O. obesus* undergo three moults after coming out from the third instar larva; adult workers represent the fourth and final stage. Biometrically all the three instars of the workers (both major and minor) of *O. obesus* were found to be significantly different (Tables VIII and XIV) except for the total body length, which in the case of third instar major worker and adult major workers vary little. Biometric studies by Akhtar and Rana (1988)on caste polymorphism in a field colony of *O. gurdaspurensis* revealed that worker passes through five instars before becoming adult.

Developmental line of soldier

The soldier developmental line is an off-shoot from the third larval instar from which also arises the developmental line of minor worker. The pre-soldiers in *O. obesus* undergoes 5 moults before becoming adult. The rectangularly oval head characteristics of the species is visible in the last (5th) stage leading to the adults. In the preceding instars the head capsule remained nearly round.

Okot-Kotber (1985) reports that in *M. michaelseni* soldiers develop from the third instar larva. In *M. bellicosus* minor soldier originate from third instar larva, whereas origin of major soldier takes place from first instar minor worker (Luscher, 1976). Akhtar and Rana (1988) stated that soldiers in *O. gurdaspurensis* develop from third instar major worker larva and after seven moults develops into adult soldiers.

Developmental line of alates

The present studies on developmental lines of *O. obesus* revealed that the origin of alate line takes place from the second instar larva and nymph after 5 significantly different instars develops into alate. In *M. bellicosus* five nymphal instars have been recorded by Luscher (1976). Okot-Kother (1985) has also reported five nymphal stages upto alate after first instar larva in *M. michaelseni*.

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BIOTECHNOLOGICAL IMPLICATION OF FLAGELLATE PROTOZOANS IN CLEANING INDUSTRIAL EFFLUENTS CONTAINING HEAVY METAL Cr^{+6}

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Abstract: A flagellate protozoan was isolated from tanneries' effluent collected from the vicinity of city Kasur. Axenic culture of the protozoans was established in 0.1% aqueous solution of molasses supplemented with antibiotic chloramphenicol. The medium supported a healthy growth of the microorganisms both in terms of number as well as size, however, these parameters decreased after one week and few days, respectively. Following Cr^{+6} exposure it was found that up to 4 µg of the metal/ml of the medium, the protozoans showed, but a significantly decreased growth throughout the study period. In case of higher concentrations; 6, 8 and 10 µg of Cr^{+6}/ml the protozoans showed a further delay of one-day in the manifestation of growth. Following 9, 7 and 6 days of incubation the protozoans were not observed in case of the culture media containing 6, 8 and 10 µg of Cr^{+6}/ml, respectively. Cultures of the flagellates exposed to chromium were found invariably devoid of dividing cells, throughout the observation period. The flagellates were found to reduce Cr^{+6}, up to a level of 6 µg/ml of the medium. Beyond this level the protozoans remained unsuccessful to reduce the metal.

Key words: Protozoan and Cr^{+6}, biological reduction of Cr^{+6}, bioremediation of heavy metals.

INTRODUCTION

In Pakistan the industrial effluents are generally discharged directly or through adjoined land areas into nearby streamlets/rivers. Besides a large variety of industrial effluents representing various kinds of pollutants, effluents from tanneries must be treated on priority basis because the waste contains heavy metal chromium and is converting cultivable land areas to highly polluted shallow ponds. A vivid example of the problem is being represented by city Kasur. The heavy metal chromium is used in the tanning process and the waste water of the study area has been found to contain 1.93 to 6.45 µg/ml of Cr^{+6} with pH values ranging from 8 to 9 (Qazi *et al.*, 1997).

Apart from very low levels of chromium, necessary for carbohydrate and fat metabolism (Anderson *et al.*, 1983; Chakraborty and Mishra, 1992), its higher amounts are highly toxic and express carcinogenic, teratogenic and mutagenic effects in a variety of organisms (Nieto *et al.*, 1989; Nair and Krishnamurthi, 1991; Lansdown, 1995). Valency of chromium determines its cytotoxic effects. In living system, chromium is usually found in trivalent state (Ohtake and Silver, 1992). Hexavalent forms of chromium

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are known to be more toxic than CrIII. Because CrVI is easily taken up by the cells as the chromate anion via the sulphate anion transport system. Following entry within living system the CrVI is reduced intracellularly through reactive intermediates such as CrV and IV to more stable and less toxic CrIII by cellular reductants such as glutathion, vitamin C and B_2, as well as flavo-enzymes. The intracellular CrIII forms tight complexes with biological ligands such as DNA and proteins (DeFlora and Wetterhahn, 1989; DeFlora *et al.*, 1990; Campos *et al.*, 1995). Regarding the mechanism of the cytotoxicity including carcinogenic and mutagenic effects of chromium, it has been explained that during the intracellular reduction of CrVI to CrIII reactive oxygen species are generated, through reactions such as CrV with H_2O_2 in a Fenton-like manner to produce hydroxyl radicals (Aiyar *et al.*, 1990; Coudray *et al.*, 1992). The reactive oxygen species including superoxide ion, hydrogen peroxide and hydroxyl radicals produced as a result of redox cycling of chromium and other transition metal ions, have been reported for their oxidative deterioration of biological macromolecules including DNA damage and lipid peroxidation (Stohs and Bagchi, 1995; Susa *et al.*, 1996). Sugden and Wetterhahn (1996) have suggested that CrIV formed upon disproportionation of CrV oxidizes (oxygen-dependent oxidation) the nucleotide deoxyribose sugar moiety at the C-4' position via a phosphate bond intermediate. These workers found that the nucleoside thymidine show no reaction, while extent of CrV induced nucleotide oxidation was greater for thymidine diphosphates.

For environmentally sound strategies to protect our environment from various pollutants, bioremediation is probably the best choice. Bioremediation is the technology of using biological agent particularly microorganism to remove toxic pollutants from the environment especially soil and water. The pollutants are decomposed to non-toxic substances through microbial metabolism. Either microorganism indigenous to the environment, improved strains, or genetically engineered species are used for bioremediation. Microbe-enhancing substances are added to promote growth and metabolic activity of microorganisms already present in the environment (Pelczar *et al.*, 1993; Campos *et al.*, 1995).

Presence of protozoan species in industrial effluents containing heavy metals has allured many workers to study these microorganisms for their metal detoxification abilities and possible role in bioremediation processes (Madoni *et al.*, 1996; Qazi *et al.*, 1997; Haq *et al.*, 1998). In this regard axenic culturing of protozoa is required to study effect(s) and/or fate of a pollutant in a particular species (Lessard, 1993; Weeker and Vogels, 1994; Qazi *et al.*, 1997). The flagellate protozoans reported here, isolated from waste-water ponds getting tanneries' effluents, have been found to tolerate and reduce Cr^{+6} up to 6 µg/ml of the medium. These protists as well as other microbial community of the habitat can be exploited for the bioremediation of industrial effluents containing the heavy metal.

MATERIALS AND METHODS

Axenic culturing of flagellates

Samples of the industrial effluents from ponds getting waste water of tanneries in Kasur city, about 54 km south-east of Lahore, were collected in sterilized containers. From one sample, most abundant protozoan flagellate was isolated mechanically by micropipette to collect individual cells directly from water sample, as described originally by Lessard (1993). For this purpose the sample was filtered through a clean, Whatman filter paper to render the filtrate free from particulate material, and one drop of it was taken on a clean, sterile slide with the help of sterile inoculating loop. While observing under microscope at 125X, the flagellates were transferred to a drop of distilled water on the same slide with the help of a narrow-nosed Pasteur pipette. The diluted sample was sucked up in a capillary tube that was gently put and lifted on the slide to make six droplets. These droplets were diluted with distilled water, to avoid rapid evaporation. The droplet(s) containing either only one flagellate or multiples of the same was picked up with Pasteur pipette and transferred to a 250 ml conical flask containing 50 ml of autoclaved 0.1% molasses solution (w/v). About 40 flagellates were introduced in this manner and the flask was kept at room temperature. To inhibit bacterial growth 1% solution of chloramphenicol (Sigma) in methanol was used to provide 20 μg of the drug per ml of the culture. Stock culture of the flagellates was maintained in the laboratory, at room temperature. Fresh stock culture was made by inoculating 1.0 ml of a previous culture into 100 ml of the medium within 15 days.

Assessment and optimization of growth

Growth of the protozoans under different experimental conditions was assessed daily, by measuring size and number of motile and dividing cells/ml of culture. The microorganisms were counted using haemocytometer at 125X. Their number excluding the dividing cells was counted in total of the sixteen squares, represented by four squares at each corner of the chamber including those crossing the right and below bordering lines of each quadrant of the squares. The corner-squares were selected to minimize the chance of recounting a motile protozoan. Relative growth was assessed by dividing number of cells/ml at a given stage by the value of the same parameter at time of inoculation. Diameter of the protozoans, was measured at 500X with the help of ocular and stage micrometers.

All cultures were grown in 250 ml conical flasks except mentioned otherwise by inoculating 0.1 ml of a stock culture to 19.9 ml medium. Various growth conditions were optimized. Oxygen requirement was assessed by growth of the flagellates in test tubes and the conical flasks at room temperature. The protozoans, were grown also in 12 hours light/dark cycle and complete darkness; provided by covering the culture flasks with black paper. The protists' growth was studied at 10°C (refrigerator), 30°C (room temperature

and 37°C (incubator), pH of freshly prepared medium ranged from 7.5 to 8.0. The cultures were also grown in medium having pH of 10 attained by adding 1% aqueous solution of $NaHCO_3$.

Microscopic observations

Living flagellates were immobilized with 1% mthylcellulose for microscopic observations as described by Cox (1981). The protozoans were also processed for chemical fixation and staining. Filtered albumen (albumen + glycerine; 1:1 ratio) was applied on one side of cover slip and a drop of the flagellate's culture was dropped from a height of about 1″ on it. The drop was subsequently spread to make a thin smear, which was allowed near to air dry and then fixed in Bouin's fixative for 20 minutes. The smears were stained as described by Cox (1981), for the staining of parasitic protozoans except for few modifications. Smears were dipped in 70% ethanol and subsequently stained in Ehrlich's haematoxylin till the protozoans appeared as dark blue-black. After a washing of 5 minutes in 70% alcohol, the stained smears were differentiated in acid alcohol (1.5% HCl in 95% ethanol), till the stain left the cytoplasm and the process was soon stopped by washing the cover slips and slides in 70% alcohol. After passing through 50% alcohol for 5 minutes, the smears were washed in tap water, till the specimens appeared blue in colour. They were then passed through 70% and 90% alcohols and stained in 1% alcoholic eosin for about two minutes. After washing the stain in 95% alcohol the smears were dehydrated, cleared in xylene and mounted in Canada balsam. Photomicrograph of the flagellates were taken on a camera-fitted microscope.

Chromium VI tolerance and its reduction by the flagellates

$K_2Cr_2O_7$ was used as source of Cr^{+6} (Bianchi and Levis, 1985) and stock solution of this salt was prepared by dissolving 0.707 gm of $K_2Cr_2O_7$ in 500 ml of glass distilled water. Culture media containing 2, 4, 6, 8 and 10 μg of Cr^{+6}/ml in 0.1% (w/v) molasses were then prepared by adding appropriate amounts of sterile chromium stock solution, tap water and 10% solution of molasses.

Cr^{+6} was measured in uninoculated culture media containing different concentrations of the heavy metal as well as in cell free 12-days old cultures by the Petrilli and DeFlora method (1977), 7.0 ml of a sample was taken in 250 ml conical flasks and mixed with 43.0 ml of glass distilled water. pH was then adjusted between 0.81 and 0.95, with concentrated H_2SO_4 and the volume was made up to 100 ml with the water. 2.0 ml of diphenyl carbazide reagent, freshly prepared by dissolving 250 mg/50 ml of acetone was added and after 10 minutes optical density of each sample was measured at 540 nm, taking glass distilled water as reference. Statistical analyses between different parameters of control and experimental cultures were made by employing Student's 't' test.

RESULTS

Axenic culturing of flagellate protozoans

At inoculation cultures contained $0.21\pm0.01x10^4$ cells/ml (n=4) growth of the flagellate protozoan was detectable on day-2 following inoculation. Maximum relative growth of the protozoans in control cultures was observed at day-3rd, while from the 7th day the number of flagellates decreased retrogressively up to the end of experimental period (Table I). At the initial stages the cells in control cultures attained average diameter of about 10 μm. The size of the flagellate decreased continuously so that at day-12th following the inoculation the figure turned out to be as 6.18 μm (Table II).

Microscopically various details of the protist such as presence of vacuoles, flagella and shape of the cell were observable, even in living stage, while observing under the microscope (Fig.1). The cells fixed in Bouin's fixative and subsequently stained with haematoxylin and eosin, also revealed structural details such as size of the flagellum, shape of the cell, location of chromatin material and relative size of the protozoans (Fig.1).

Optimization of growth conditions

Relative growth of the protozoan grown in test tubes (little dissolved O_2) remained significantly lower than the values for the cultures in conical flasks (Fig.2). Similarly, the number of dividing cells $x10^4$/ml was found significantly less in cultures grown in test tubes and at day 7th and 9th the values turned out to be 0.67 ± 0.57 and 1.00 ± 0.00 as compared to the control figures of 2.67 ± 0.57 and 2.33 ± 0.57, respectively. However, size of the cells did not show difference between two types of the cultures (Fig.2).

The protozoans growth increased significantly in cultures kept away from light, sparing the initial phases (Fig.2). At days 6th and 7th post-inoculation values indicating relative growth of the protozoans cultured under darkness were 404.93 ± 5.67 and 485.22 ± 11.11 as compared to the figures 324.69 ± 20.41 and 329.63 ± 22.56, respectively for the cultures grown in usual light/dark cycle. Similarly, numbers of dividing cells in cultures kept in darkness for the said stages, 1.33 ± 0.57 and 2.67 ± 0.57 $X10^4$/ml were significantly higher than the value 1.00 ± 0.00 for each of the corresponding study periods for the protists grown in usual light/dark cycle. Regarding the size of the cell at day 4th cells' diameter of cultures grown under darkness decreased significantly than the values for the cultures kept under normal light/dark cycle (Fig.2).

The flagellates did not show growth at all in inoculated medium kept at 10°C, grew well at room temperature, while at 37°C significant reduction in growth as well as cells' size was observed (Fig.2). Concerning the number of dividing cells, except at day-2 with a figure of 1.00 ± 0.00 no dividing cells were observed in cultures incubated at 37°C.

Table I: **Effect of Cr^{+6} on relative growth of the flagellates, grown in control (A) containing 2 (B), 4 (C), 6 (D), 8 (E) and 10 µg (F) of Cr^{+6}/ml of medium.**

Treat-ment	DAYS AFTER INOCULATION										
	2[a]	3	4	5	6	7	8	9	10	11	12
Control[c]	131.71[b] ±9.23	186.86• ±8.09	152.09 ±9.23	143.62 ±9.23	161.90• ±37.90	126.19 ±22.04	88.09 ±5.23	71.04• ±7.23	69.04 ±10.76	34.47 ±3.23	26.90 ±3.23
2 µg	6.72*** ±2.38	7.58•*** ±3.77	5.96*** ±2.38	4.38*** ±0.77	5.53•*** ±2.77	7.14*** ±4.76	6.33*** ±3.19	4.76•*** ±0.00	4.38*** ±0.77	4.38*** ±0.76	4.00*** 1.57
4 µg	1.19*** ±2.38	6.33•*** ±1.80	5.52*** ±0.90	4.76*** ±0.00	4.38•*** ±0.81	1.19*** ±2.38	4.76*** ±0.00	4.00•*** ±0.90	4.76*** ±0.00	4.00*** ±0.90	2.38*** ±2.76
6 µg	N.D.	7.93•*** ±2.85	7.14*** ±2.04	4.42*** ±0.76	4.76•*** ±0.00	2.38*** ±2.71	3.61*** ±2.50	3.19•*** ±1.28	N.D.	N.D.	N.D.
8 µg	N.D.	8.76•*** ±4.00	2.38*** ±0.90	1.19*** ±2.38	3.57•*** ±2.38	4.76*** ±0.00	N.D.	N.D.	N.D.	N.D.	N.D.
10 µg	N.D.	3.57•*** ±2.38	N.D.	N.D.	1.57•*** ±3.14	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

a. Protozoans were detectable on day-2; b: Mean of four replicates ± S.D; N.D.: Not detected; •: Chloramphenicol supplied (20 µg/ml); Values with asterisks are significantly different from those in A; Student's 't' test ***: $P<0.001$.

Table II: Diameter of the flagellates in µm grown in media containing various concentrations of Cr^{+6}/ml.

Treat-ment	DAYS AFTER INOCULATION										
	2[a]	3	4	5	6	7	8	9	10	11	12
Control[c]	10.23[b] ±0.36	9.42 ±0.70	9.67 ±0.44	7.65 ±0.30	7.65 ±0.32	7.42 ±0.00	6.47 ±0.74	6.78 ±0.47	7.32 ±0.07	7.32 ±0.07	6.18 ±0.81
2 µg	10.91 ±0.07	10.98 ±0.07	11.27 ±0.10	11.16*** ±0.21	10.54** ±0.74	10.65*** ±0.74	11.05* ±0.03	11.05 ±0.03	10.98 ±0.07	10.95 ±0.03	10.95** ±0.10
4 µg	10.98 ±0.03	11.05 ±0.10	11.08 ±0.07	10.98 ±0.14	11.27*** ±0.43	11.05* ±1.94	11.01 ±0.03	11.01 ±0.03	10.32 ±1.24	10.66 ±0.59	11.14 ±6.40
6 µg	N.D.	10.98 ±0.03	11.08 ±0.07	10.94 ±0.10	11.05*** ±0.07	11.09 ±6.40	11.05 ±5.49	10.98 ±0.03	N.D.	N.D.	N.D.
8 µg	N.D.	10.95 ±0.10	11.01 ±0.18	11.09*** ±0.54	10.25* ±5.23	10.76*** ±0.58	N.D.	N.D.	N.D.	N.D.	N.D.
10 µg	N.D.	10.94 ±5.34	N.D.	N.D.	11.35 ±6.51	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

a: Protozoans were detectable on day-2; b: Mean of four replicates ± S.D; N.D.: Not detected; Values with asterisk(s) are significantly different from those in A; Student's 't' test *: P< 0.05; **: P<0.01; ***: P<0.001.

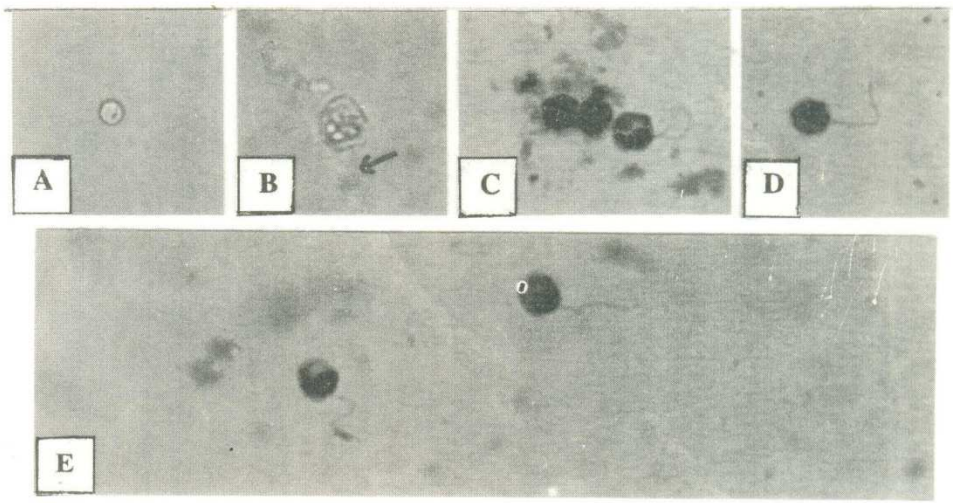

Fig. 1: Photomicrograph of unstained, living flagellates, immobilized with 1% methyl cellulose at 400X (A), at 1000X (B). Note various vacuoles within the cell, a flagellum (arrow), round bottom and flattened area at the base of flagellum are evident. The flagellates from a 10-day old culture (C): chromatin material and a flagellum areprominent. A flagellate from a 3-day old culture kept at usual light/dark cycle (D). Another view of the protist (E). Photomicrograph from C to E (1000X) represent Haematoxylin and eosin stained preparations.

Table III: Reduction of Cr^{+6} in the culture medium after 12 days of the flagellate's growth.

Cr^{+6} µg/ml of culture fluid		% reduction of Cr^{+6}
At inoculation	After 12-days	
2	0.08±0.17(4)[a]	95.82±8.36
4	0.92±1.26(4)	77.25±31.15
6	2.74±0.47(4)	54.43±7.79
8	8.89±0.65	-[b]
10	10.10±0.20(3)	-

[a]: Mean ± S.D; [b]: No decrease was observed. Number in parenthesis indicates sample size.

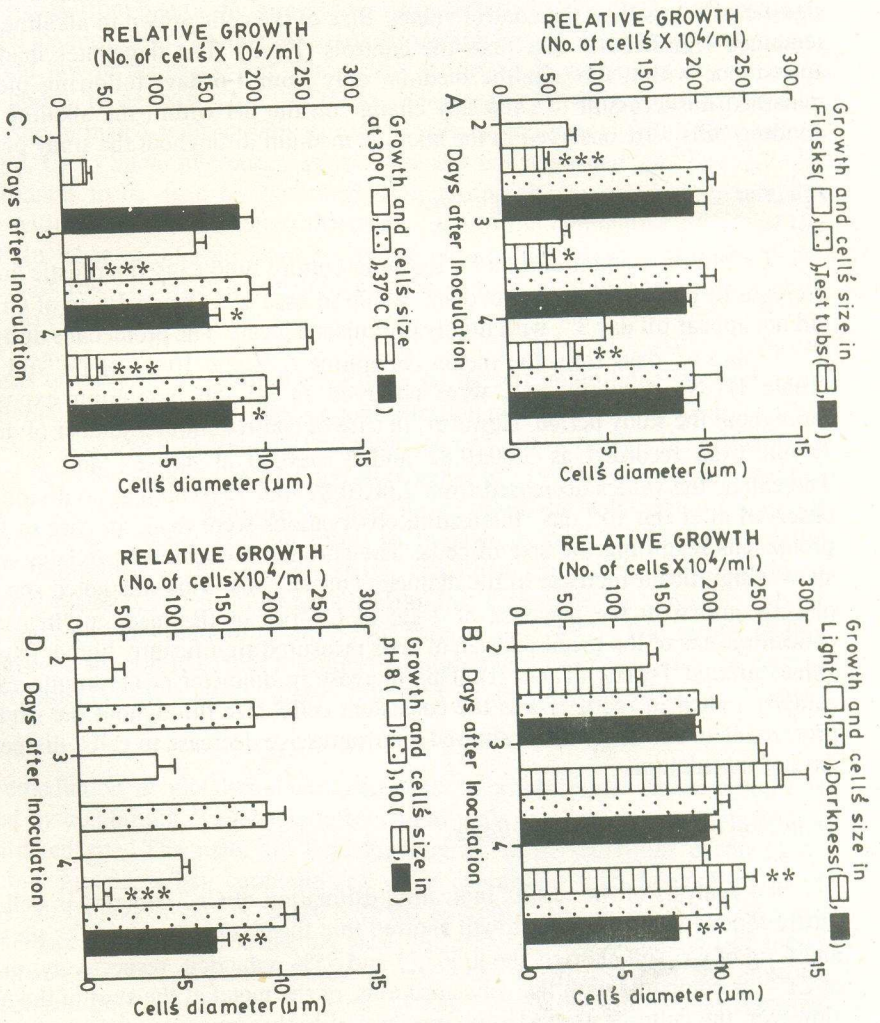

Fig. 2: Optimization of growth conditions of the flagellates: Effect of amount of dissolved oxygen (A), light (B), temperature (C) and pH (D). Values with asterisk(s) are significantly different from respective stage of first treatment. Student's 't' test *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$.

pH of freshly prepared medium was found to be about 8. Growth of the protozoans turned down this pH to a value around 7 after two days of inoculation, which then remained almost consistent for rest of the period. Appearance of growth was delayed in the alkaline medium (pH 10) till the day 4 after inoculation with number of the protozoans significantly less than the control values. Size of the cells grown in alkaline medium also remained significantly less than the controls (Fig.2). The flagellates made their weak appearance within the alkaline medium only from 4-6 days following inoculation and remained unsuccessful to exert any change on the pH within the alkaline medium. No dividing cells were observed in the alkaline medium throughout the study period.

Effect of Cr^{+6} on growth of flagellates

Cr^{+6} in an amount of 2 and 4 μg/ml of culture fluid caused a drastic and significant decrease in growth of the protozoans. While in case of 6, 8 and 10 μg of Cr^{+6}/ml growth did not appear till day 3rd, with highly diminished levels. The protozoans disappeared after 9th, 7th and 6th days from the media containing 6, 8 and 10 μg of Cr^{+6}/ml, respectively (Table I). No dividing cells were observed in all the chromium exposed cultures, throughout the study period. However, in case of control culture number of dividing cell x 10^4/ml were recorded as 3.00±0.82 and 3.75±0.50 at day 2nd and 3rd, respectively. Thereafter, the values decreased from 2.00±0.82 to 0.75±0.50 and no dividing cells were observed after the 10th day. Interesting observations were made in case of Cr^{+6} exposed protozoans regarding the size of cells. The chromium exposed flagellates were found to show a significant increase in the diameters of the cells. This was noted specially in case of cells grown in the presence of 2 μg of Cr^{+6}/ml of the medium. In case of higher concentrations of the metal the size of cells measured significantly higher than the control values around 1-week (Table II). This increase in diameter of chromium exposed cells, actually indicated more or less the consistent cells' size throughout the study, while the protozoans in control cultures showed a retrogressive decrease in cells' diameter after few days of inoculation.

Reduction of Cr^{+6} by the flagellates

An analysis of the results indicating estimation of Cr^{+6} contents in cell free culture media following 12-days of growth showed that flagellates grown in the presence of 2, 4 and 6 μg of Cr^{+6}/ml showed about 96, 77 and 55% reduction, respectively, in the amount of Cr^{+6}, as compared with the concentrations, of the metal at the start of the experiments. However, the cultures grown in the presence of higher amounts of the heavy metal i.e., 8 and 10 μg/ml were found unable to exert any reducing effect. Rather, some slightly higher amounts of the metal possibly due to death of the protists and subsequent release of Cr^{+6} into the culture fluids, were recorded (Table III).

DISCUSSION

Effluents of the study area were found to harbour a number of microorganisms. A flagellate protozoan from one of the samples was successfully grown under the laboratory conditions. The axenic culture of the protozoan responded negatively to alkaline nature of the medium and low levels of oxygen. The axenic culturing of the protist was aimed at exploring its ability to detoxify Cr^{+6}. As these microorganisms were obtained from an environment contaminated with varying concentrations of the heavy metal, they indeed were tolerating Cr^{+6}. Such microorganisms from the contaminated environment are potential candidates to be used for bioremediation processes. An organism essentially plays its role within its natural habitat differently, due to the cumulative effects of the community, than what is inferred from its axenic/pure culture study. However, if the purpose is to study role played by a particular microorganism, establishing axenic culture then becomes necessary.

The results of the present study indicate that the heterotrophic flagellate protozoan was able to tolerate and detoxify Cr^{+6} upto a concentration of 6 μg/ml in the medium. This tolerance level corresponded to the concentrations of Cr^{+6} prevailing in the effluents of study area, that have been found from 3-6 μg/ml (Qazi *et al.*, 1997). But in case of higher concentrations of the metal i.e., 8 and 10 μg/ml of the medium, the isolated protozoans did not show considerable growth, both in terms of number as well as size of the cells. Likewise, the protozoans, in the present study, remained completely unsuccessful to reduce the heavy metal (Cr^{+6}) in higher concentrations. Chromium is known to inhibit ciliate protozoans' growth (Madoni *et al.*, 1996; Qazi *et al.*, 1997). The results of this investigation bring a support to earlier findings that higher levels of chromium are toxic to a variety of organisms (Nieto *et al.*, 1989; Nair and Krishnamurthi, 1991; Lansdown, 1995).

For bioremediation of metal-contaminated sites, microbial mats' construction has been suggested by combining cyanobacteria inoculum with a sediment inoculum from a metal contaminated site. The mats are held together by slimy secretions produced by various microbial groups. When contaminated water containing high concentrations of metals such as Cd, Pb and Cr etc., is passed over the microbial mats immobilized on glass wool, there is rapid removal of the metals from the water due to deposition of metal compounds outside the cell surfaces as well as chemical modification of the aqueous environment surrounding the mats (Bender *et al.*, 1995). The flagellate protozoan reported here can be employed for the development of such strategies to control the water pollution through bioremediation. This notion is supported from previous studies on the same area, which have indicated many microbes resisting varying levels of concentrations of Cr^{+6} (Qazi *et al.*, 1997; Haq *et al.*, 1998). Further work is needed to optimize growth conditions and thus to enhance the efficiency of the microorganisms, in term of detoxification (reduction) of Cr^{+6}, inhabitants of the tanneries' effluents, including bacteria and protozoa.

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BLOOD BIOCHEMISTRY OF RABBITS AFTER INTRAVENOUS INOCULATION OF UNILOCULAR HYDATID CYST FLUID OF SHEEP ORIGIN

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Abstract: Unilocular, hydatid cyst fluid of sheep origin was inoculated to five group of rabbits. The doses given were (1) Crude Low Dose (CLD), (2) Filtered Low Dose (FLD), Crude Medium Dose (CMD), (3) Filtered Medium Dose, (FMD) and (4) High Dose (HD of filtered hydatid cyst fluid). Control group was inoculated with similar doses of distilled water. Blood samples were pooled fortnightly and biochemical analysis made were glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) bilirubin, glucose, and plasma protein content. To face stress conditions through energy production, all the groups showed elevation in their GOT, GPT activities and depletion in glucose and protein contents of blood. High dose group, however, behaved differently, which showed depletion in both of these transaminases, probably due to interference in the synthesis or post synthetic destruction of these enzymes. Bilirubin content increased significantly only in the "high dose" group probably due to enhanced degradation of haemoglobin content.

Key words: Hydatidosis, blood biochemistry, rabbit.

INTRODUCTION

Hydatidosis adversely affect the normal body functioning by producing haematological changes (Amizhanov, 1977; sheep, Hinz and Gehring, 1987; mice, Alkarmi and Behbiani, 1989; Bressen-Handi *et al.*, 1989; man, Wangoo *et al.*, 1989; mice, Tanveer *et al.*, 1996, a,b, rabbits). In addition to these biochemical alterations have also been reported by Davydov and Smirnov (1982, pigs), Kroeze and Tanner (1985, rats) and Tanveer *et al.* (1997, 1998, 1998a).

Considering the medical, veterinary and economic importance of hydatidosis (FAO, 1985) the present study is aimed to work out the effects of hydatid cyst fluid on blood biochemistry of rabbits as a mammalian model.

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MATERIALS AND METHODS

Rabbits (*Oryctoragus cuniculus*) maintained in the optimal condition of animal house were acclimatized for two weeks prior to inoculation with hydatid cyst fluid of sheep origin (Tanveer *et al.*, 1996). Different doses of hydatid cyst fluid were given according to following schedule.

Time (day)	GROUPS			
	CLD (n=7) FLD (n=7)	CMD (n=7) FMD (n=7)	HD (n=7)	
	Dose (ml/day)	Dose (ml/day)	Dose (ml/day) 1st week	2nd week
1-14	10	0.1	0.1	0.15
15-28	20	0.15	0.2	0.25
29-42	30	0.2	0.3	0.35
43-56	40	0.25	0.4	0.45
57-70	50	0.3	0.5	0.55
71-84	50	0.3	0.55	0.55
85-98	50	0.3	0.55	0.55

Abbreviatinos used:
CLD, Crude low dose; FLD, Filtered low dose; CMD, Crude medium dose; FMD, Filtered medium dose; HD, High dose filtered hydatid cyst fluid.

About 4.0 ml of blood pooled out fortnightly in small sterilized test tubes was allowed to clot at 4°C and centrifuged at 3500 RPM for 20 minutes. Clear serum was separated and was further used for various biochemical analyses like glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), bilirubin, glucose, and plasma protein content. GOT and GPT activity was estimated by using RANDOX Kit based upon the method of Reitman and Frankel (1957), Bilirubin by Jendrassik and Grof (1938), Glucose by Glucose oxidase per oxidase method of Trinder (1969), modified by Teuscher and Richterich (1971), and Barham and Trinder (1972). Plasma protein content was estimated by using Biuret method designed by Henry *et al.*, (1974). Statistical significance was computed according to Student's 't' test (Steel and Torri, 1981).

RESULTS AND DISCUSSIONS

Fig.1 showed that GOT activity remained almost unchanged in control group through out the study period. The increase in GOT activity was statistically non significant through out crude hydatid cyst fluid (CHCF) treatment in CLD group. FLD group also showed an average increase in GOT activity that was statistically significant only after 42 ($P<0.05$), 56 ($P<0.01$) and 70 ($P<0.001$) days. GOT activity increased with dose and time in CMD group and was found statistically significant after 14 ($P<0.05$), 28 ($P<0.05$), 56 ($P<0.05$), 70 ($P<0.05$) and 98 ($P<0.01$) days in this group. Increasing trend in GOT activity was also observed in FMD group but it was found statistically non significant through out the HCF treatment in this group. HD group however, showed an average decrease of GOT activity after CHCF

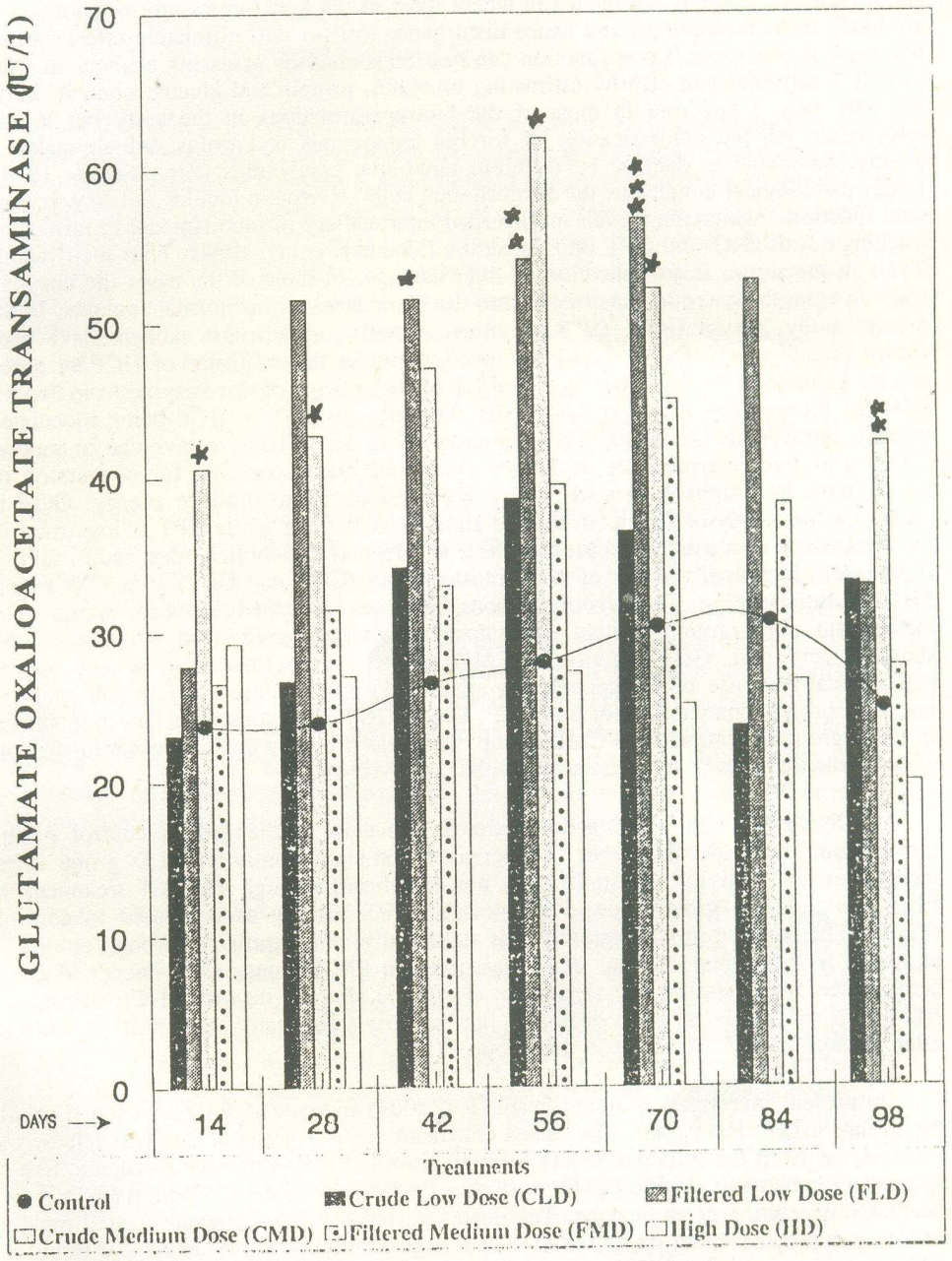

Fig. 1: Change in Glutamate Oxaloacetate Transaminase (U/l) of rabbit blood after administrating different doses of sheep originated unilocular hydatid cyst fluid in various forms and doses like crude low dose (CLD), filtered low dose (FLD), crude medium dose (CMD), filtered medium dose (FMD) and high dose (HD) (please see materials and methods). Statistical significance has been determined by student's "t" test and the probability represented by stars; * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

administration. Liver is the main site where most of the xenobiotics are metabolized or are likely to be metabolized and hence disturbance to liver will ultimately effect various biochemical processes. Liver function can best be studied by assessing changes in GOT and GPT activities and also by estimating bilirubin, protein and glucose content. Liver not only play a key role in most of the biotransformations in the body but is also responsible for the detoxification of foreign compounds and drugs, which makes it greatly vulnerable to damage by different xinobiotic compounds (Zimmerman, 1974). Under pathological conditions the parenchymal cells of hepatic lobules fail to carry out vital functions, which may result in disturbed intermediary metabolism and in turn cause leaching out of GOT and GPT into the serum (Shakoori *et al.*, 1984). Thus their raising levels in the serum is an indication of liver damage. In most of the cases the enzymes leak out from the necrotic hepatocytes into the blood stream in abnormal amounts. In the present study, serum GOT, GPT activities, protein and bilirubin content have been shown as indicators of disturbed liver functions under the influence of HCF of sheep origin. Increase in GOT activity is either due to (i) Leakage of this enzyme from hepatic cells (ii) Increased synthesis of GOT (iii) Presence of GOT in HCF being inoculated through intravenous injections. Transaminases (GOT and GPT) are involved in routing amino acid into intermediates of Kreb's cycle and are responsible for conversion of amino acids into alpha keto acids which are metabolized to produce energy. GOT is important for metabolizing glutamate and alpha ketoglutarate while GPT is important in the metabolism of alanine and pyruvate these in tern may metabolize other amino acid of importance. Increased activity of both transaminases (GOT and GPT) after CHCF and FHCF administration through intravenous injection indicated elevated amino acid metabolism after protein content to compensate energy requirement. Present results showed, very high GOT activity in CMD treated rabbits that may be due to the pathological response of the hepatocytes against (1) protoscoleces, (2) soluble and (3) insoluble protein content present in CHCF. Serum GOT elevations were less pronounced in FMD group as compared to CMD group. This was probably due to the administration of only soluble types of antigens to the rabbits of FMD group.

Fig.2 showed that GPT activity almost remained unchanged in control group through out the study. However, it decreased non-significantly in CLD group. The increase in GPT activity was statistically non-significant through out HCF treatment in FLD group. CMD group showed an elevated GPT activity as compared to control (Fig.2). The increase in GPT activity was statistically non significant through out HCF treatment in this group. Similar trend was noted in FMD group where increased GPT activity was found statistically significant ($P<0.05$) after 70 days of FHCF treatment. HD group showed an average decrease, that became statistically significant ($P<0.01$) after 70 days of HCF treatment in this group.

In present investigation a significant ($P<0.001$) increase in GPT was noted in all the groups except HD group. The raised enzymatic levels may be a result of release of this enzyme from the cells due to (1) Leaking from the liver cells due to hepatic tissue necrosis (Zimmerman, 1974). (2) Or enzyme induction and release (Street, 1969) (3) Or decreased deposition of an enzyme. The decreased level of GPT in experimental rabbits of HD group could either be due to (1) Great regeneration power of liver as a result of which leaking out of the GPT in the serum become minimal (2) The biosynthetic activity which implies routing of all the biochemical components towards this activity in liver (Knox and Greengard, 1965; Bhatia *et al.*, 1972) (3) Enzymatic inhibition (Hendrickson and Bowden, 1976; Meany and Pocker, 1979) (4) Development of the host resistance against the foreign and excessive GPT.

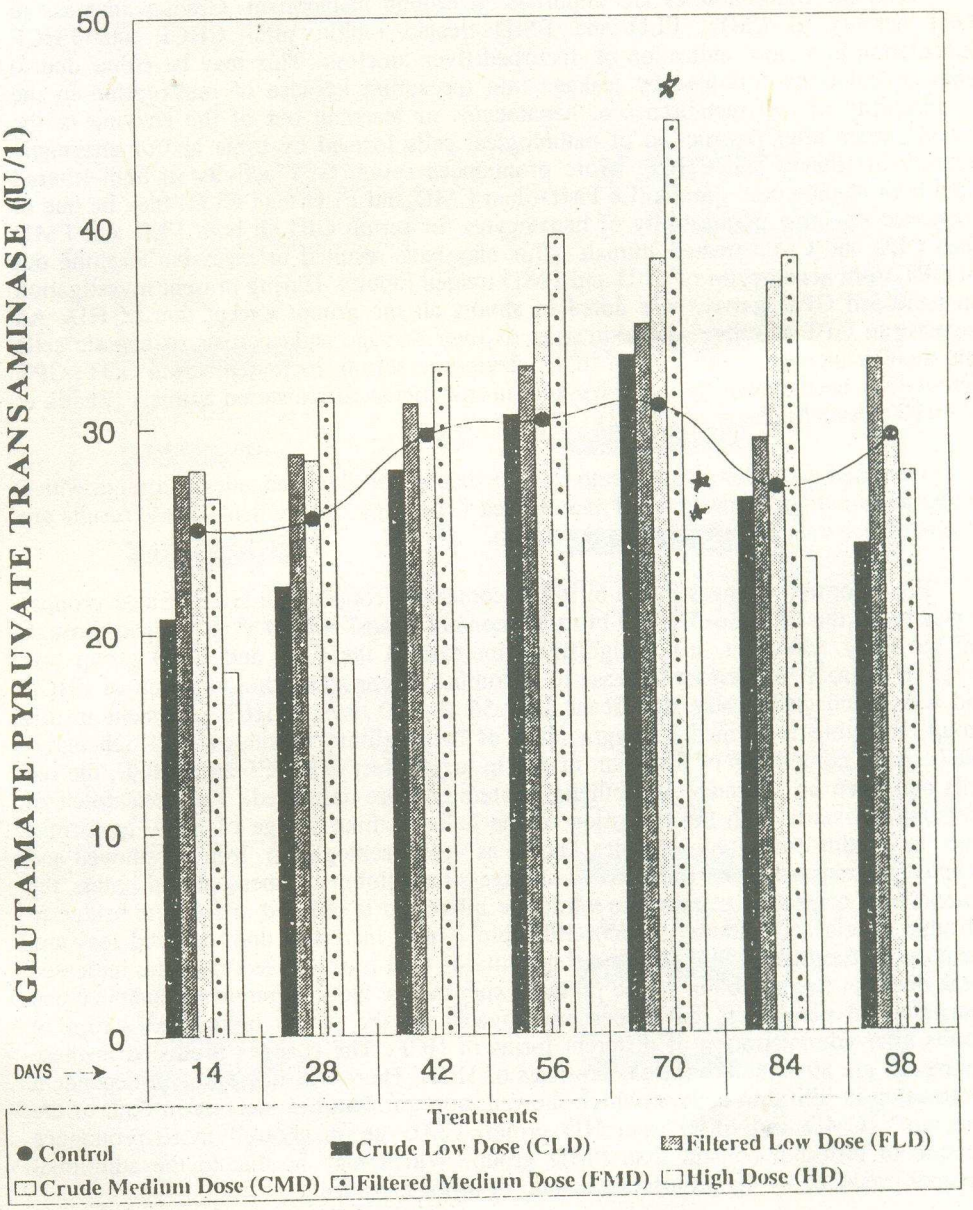

Fig. 2: Change in Glutamate Pyruvate Transaminase (U/l) of rabbit blood after administrating different doses of sheep originated unilocular hydatid cyst fluid in various forms and doses like crude low dose (CLD), filtered low dose (FLD), crude medium dose (CMD), filtered medium dose (FMD) and high dose (HD) (please see materials and methods). Statistical significance has been determined by student's "t" test and the probability represented by stars; * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Both the transaminases are important in protein metabolism. Gradual increase in GPT activity in CMD, FLD and FMD treated rabbits after CHCF and FHCF inoculation is a clear indication of disturbed liver function. This may be either due to enzyme induction followed by leakage into the serum because of interruption in the permeability of cell membranes of hepatocytes or leaching out of the enzyme in the blood stream after destruction of pathological cells formed by toxic and/or enzymatic activity of filtered and CHCF. More pronounced serum GPT activity in both filtered then both of the crude groups (i.e FMD than CMD and FLD than CLD) may be due to increased selective permeability of hepatocytes for serum GPT in both FLD and FMD than CLD and CMD treated animals. This may have resulted in excessive leaching out of GPT from hepatocytes of FLD and FMD treated rabbits. During present investigation an increased GPT activity was noted in almost all the groups except that of HD. An increase in GPT activity is an indication of liver damage and necrosis of hepatic cells and thereby increasing the level of these enzymes in serum. Increased serum GOT, GPT activity has been shown by many workers in insecticide administered animals (Bhatia *et al.*, 1973; Abdelsalam *et al.*, 1982).

Increased activities also indicate cellular damage or gluconeogenesis through which amino acids may be transaminated and utilized for energy requirement. These results are in accordance with Kroeze and Tanner (1985).

Fig.3 showed changes in the bilirubin content of control and HCF treated groups. It was noted that in control group bilirubin content almost remained unchanged through out the study. However, a non-significant increase in the CLD and FMD group was noted. HD group showed an increase in bilirubin content after administration of FHCF and was found statistically significant after 56 and 70 days of HCF treatment in this group. Bilirubin is formed by degradation of haemoglobin (Cantarow and Schepartz, 1967). After completion of life span, or due to toxic effect of CHCF and FHCF, the red cells are taken up by reticuloendothelial system and are destroyed. The breakdown of haemoglobin starts with the oxidation of the delta-methine bridge of heme to form a green biliverdin-iron globin complex known as verdohaemoglobin. Iron is removed and attached to transferrin for transport to storage sites, globin is liberated and enters the general pool of protein metabolism while the biliverdin is reduced at methine bridge to bilirubin (Datta and Ottaway, 1965). Bilirubin is also increased due to blood loss and haemolysis (Eastham, 1985). Increased quantity of total bilirubin indicates the increased break down of haemoglobin. In the present experimental work different biochemical and hematological parameters have been investigated on the whole blood and serum of rabbits after administration of different forms of HCF. The changes produced in these parameters are attributed to the toxic effects of HCF. There was noted a dose dependent relationship in bilirubin content which showed more pronounced increase in both of the "medium" (CMD and FMD) and HD group. CMD group showed more pronounce increase in bilirubin content than FMD group. Which may be due to the additional enzymes produced by the protoscoleces and/ or brood capsules.

Fig.4 showed changes in glucose content of control and HCF treated groups. It was noted that these content remained unchanged through out the study in control group. CLD group showed an average decrease in glucose content after administration of CHCF. When analyzed by Student's "t" test decrease in glucose content was found statistically significant ($P<0.05$) after 14 and 56 days of CHCF treatment in CLD group. Decrease in glucose content was found statistically significant ($P<0.05$) after 56 and 70 days of HCF treatment in FLD group. FMD group also showed a general decrease that was found statistically significant ($P<0.05$) after 56 days of HCF treatment in this group. In the HD group glucose content decrease was more pronounced

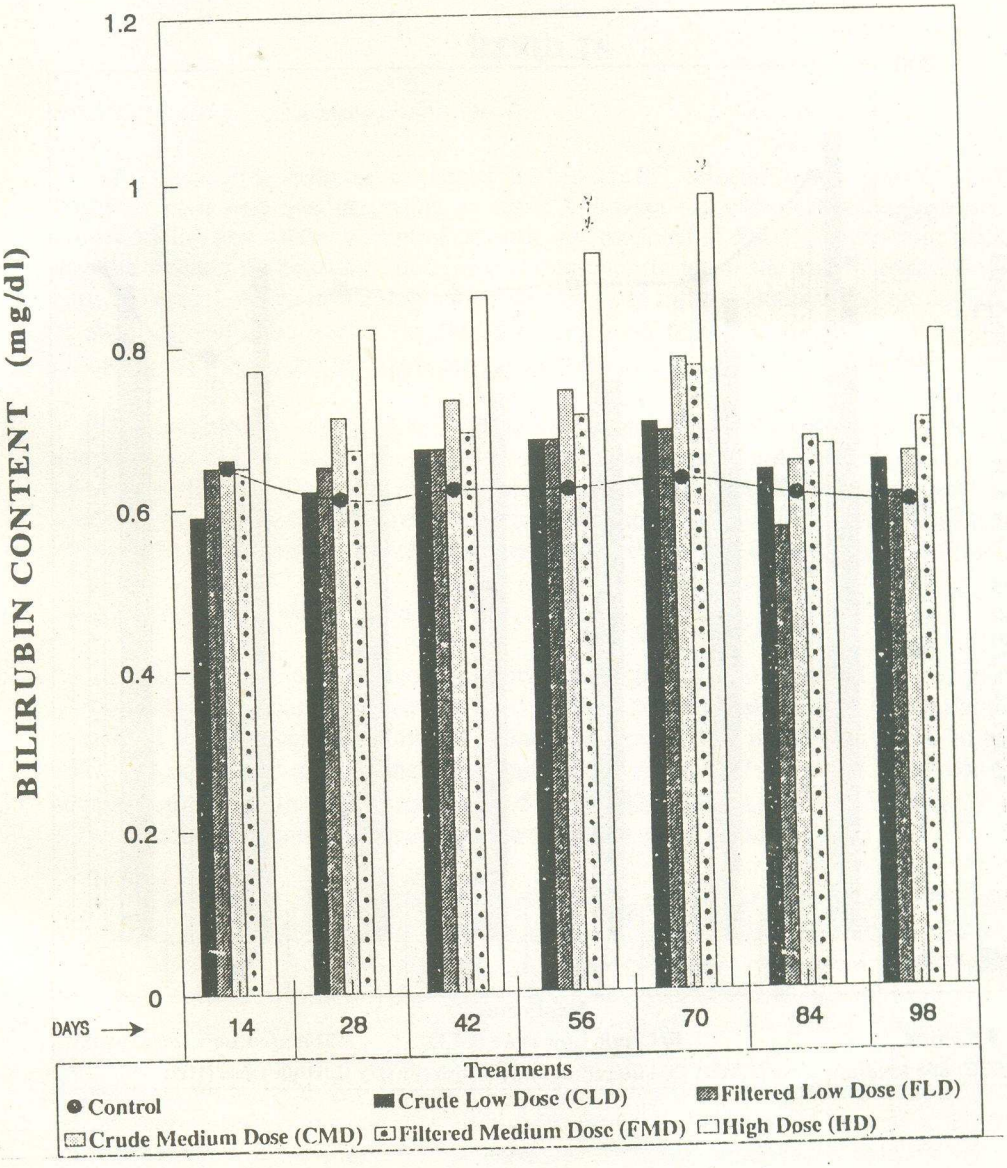

Fig. 3: Change in Bilirubin Contents (mg/dl) of rabbit blood after administrating different doses of sheep originated unilocular hydatid cyst fluid in various forms and doses like crude low dose (CLD), filtered low dose (FLD), crude medium dose (CMD), filtered medium dose (FMD) and high dose (HD) (please see materials and methods). Statistical significance has been determined by student's "t" test and the probability represented by stars; * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

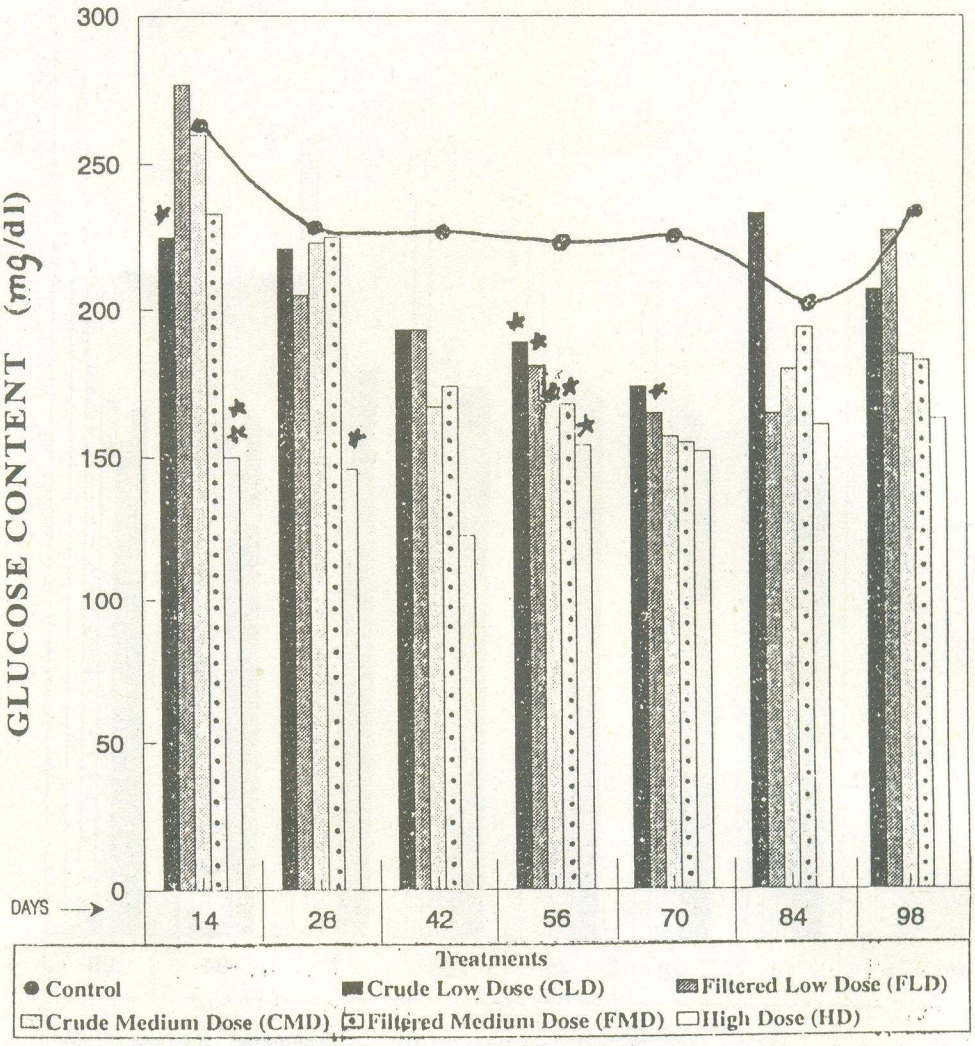

Fig. 4: Change in Glucose Contents (mg/dl) of rabbit blood after administrating different doses of sheep originated unilocular hydatid cyst fluid in various forms and doses like crude low dose (CLD), filtered low dose (FLD), crude medium dose (CMD), filtered medium dose (FMD) and high dose (HD) (please see materials and methods). Statistical significance has been determined by student's "t" test and the probability represented by stars; * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

and found statistically significant after 14 ($P<0.01$), 28 ($P<0.05$) and 56 days of HCF treatment in this group. Glucose is the main source of carbon for making different compounds in the body. It is the instant source of energy. The sequence of reactions by which glucose is degraded anaerobically is called glycolysis which produces ATP molecules, which is the high energy compound. The reverse process of glycolysis is called gluconeogenesis during which glucose molecules are produced from amino acids and other macromolecules. Surplus amount of glucose is also stored in the body in liver of animals as glycogen. Whenever there is deficiency of glucose in body then animal utilizes its stored glycogen for producing energy and making different compounds (Conn and Stumps, 1987). In the present findings, depletion in glucose content is common in all the groups and that the depletion was dose dependent. Glucose depletion in the present study may be due to (1) Decrease gluconeogenesis or increased glycolysis (2) Liver damage (3) Inhibition of liver enzymes (4) Stressed conditions (5) Tubular dysfunctioning in kidney, which resulted in reduced glucose reabsorption from nephron. Tufail *et al.*, (1984) have suggested that glucose level decreases because it might be forcefully catabolized by the aerobic and anaerobic enzymes which are also present in the protoscoleces (Agosin *et al.*, 1957). They have found kinases, myokinase, mannose, isomerase and phosphate in protoscoleces. In the present investigation FHCF contained enzymes of protoscoleces that are toxic. McManus and Smyth (1982) measured the substrates and enzymes of glycolysis and associated enzymes both in *Echinococcus granulosus* and *E. multilocularis*. They have determined that phosphorylase, hexokinase, phosphofructokinase and pyruvate kinases, were regulators of carbon flow in glycolysis. Glucose depletion in this study may be due to the aerobic and anaerobic enzymes present in the HCF. Agosin and Repetto (1963, 1965) found that scoleces and whole homogenate oxidize a range of tricarboxylic acid intermediates as well as of glutamate, glyoxylate, glycolate, acetate and lactate. Continuously low glucose content of CHCF and FHCF treated rabbits was noted with increase in time and dosage. This also indicated the stress condition of treated animals. Glucose is mostly oxidized for energy production to cope with the stress impacts. Similar findings have also reported by Tanveer *et al.* (1997, 1998, 1998a).

Fig.5 showed changes in Plasma protein content of control and HCF treated groups. It was noted that plasma protein content remained almost unchanged through out the study in control group. The decreased protein content in CLD group were found non significant through out this treatment. FLD group showed a slight increase that was found statistically significant ($P<0.05$) after 84 days of FHCF treatment. CMD and FMD groups showed decreased protein contents after administration of CHCF. HD group also showed decrease in protein content after FHCF administration. Decrease in protein content was found statistically significant ($P<0.05$) after 56 and 70 days of FHCF treatment in this group. Low protein content recorded in the eperimental rabbits (as compared to that of control) is an indication of direct proteolytic effect of CHCF and FHCF as they contained many lytic enzymes as reported by Faryha and Haddad (1980). Benjamin (1985) suggested that decrease in protein might be due to increased protein catabolism from strong condition like fever, infection etc. GOT and GPT activities are present in cystic fluid and are also involved in the interconversion of amino acids (Sanchez and Sanchez, 1971; Frayha and Haddad, 1980). These amino acids then can move to oxidative pathway to cope with energy requirement. *In vitro*, hydatid cyst took protein from surroundings and catabolize (Coltorti and Varela-Dias, 1975; Hustead and Williams, 1977). Increased protein content, in all the groups from 84 to 98 days, may be due to the formation of antibodies against the antigens present in hydatid cyst fluid. Faryha and Haddad (1980) indicated the presence of albumin, globulin, many enzymatic proteins, lactate dehydrogenase, phosphatase activity GOT and GPT in hydatid cyst fluid, which may also be the cause of elevated protein content at the end of experiment

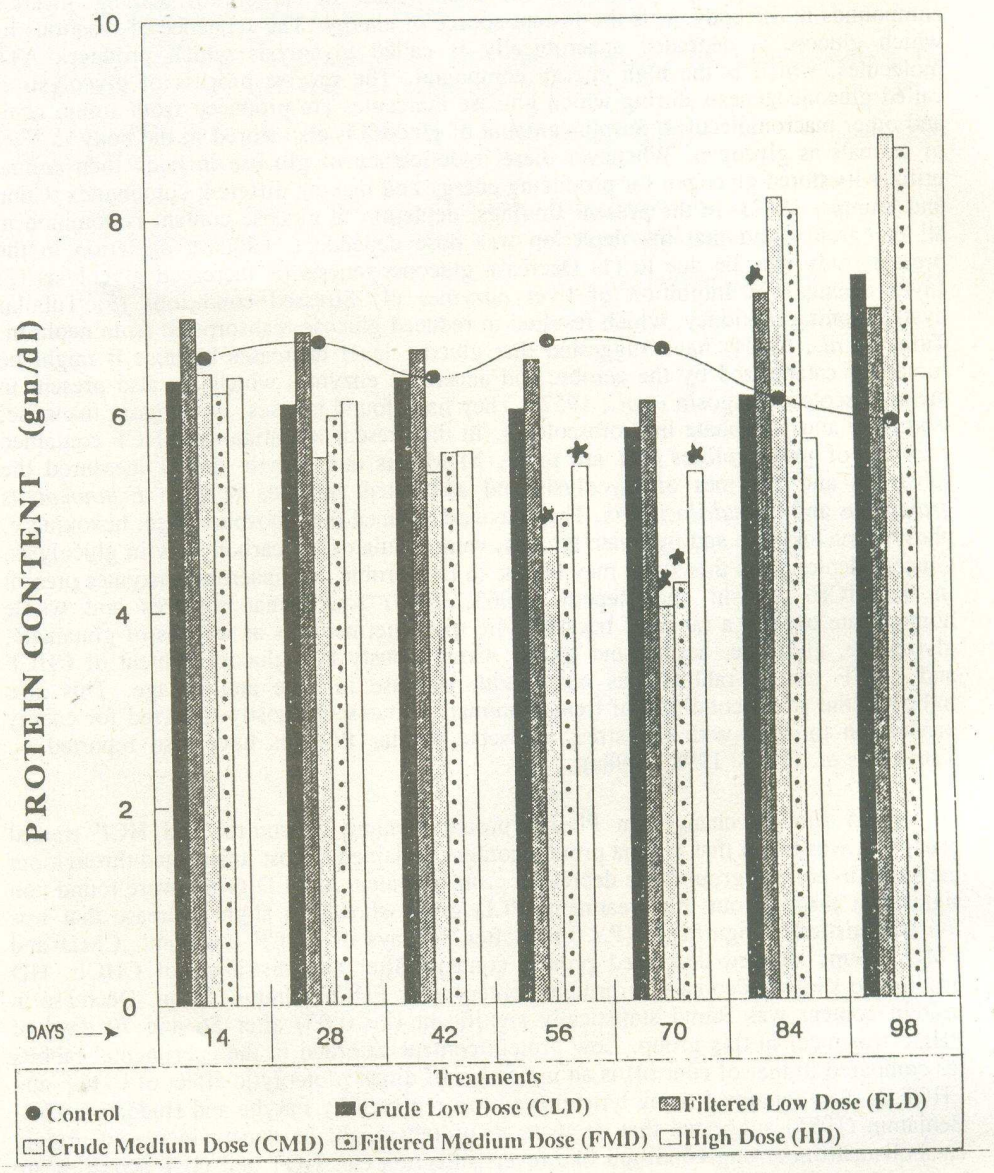

Fig. 5: Changes in Protein Content (mg/dl) of rabbit blood after administrating different doses of sheep originated unilocular hydatid cyst fluid in various forms and doses like crude low dose (CLD), filtered low dose (FLD), crude medium dose (CMD), filtered medium dose (FMD) and high dose (HD) (please see materials and methods). Statistical significance has been determined by student's "t" test and the probability represented by stars; * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

in all the treated groups. Nineteen protein components have been isolated from HCF of which ten were antigen of parasite origin (Biguet *et al.*, 1962; Capron *et al.*, 1962; Chordi and Kagan, 1965; Castagnari and Pozzuoli, 1969). Host immunoglobulins have also been reported in cyst wall, fluid and on the surface of the protoscoleces (Kassis and Tanner, 1977).

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TOXICITY OF BAYTHROID-TM : HEMATOLOGICAL EFFECTS IN GROWING CHICKS OF *GALLUS DOMESTICUS*

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Abstract: Baythroid (TM), a mixture of methamidophos and cyfluthrin, was administered orally to chicks as two concentrations (strong and weak) for 40 hours and 9 days, respectively. In strong dose (10 mg/kg body wt.) experiment the blood samples were collected from control and treated groups at 5, 10, 20 and 40 hours following administration of a single dose of insecticide. In weak dose experiment (5 mg/kg body wt./day), the blood was collected at 3, 6 and 9 days of insecticide administration and used to study various hematological parameters such as hemoglobin (Hb), total erythrocytic count (TEC), total leukocytic count (TLC) and hematocrit (Hct) estimation. The above data were used to evaluate mean corpuscular volume (MCV), mean corpuscular Hb (MCH) and mean corpuscular Hb concentration (MCHC). Baythroid-TM treatment as strong dose produce significant increase in Hb (32%, 41%, 28% and 30%) and MCH (22%, 32%, 25% and 27%) contents at 5, 10, 20 and 40 hours, respectively. Rise in case of Hct (29%) and TEC (9%) was also observed, which was significant only at 5 hours treatment. The TLC also showed an increase, which was 39%, 48%, 34% and 20% at 5, 10, 20 and 40 hours treatments, respectively. Weak dose of insecticide produced significant decrease in TEC (16%, 15% and 11%) and Hb content, which was 24%, 16% and 21% at 3, 6 and 9 day treatments, respectively. MCV and TLC showed an increase only at 3 and 9 days insecticide feeding durations, respectively. It is concluded that 10 mg dose level of Baythroid-TM, administered for short duration has somewhat stimulatory effect on hemopoietic tissue in chicks, which may be a protective response but weak dose administered for relatively long duration indicated suppression of blood components.

Key words: Insecticides, pesticides, pyrethroid, organophosphate blood cells, RBC, WBC, hematological indices, hemopoietic, vertebrate, aves.

INTRODUCTION

The present pesticides, in addition to their outstanding role in insect pest control, are also responsible for induction of variety of toxic effects in the environment and its various components (Gomes *et al.*, 1999; Khalaf-Allah, 1999; Stiller *et al.*, 1999). The pesticides currently in use are mostly organophosphates (OP) pyrethroids and their mixture. As a result of prolong unmanaged use, these pesticides are gradually becoming ineffective against insects due to induction of defence systems (Ben-Cheikh *et al.*, 1998; Chandre *et al.*, 1998; Park and Kamble, 1998; Wirth, 1998; Koffi *et al.*, 1999; Lee *et al.*, 1999). Gradually higher concentrations of these chemicals are required to kill the insect pests, the practice, which is environmentally more hazardous

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significant ($P<0.001$) after 5 hours of toxicant feeding (Tables I-II). In long term (weak dose) experiment, significant decline (24, 16 and 21%) in Hb content was observed at 3, 6 and 9 days ($P<0.05$), respectively (Tables III-VI). The Hct in both strong (10 mg/kg body weight) and weak dose (5 mg/kg body weight/day) Baythroid-TM treatments, did not show any severe alteration except 29% rise within 5 hours in case of strong dose (Tables I-II) and 19% decline at 9 days in weak dose treatment (Tables III-VI), while non-significant changes were observed at 3 and 6 days.

Table I: Effect of Baythroid-TM *(10 mg/kg/body weight)* administered for a total period of 40 hours on some hematological parameters of chick, *Gallus domesticus*.

Parameters[b]	Control (n=4)	Insecticide Treatment (Hours)			
		5 (n=5)	10 (n=5)	20 (n=5)	40 (n=5)
TEC	2.46[a]	2.67**	2.60	2.54	2.51
($X10^6/\mu l$)	±0.03	±0.03	±0.03	±0.06	±0.04
Hb content	5.94	7.86***	8.40**	7.63*	7.74**
(g/dl)	±0.17	±0.24	±0.54	±0.55	±0.38
Hct (%)	30.08	38.80*	35.47	32.49	31.64
	±2.67	±1.56	±1.69	±1.42	±1.01
MCV (fl)	120.73	144.38	138.03	127.79	125.78
	±9.22	±5.46	±5.13	±6.10	±3.12
MCH (pg)	24.07	29.46**	31.73**	30.04***	30.56***
	±0.80	±0.95	±1.79	±0.87	±1.50
MCHC (%)	20.70	20.79	22.71	23.48	23.85
	±1.11	±0.74	±0.55	±2.33	±1.50
TLC	20.30	28.20***	29.95***	27.10**	24.25**
($X10^3/\mu l$)	±0.57	±0.32	±0.44	±0.63	±0.82

a: Mean ± SEM; *: P<0.05; **: P<0.01; ***: P<0.001 (Student's 't' test).

Abbreviations used: Hb, hemoglobin; Hct, hematocrit; TEC, total erythrocytic count; TLC, total leukocytic count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; fl, femtolitre = 10^{-15} litre; pg, picogram = 10^{-12}g.

Total erythrocytic count did not exhibit any severe change in strong dose experiment, however weak dose of toxicant administered for 9 days proved more toxic then strong dose. A significant decrease of 15.7%, 15.5% and 11% was observed in TEC at 3, 6 and 9 days treatments, respectively (Tables III, VI).

Experimental procedure

Two doses of Baythroid TM were administered to chicks (orally) for two different durations *i.e.*, for 40 hours in short-term experiment and for 9 days in long-term experiment.

In short-term (strong dose) experiment a group of twenty-four chicks were used. Twenty chicks were divided into four groups of five birds each while a group of four animals were used for control experiments. All chicks were weighed and a strong dose of Baythroid-TM 10 mg/kg body wt. was administered orally to 20 mice with the help of blunt glass pipette. After the stipulated periods of 5, 10, 20 and 40 hours, a group of five treated birds were dissected, their blood samples were collected in anticoagulant (EDTA) containing eppendorfs.

For weak dose (long-term) experiment another group of 24 chicks was administered with Baythroid-TM @ 5 mg/kg body wt./day for 9 days. After regular intervals of 3, 6 and 9 days, a group of five chicks was taken out and dissected. The blood samples were collected as above for hematological studies.

Methodology used

The hemoglobin (Hb) content of the blood was estimated according to Vankampen and Zijlstra (1961). The hematocrit (Hct) was analyzed by microhematocrit method of Strumia *et al.* (1954), while the total erythrocytic count (TEC) and total leukocytic count (TLC) was performed according to routine clinical methods as described in Dacie and Lewis (1986). The above hematological values were then used to determine different hematological indices *i.e.*, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) as mentioned in Dacie and Lewis (1986).

RESULTS

Mortality

Administration of Baythroid-TM as weak dose (5 mg/kg body wt./day) produced 20% mortality in chicks at 3 and 9 days treatment groups, separately. The overall mortality of experimental animals in long-term experiment was 13.3%. No mortality was observed in short-term (10 mg/kg body wt./day) experiment.

Hematological effects

Baythroid-TM as strong dose produced significant increase of 32%, 41%, 28% and 30% in Hb content after 5, 10, 20 and 40 hours, respectively. The difference was highly

significant ($P<0.001$) after 5 hours of toxicant feeding (Tables I-II). In long term (weak dose) experiment, significant decline (24, 16 and 21%) in Hb content was observed at 3, 6 and 9 days ($P<0.05$), respectively (Tables III-VI). The Hct in both strong (10 mg/kg body weight) and weak dose (5 mg/kg body weight/day) Baythroid-TM treatments, did not show any severe alteration except 29% rise within 5 hours in case of strong dose (Tables I-II) and 19% decline at 9 days in weak dose treatment (Tables III-VI), while non-significant changes were observed at 3 and 6 days.

Table I: Effect of Baythroid-TM *(10 mg/kg/body weight)* administered for a total period of 40 hours on some hematological parameters of chick, *Gallus domesticus*.

Parameters[b]	Control (n=4)	Insecticide Treatment (Hours)			
		5 (n=5)	10 (n=5)	20 (n=5)	40 (n=5)
TEC	2.46[a]	2.67**	2.60	2.54	2.51
($X10^6/\mu l$)	±0.03	±0.03	±0.03	±0.06	±0.04
Hb content	5.94	7.86***	8.40**	7.63*	7.74**
(g/dl)	±0.17	±0.24	±0.54	±0.55	±0.38
Hct (%)	30.08	38.80*	35.47	32.49	31.64
	±2.67	±1.56	±1.69	±1.42	±1.01
MCV (fl)	120.73	144.38	138.03	127.79	125.78
	±9.22	±5.46	±5.13	±6.10	±3.12
MCH (pg)	24.07	29.46**	31.73**	30.04***	30.56***
	±0.80	±0.95	±1.79	±0.87	±1.50
MCHC (%)	20.70	20.79	22.71	23.48	23.85
	±1.11	±0.74	±0.55	±2.33	±1.50
TLC	20.30	28.20***	29.95***	27.10**	24.25**
($X10^3/\mu l$)	±0.57	±0.32	±0.44	±0.63	±0.82

a: Mean ± SEM; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$ (Student's 't' test).

Abbreviations used: Hb, hemoglobin; Hct, hematocrit; TEC, total erythrocytic count; TLC, total leukocytic count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; fl, femtolitre = 10^{-15} litre; pg, picogram = 10^{-12}g.

Total erythrocytic count did not exhibit any severe change in strong dose experiment, however weak dose of toxicant administered for 9 days proved more toxic then strong dose. A significant decrease of 15.7%, 15.5% and 11% was observed in TEC at 3, 6 and 9 days treatments, respectively (Tables III, VI).

Table II: Percent increase (+) or decrease (-) in different hematological parameters of chick, *Gallus domesticus*, after Baythroid-TM administration *(10 mg/kg body weight)* for a total period of 40 hours.

Parameters[a]	Insecticide Treatment (hours)			
	5	10	20	40
TEC	+8.53**	+5.69	+3.25	+2.03
Hb Content	+32.32***	+41.41**	+28.45*	+30.30**
Hct	+28.65*	+17.91	+8.01	+5.18
MCV	+19.58	+14.32	+6.49	+4.18
MCH	+22.39**	+31.90**	+24.80***	+26.96***
MCHC	+0.43	+9.71	+13.43	+15.21
TLC	+38.91***	+47.53***	+33.49**	+19.45**

*P<0.05; **P<0.01; ***P<0.001 (Student's 't' test).

For abbreviations, see Table I

Table III: Effect of Baythroid-TM *(5 mg/kg/body weight/day)* administered for a total period of 3, 6 and 9 days on some hematological parameters of chick, *Gallus domesticus*.

Parameters[b]	3 days		6 days		9 days	
	Control (n=3)	Treated (n=5)	Control (n=3)	Treated (n=5)	Control (n=3)	Treated (n=5)
TEC	2.09[a]	1.72*	1.98	1.60**	1.97	1.70*
($X10^6/\mu l$)	±0.09	±0.07	±0.08	±0.06	±0.07	±0.05
Hb content	7.92	6.02*	8.26	6.95*	8.38	6.61*
(g/dl)	±0.56	±0.47	±0.33	±0.41	±0.44	±0.31
Hct (%)	40.83	43.20	42.30	37.51	41.13	33.54**
	±1.62	±2.02	±1.88	±1.79	±1.52	±0.87
MCV (fl)	195.36	251.16*	238.04	228.72	213.11	195.00
	±9.66	±10.23	±14.62	±9.11	±14.97	±14.52
MCH (pg)	38.82	35.00	42.58	42.38	43.42	38.43
	±2.71	±1.42	±2.36	±3.27	±3.37	±4.25
MCHC	19.40	13.94*	19.53	18.53	20.37	19.71
(g/dl)	±1.44	±1.20	±2.15	±1.32	±0.81	±1.33
TLC	32.67	38.60	25.50	36.50**	31.83	39.25*
($X10^3/\mu l$)	±1.97	±3.71	±2.02	±1.46	±2.32	±1.97

a: Mean ± SEM (Student's 't' test) *P<0.05; **P<0.01; ***P<0.001.

For abbreviations, see Table I.

The only hematological parameter which showed prominent change in both insecticide treatments was TLC, which was increased with high significance by 39%, 48%, 34% and 20% at 5, 10, 20 and 40 hours treatments in strong dose (Tables I-II). In weak dose insecticide treatment, a significant increase of 43% and 23% was noticed at 6 and 9 day treatments, respectively, while a non-significant rise of 18% was observed at 3 days (Tables III-VI).

Table IV: Percent increase (+) or decrease (-) in different hematological parameters of chick, *Gallus domesticus*, after Baythroid-TM administration *(5 mg/kg body weight)* for the total period of 9 days.

Parameters[a]	Cyfluthrin Treatment (days)		
	3 (n=5)	6 (n=5)	9 (n=5)
TEC	-15.69*	-15.46**	-10.88*
Hb content	-23.89*	-15.85*	-21.12*
Hct	+5.80	-11.32	-18.49**
MCV	+28.58*	-4.89	-8.49
MCH	-9.99	-0.46	-11.49
MCHC	-28.14*	-5.12	-3.24
TLC	+18.15	+43.14**	+23.31*

*P 0.01; **P 0.01; ***P 0.001 (Student's 't' test)
For abbreviations, see Table I.

Hematological indices almost reflected the same pattern of changes as were observed in case of TEC, Hb and Hct. Tables I-VI indicate the effect of Baythroid-TM as strong and weak doses, on hematological indices values of chick. The MCV and MCHC did not show any sign of toxicity throughout the experiment. In contrast MCH exhibited the prominent rise of 22%, 32%, 25% and 27% at 5, 10, 20 and 40 hours after insecticide feeding. The last two values were highly significant (Tables I-VI).

DISCUSSION

Two different doses of Baythroid-TM administered for various durations produced variable effects on hematological parameters. The TEC and Hct did not show any significant shift at 10 mg dose level, administered for short duration except 9% and 29% increase at 5 hours treatment respectively. Haemoglobin content on the other hand, showed 28-41% increase from 5 to 40 hours treatment. The rise in TEC and Hct was also found in rabbits administered with Karate (cyhalothrin) for 15 days @ 10 mg/kg body wt./day (Shakoori *et al.*, 1992). Ali *et al.* (1997) showed increase in Hb and MCHC following malathion toxicity in chick of *Gallus domesticus*. This increase may be a protective response in chicks to counter the toxic effects and for induction of defense system of the body. In 9 day insecticide treatment, however, TEC and Hb decreased

significantly, which were 11-16% in former case and 16-24% in later. Decrease was also found in Hct (19%) and MCHC (28%) at 9th and 3rd day, respectively. The results indicated that Baythroid-TM administration at 10 mg dose level for short duration has somewhat stimulatory effect on hemopoietic system at least for initial few hours, while significant alteration were observed in 5 mg dose level administered for 9 days.

This type of data showed that this dual target site mixture of OP and pyrethroid insecticides, induced its toxicity gradually after few days exposure even at low dose (5 mg/kg body wt./day). Toxicant induced hematologic disorders such as RBC, WBC counts, Hct, MCH, MCV porphyries, porphyrinurias, aplastic anemia and increased risks of leukemia or lymphoma has been reported (Shakoori *et al.*, 1988; Ali and Shakoori, 1981, 1990; Lisiewicz, 1993; Khalaf-Allah, 1999; Ali *et al.*, 1997, 2000). In another study mixture of endosulfan, dimethoate and carbaryl, given to rats orally induced significant alteration in red cell count, white cell counts and Hb content, while no change was observed when these pesticides were administered alone at 100 fold ADI level (Akay *et al.*, 1999), while MCV, MCH, MCHC and erythrocyte sedimentation rate remained unaffected.

The findings in the present experiment differ from the studies of Shakoori *et al.* (1990a,b) with bifenthrin (a pyrethroid) in rabbits, which showed the significant decrease in Hb content, TEC and MCHC in 30 days study with sublethal dose. Similar decrease was also observed in rat and fish with other pesticides like malathion, aldrin, gamma-BHC, endrin and cypermethrin (Ali *et al.*, 1988; Ali and Shakoori, 1990; Shakoori *et al.*, 1988; Reedy and Bashamohideen, 1989; Guilhermino *et al.*, 1998). Ahmad *et al.* (1995) reported decrease in Hb and Hct values following administration of fenpropathrin (Danitol) for 30 days in Chinese grass carp. Similar decrease in Hb, TEC and Hct was also shown in another study with DDT in rats administered @ 10, 20 and 100 mg/kg body wt. for 48 hours, 15 days and 18 months, respectively (Ali and Shakoori, 1994). There was a significant increase in TLC in both treatments, which was more prominent in 10 mg dose level administered for 40 hours duration. Increase in TLC is a typical protective response of vertebrate systems against toxic insult (Ali and Shakoori, 1990, 1994; Shakoori *et al.*, 1990a,b). Ahmad *et al.* (1995) also reported the significant increase in WBC count with sublethal dose of Danitol (a pyrethroid) in fish, which may enhance the detoxification process.

The present study revealed that administration of double target site mixture of OPs and pyrethroid (Baythroid-TM) insecticides to chicks, slight stimulatory effects in case of 10 mg dose level fed for 40 hours, while low dose fed for 9 days showed significant inhibitory effects on hemopoietic system in chicks.

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STREPTOZOTOCIN INDUCED HYPOINSULINEMIA ON STRUCTURAL ASPECTS OF THYROID GLAND IN DWARF GOAT

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Abstract: A successful hypoinsulinemia (experimental diabetes type 1) was induced with two consecutive doses of 33mg/kg body weight and 40mg/kg body weight streptozotocin (STZ), at the gap of four days, in male dwarf goats. Marked hyperglycemia >250mg/dl (average increase of 278%, $P < 0.001$) and significant glycosuria up to 1.7 to 7 g/dl confirmed the induction of the syndrome. The hypoinsulinemic and control goats were maintained for 14 weeks before slaughtered for obtaining tissues. Marked effects on the morphology of the hypoinsulinemic compare to the control goats have been observed. Increased follicular size with fewer interfollicular stroma and excessively flattened follicular epithelium with the evident indications of inactive state of the gland are the characteristic of the thyroid in insulin insufficiency. The weight of thyroid did not exhibit a noticeable variation in the comparable groups, however, average follicular epithelial height was measured at 7.26±0.09 μm and 4.22±0.10 μm in the control and hypoinsulinemic goats respectively. Calcitonin cells remained unaffected in hypoinsulinemia.

Key words: Goat, diabetes, thyroid.

INTRODUCTION

Interrelationships between endocrine glands maintain the crucial role of physiological homeostasis. Almost four decades ago endocrine pancreas-thyroid interaction received preliminary attention. Since then numerous studies have been reported to show sub-normal thyroid function in experimentally induced diabetes mellitus (Serif and Sihotang, 1962; Kumareson and Turner, 1966; Cheema and Matty, 1972; Zaninovich et al., 1977 and Jolin and Gonzalez, 1978). Similarly in clinical observations reduced thyroid function in diabetes (Zaripova, 1970; Inada, 1973; Saunders et al., 1978; Solar and Nicholson, 1979 and Gray et al., 1980) and stimulated thyroid activity following insulin administration (Blum 1973) have been observed. Induced diabetes creating hypoinsulinemia have been employed also in ruminants to see the effects of insulin deprivation on various physiological targets such as metabolites in pregnant and lactating ewe (Leenanuruksa and McDowell, 1988; Lips et al., 1988 and Miodovnik et al., 1989). However, a study in such induced state on thyroid function in ruminants has not appeared.

The role of insulin in ruminants is very intriguing as these exhibit insulin resistance. This is an adaptation, in ruminants to manage the poor availability of glucose directly

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from gastrointestinal tract (GIT) and to promote gluconeogenesis in liver and kidney (Brockman, 1990; Wastney *et al.*, 1983)). Thus the role of insulin in endocrine pancreas-thyroid interaction, in ruminants, may be expected deviated from non ruminants. Thus the present study is performed to investigate endocrine pancreas and thyroid interaction in goat model of the ruminants to compare with reported effects of these axis in non ruminants.

MATERIALS AND METHODS

A group of adult four male dwarf goats tagged as 16, 17, 19 and 20 were shifted from Farm house to animal house for experimentation and round the clock care. Similarly another batch of 4 male goats numbered as 36, 37, 38 and 39 were kept as control group. The goats were confined to the animal house and were provided with green fodder and concentrate dry ration. An injection of streptozotocin (Sigma Chemical Company, USA) was prepared at a concentration of 33mg/ml in sterile saline citric acid buffer of pH 4.3 and administered to the experimental goats in a dose of 33mg/kg body weight. Subsequently the goats were monitored for glycemia and glycosuria. Goats were not rendered diabetic with this dose, therefore administered with another dose of 40mg/dl 4 days after the first injection. The goats developed significant hyperglycemia, three days after the second injection goats were suffering with severe hyperglycemia and glycosuria. The goats were maintained for 14 weeks. The control goats were also maintained in the similar conditions of diet and housing condition and sham injections of sterile isotonic saline were given on the same pattern as for experimental goats.

Following slaughtering tracheal tube was excised carefully and thyroid lobes along the isthmus were separated. The gland of each goat was weighed and of two goats from each batch of experimental and controls was fixed in Bouin's fixative. The tissues were dehydated in grades of ethyl alcohol, cleared in cedar wood oil and the oil was removed with xylene. Tissues were ultimately fixed in high grade paraffin wax for serial sections on a rotary microtome at a thickness of 6μ. Tissues were fixed on glass slides and stained with Ehrlich's haematoxylin and eosine as prepared by Drury et al. (1973). Tissues were studied under the light microscope at various magnifications for histological and histometric observations.

RESULTS AND OBSERVATIONS

General

The surface of the gland in control goats was smooth in contrast to uneven surface with tiny out bulgings form the glandular surface in the hypoinsulinemic goats. The mean weight of the gland was 1.676± 0.101g in the diabetic and 1.800±0.083g in the control goats. The weight of the gland did not show any noticeable difference in both the comparable groups (Fig.1).

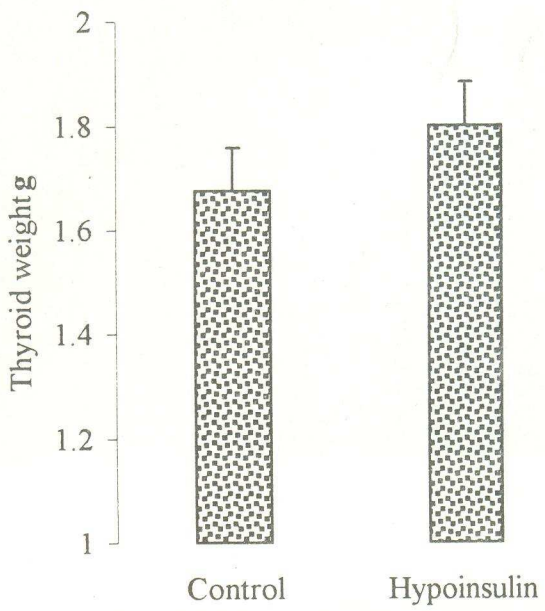

Fig. 1: Thyroid weight in intact and induced hypoinsulinemic goats.

Histological study

The follicles with sufficient interfollicular stroma clearly represent the picture of thyroid in intact goats. The follicles of varying size and shapes are observed. There is a trend of distribution of larger size follicles on the periphery and smaller size follicles towards the center of the glandular geography. Colloid in the follicles fills, adequately, intrafollicular space with striations and spaces in between hence clearly explicating optimum or moderate secretory capacity of the gland (Fig. 2)

In diabetic goats the substance of the gland is occupied by follicular spaces. The follicles of the glands are randomly distributed with out any specific pattern. Colloid is significantly reduced in the follicular cavity, as there is large space between follicular epithelial and colloidal boundaries. The epithelium of the follicles is comparatively flattened and appears that fewer interfollicular stroma is the result of enlarged follicles (Fig.3).

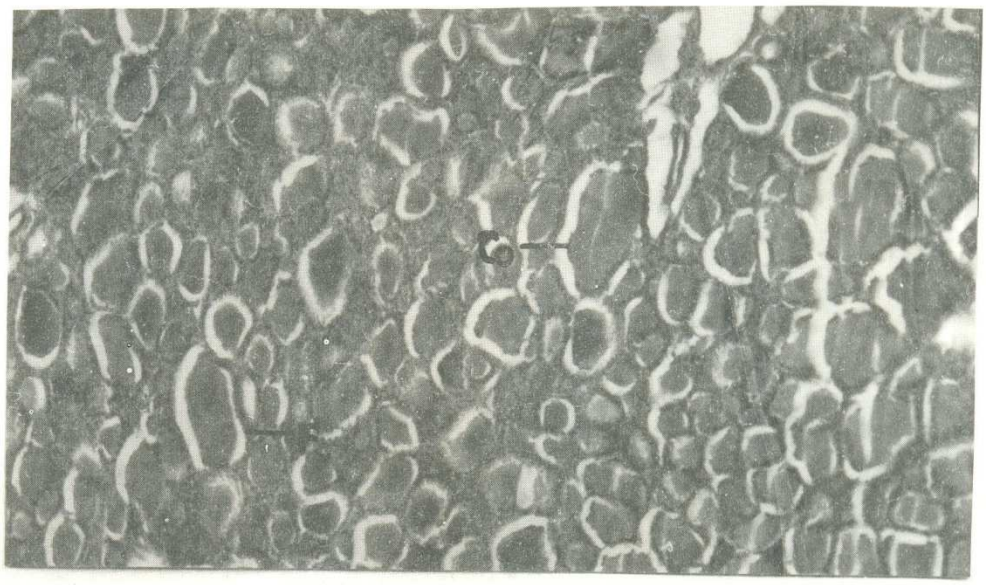

Fig. 2: Low power photomicrograph (x100) of a portion of thyroid gland in intact goat. F, Follicles; Co, Colloid.

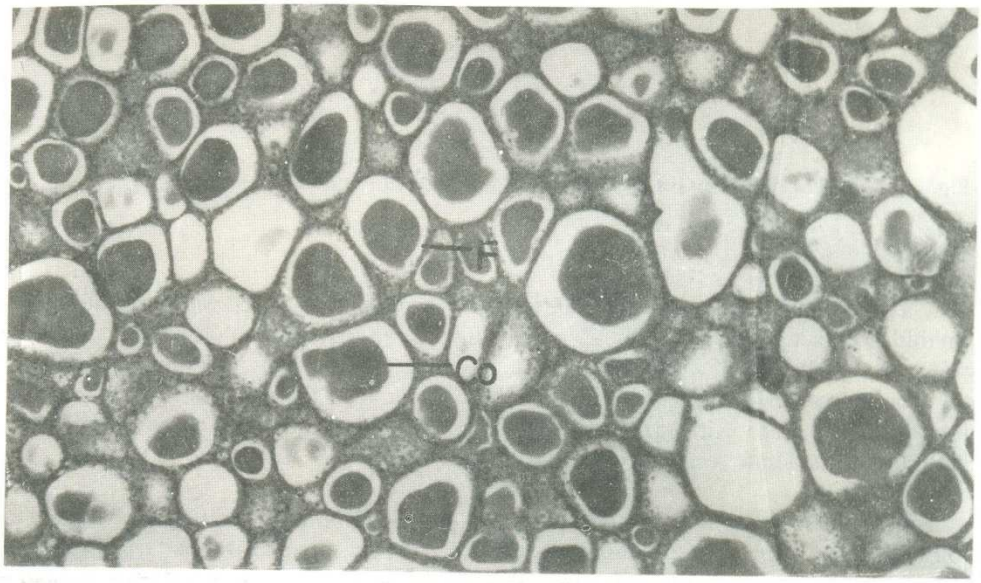

Fig. 3: Low power photomicrograph (x100) of a portion of thyroid gland in hypoinsulinemic goat. F, Follicles; Co, Colloid.

In the control goats, follicular epithelium is cuboidal, with nucleus occupying the central position in the epithelial cells. The microvillous extension form the inner membrane of the epithelium and intrafollicular droplets are clearly demonstrated (Figs 4 and 5). The general status of the gland is evidently in healthy secretory phase with replenishing synthesizing capacity.

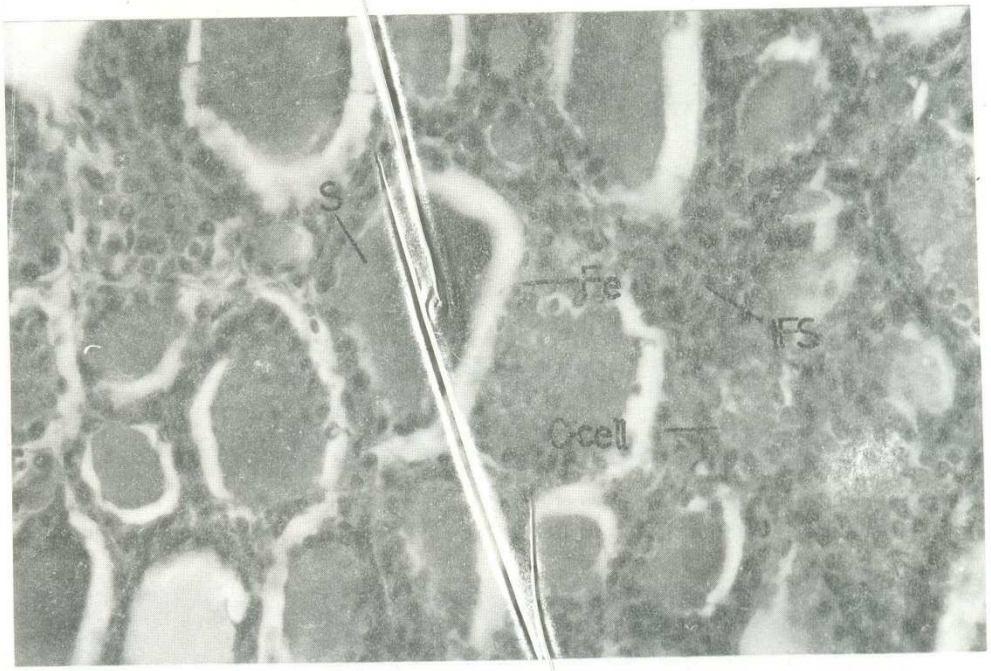

Fig. 4: High power photomicrograph (x400) of a portion of thyroid gland in intact goat. Fe, Follicular epithelium; ICD, Intrafollicular colloid droplets; C Cell, Calcitonin cells.

In diabetic goats, in contrast, has conspicuous flattened follicular epithelium and their nuclei are squeezed in the cellular membranes facing basement membrane and the follicular cavity. In the most of the follicles microvillous extension are absent or sparsely present in some. Similarly intrafollicular droplets are few. These observations demonstrate reduced synthetic ability in the gland (Figs. 6 and 7). Also the secretory capacity with reduced replenishing synthetic activity had depleted the amount of colloid in the follicular substance. Calcitonin cells did not exhibit any noticeable difference in the comparable groups.

Morphometric study

The average follicular epithelial height in the control goats was determined at 7.26±0.09 μm and 4.22±0.10 μm in the control and hypoinsulinemic goats respectively.

The follicular height was 72% ($P<0.001$) lower in hypoinsulinemic compare to the control goats (Fig. 8).

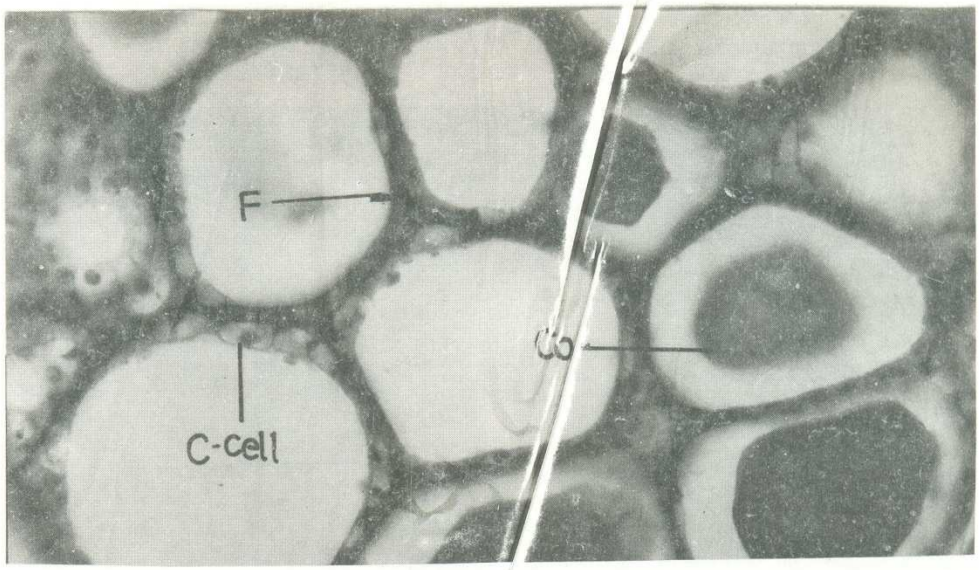

Fig. 5: High power photomicrograph (x100) of a portion of thyroid gland in intact goat. Fe, Follicular epithelium; ICD, Intrafollicular colloid droplets; C Cell, Calcitonin cells.

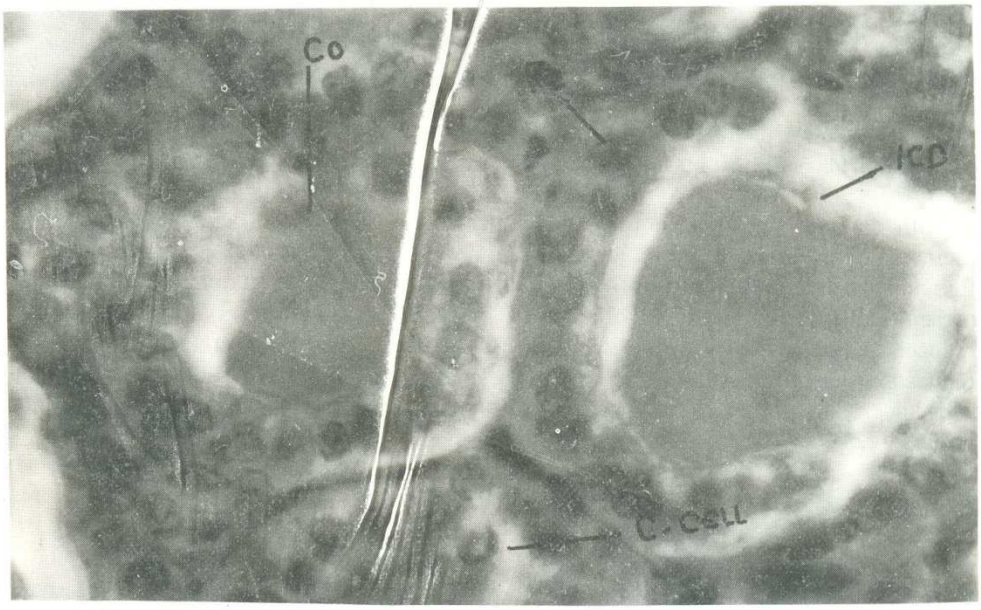

Fig. 6: High power photomicrograph (x400) of a portion of thyroid gland in hypoinsulinemic goat. F, Follicles; Co, Colloid; C Cell, Calcitonin cells.

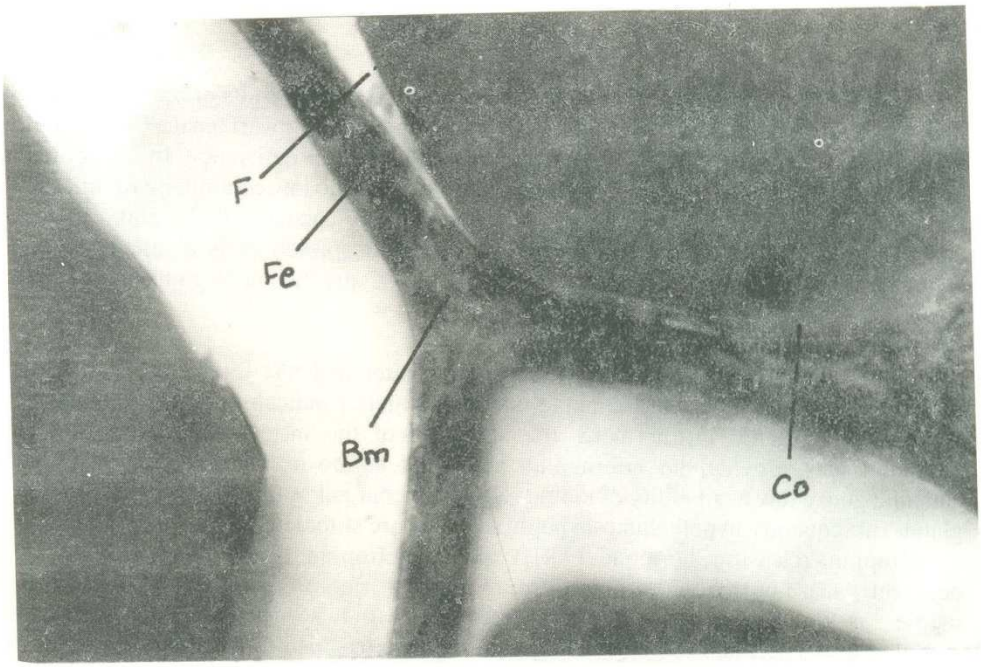

Fig. 7: High power photomicrograph (x100) of a portion of thyroid gland in hypoinsulinemic goat. F, Follicles; Fe, Follicular epithelium; C, Colloid; Bm, Basement membrane.

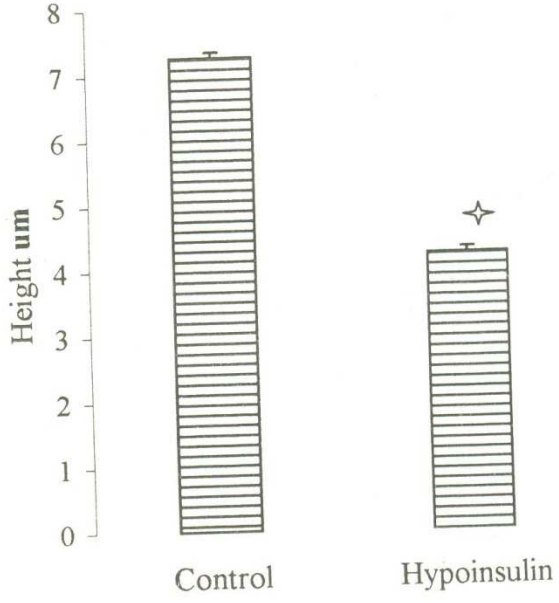

Fig. 8: Follicular epithelial height in intact and induced hypoinsulinemic goats. *P<0.0001.

DISCUSSION

Induced hypoinsulinemia, as clearly demonstrated in the results of the present study, markedly affects functional aspects of thyroid gland in the dwarf goat. The structural alterations in the gland are the manifestation of the insulin deficiency. In the follicular picture of the glandular histology, epithelium is squamous to low columnar with microvilli and intracolloidal droplets exhibiting efficient secretory capacity of the gland in intact goats. In contrast in hypoinsulinemic goats, follicular epithelium is distinctly low and cuboidal. The amount of colloid is also in decreased quantity in hypoinsulinemic goat compare to the control goats.

Morphology and histology of thyroid gland alter with the functional status of the gland. Hence enlargement of the gland in endemic goiter indicates the failing functional ability of the gland. Furthermore, the attributes of the histological and histometric alteration may provide the information about the nature of thyroid clinical state. In endemic goitre low availability of iodine reduces the out put of thyroid hormones from the gland, subsequently hypothalamo-hypophyseal axis are stimulated to enhance secretion of thyrotrophin releasing hormone (TRH) and thyrotrophin (TSH) to compensate the deficient release of thyroid hormones from the gland. Because of very low availability of iodine, as the precursor in the synthesis of thyroid hormones, the compensatory stimulation persists and follicular epithelium is hypertrophied to increase the size of the gland (Medeiros-Neto, 1989 and Greenspan, 1997). In experimental hypophysectomy in rats, follicular cells are flattened and lamina are distended by colloid (Halmi, 1978). Low cuboidal follicular epithelium is the characteristic of hypoactive follicles and consequently reduced functional capacity of the gland. (Ekholm, 1989).

The alteration of follicular epithelium, following induction of hypoinsulinemia, to low cuboidal clearly demonstrate hypoactivity in the gland and has resulted from reduced stimulation by TSH. However, in a typical reduced TSH mediated thyroid hypoacitivity, the distension of colloid in the follicular lamina is expected. Unlike to it the normal releases of thyroid hormones, as reflected by lower amount of colloid, are observed. Pericas and Jolin (1977) in their study on streptozotocin induced diabetic rats have concluded that lack of insulin diminishes the full response of thyroid to TSH and affect the pituitary TSH secretion. In light of this observation, hypoactive thyroid following induced hypoinsulinemia seems to result from diminished TSH secretion and its response on thyroid cells. However, it appears that response of TSH in thyroid hormones' synthesis is more adversely affected than the secretory capacity of the gland.

The alterations in histological results of thyroid following induced hypoinsulinemia are marked and evidently demonstrate shifts in pituitary-thyroid axis in insulin deficiency in the dwarf goat. The assay of TRH, TSH and thyroid hormones shall certainly elucidate the nature of this shift.

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ISOLATION OF *BACILLUS* SP. FROM WATER SAMPLES COLLECTED FROM SNAIL HABITATS AND ITS EVALUATION AS BIOMOLLUSCICIDE

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Abstract: *Bacillus subtilis* isolated from water samples collected from snail habitat was used as biomolluscicide against the freshwater snail *Lymnaea acuminata*. The snails were treated with bacteria. The histopathology induced by bacteria was noted. The LT_{50} and LT for control as well as treated snails were determined. These findings showed that the *Bacillus subtilis* has significant effect ($P<0.001$) on survival of the snails.

Key words: Bacteria as snail controlling agent.

INTRODUCTION

Snail intermediate hosts of various trematodes of medical and veterinary importance have caused several problems in Pakistan and thus threat imposed by them needs their fresh evaluation (Tanveer *et al.*, 1990). In a country, like Pakistan, where livestock and fisheries is steadily assuming great importance, the diseases influencing these animals have a significant bearing on the economy of the country (Tanveer and Khan, 1991). The control of snail population is, therefore, of a great value in minimizing the economic loss associated with these diseases. Both chemical and biological control methods have employed for this purpose in the past.

Chemical control is brought about by the use of molluscicides such as Nicolsamide (Cheesbrough, 1987) and $CuSO_4$ (Tanveer *et al.*, 1995; Hussain *et al.*, 1996). But Nicolsamide is cost effective (Cheesbrough, 1987) and $CuSO_4$ is reported to have adverse effects on fish and mouse (Ebele *et al.*, 1990; Benthein *et al.*, 1995). For these reasons, biological control method have been preferred over chemical control (PaurtdeBoch, 1961). Biological control of snails have been brought about by using prawn (Roberts and Kuris, 1990), molluscivorous fish (Slootweg *et al.*, 1993; Shelton *et al.*, 1995) competitive snail species (Cazzaniza, 1990; Tanveer and Khan, 1991; Hofkin *et al.*, 1991; Kinzie, 1992; Tanveer, 1995) and nematodes (Agricultural Genetics Company, 1996).

Bacteria have also been used as biocontrol agents against snails (Cheng, 1986; Agricultural Genetics Company, 1996). Bacteria have normally been found to be associated with snails as commensals or pathogens (Cheng, 1986; Watkins and Simkiss, 1990). These bacteria include various genera like *Bacillus, Pseudomonas, Staphylococcus, Acinotobacter, Micrococcus, Xanthomonas* etc. Bacteria belonging to genus *Bacillus* have

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been vigorously used to control pests (Berkley and Goodfellow, 1981; Fedianina *et al.*, 1993).

In present study *Bacillus subtilis* isolated from snail habitats was used as biomolluscicide against freshwater snail, *Lymnaea acuminata*.

MATERIALS AND METHODS

Collection and maintenance of snails

Freshwater snails *Lymnaea acuminata* were collected from different areas of Lahore including Botanical Garden, University of the Punjab, Lahore; Department of Zoology, University of the Punjab, Lahore; Botanical Garden, Government College, Lahore; and Jinnah Garden, Lahore. They were maintained in laboratory in pots and feds on fresh mulberry and Spinach leaves and checked for trematode infection. Snails free of any trematode infection were further used.

Histology of normal snails

The snails were removed off from their shells, fixed and dehydrated in alcohol, infiltered with xylene-wax mixture (1:1) and embedded in wax for 1-3 hours at 57°C. Slides of normal snail tissues were stained using Haematoxylin-Eosin method (Humason, 1967). Histological preparations were studied under microscope.

Isolation and identification of bacteria

Bacteria were isolated from water samples collected from snail habitats on Nutrient Agar medium (Rhode, 1973) by Streak-plate Method (Seeley and Vandermark, 1962). Isolated strains were checked for Gram reaction (Cheesbrough, 1993) and strain was identified by growing it on Blood Agar and MacConkey Agar media (Stokes and Ridgeway, 1980) and by performing biochemical tests (Holt *et al.*, 1994) such as Motility Test, Catalase Test, Indole Test, Voges-Prokaur Test, Nitrate Reduction Test, Trosin Decomposition Test, Citrate Utilization Test and Acid Releasing Test.

Treatment of snails and histology

Bacteria were grown in Luria Bertani (L.B.) broth medium (a liquid medium). Ten snails were taken in jars and treated with bacteria for 24, 48 and 72 hours, respectively. The tissues of treated snails were stained using MacCallum Good Pasture method (Mallary, 1944). The histological preparations were studied under microscope to observe histopathology induced by bacteria LT and LT_{50} for both control (untreated) and treated snails were determined.

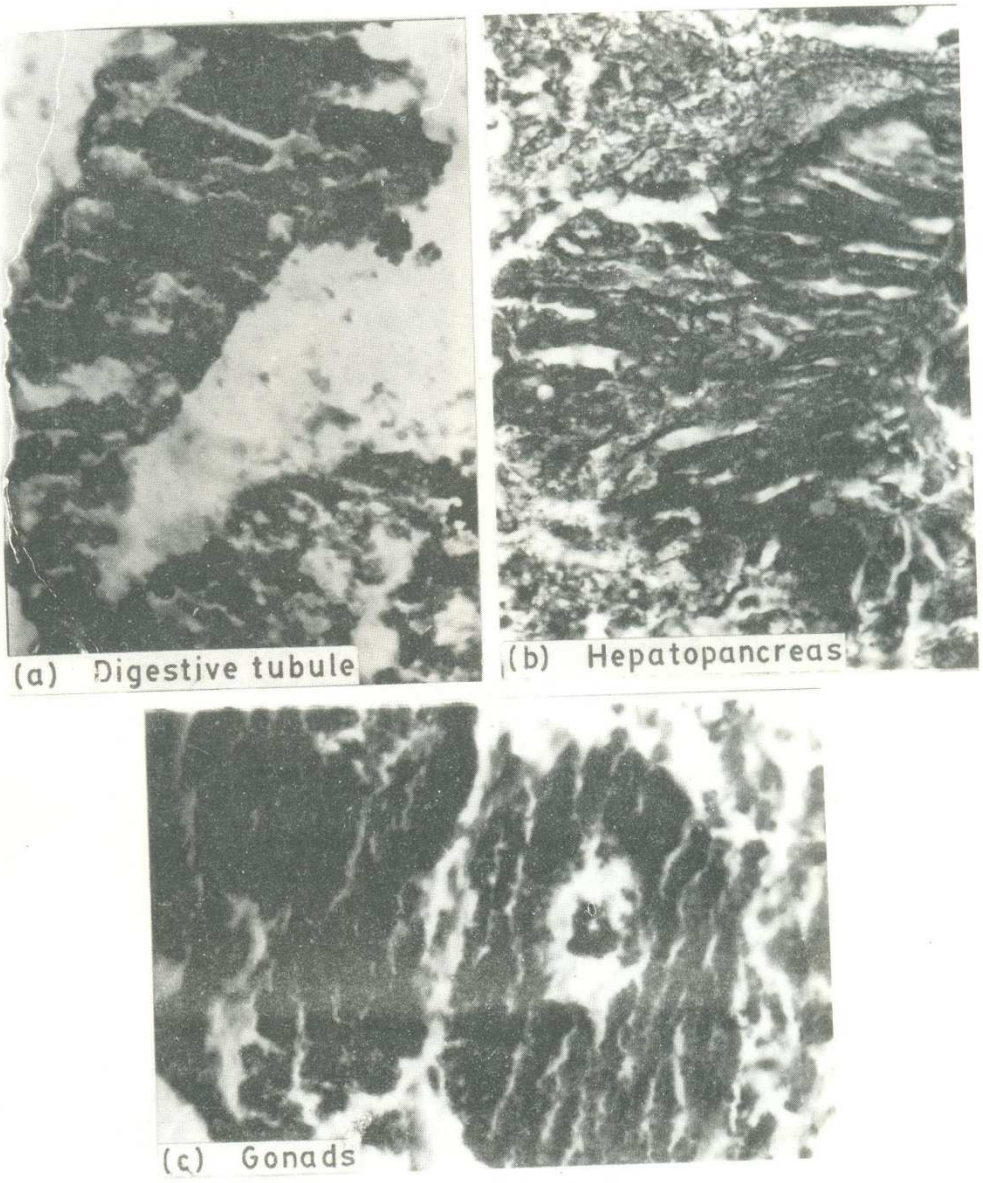

Plate I: Histology of normal *Lymnaea acuminata*.

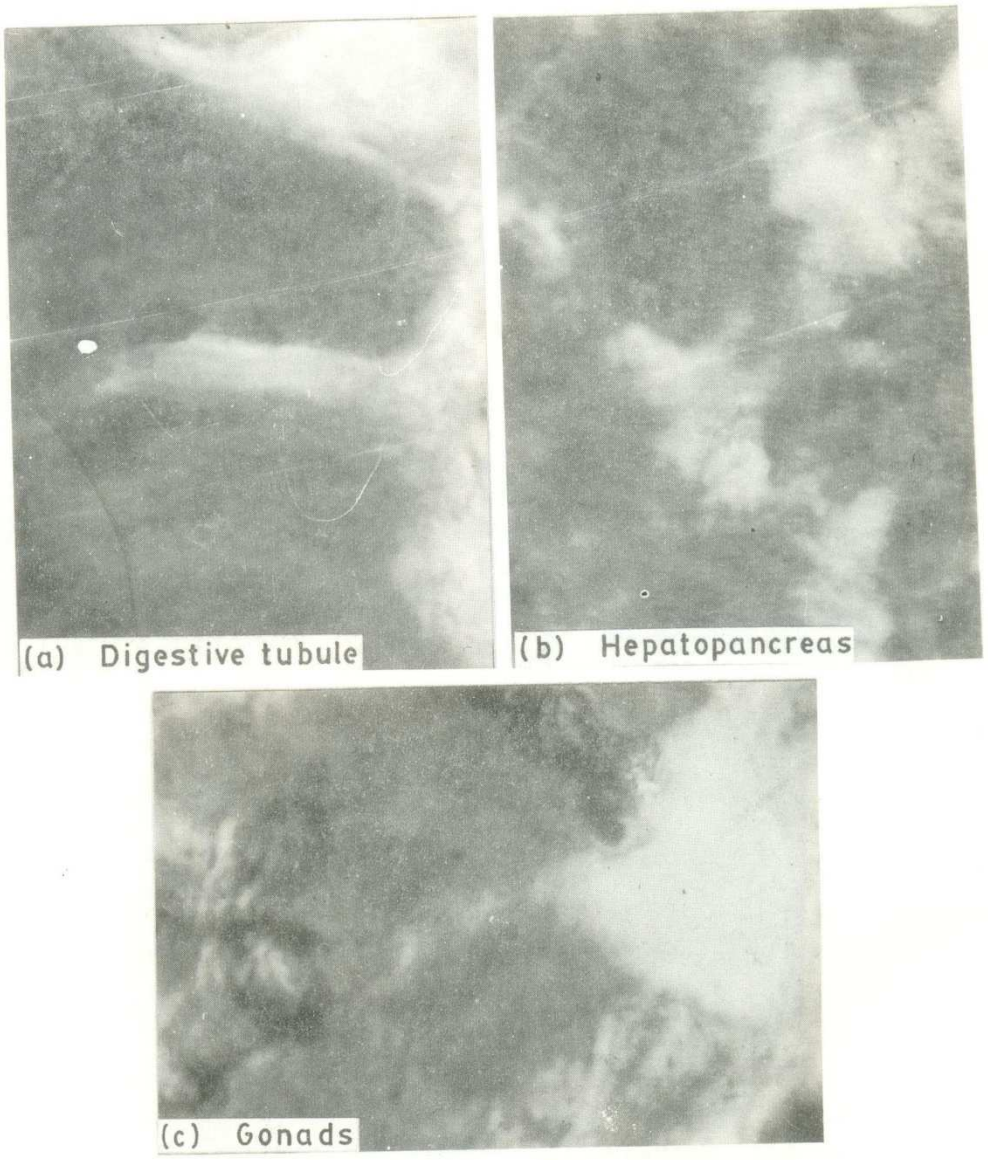

Plate II: Histology of *Lymnaea acuminata* treated with *Bacillus subtilis*.

Histological preparation of both normal and treated snails were made and photographs were compared to show the effect of bacteria on snail tissues.

RESULTS

Bacteria were isolated from water samples collected from snail habitats and tested for gram staining. Gram positive strain was identified by different growth tests and biochemical tests.

Identification of isolated strain

The results of gram staining, growth and biochemical tests were employed for identification of strain. Gram staining revealed that bacteria were gram-positive rods. Growth on blood agar showed that bacterial colonies were more than 1 mm in diameter. The strain did not show growth on MacConkey agar medium. This revealed that bacteria belonged to genus *Bacillus*.

Different biochemical tests were performed in order to confirm the bacterial species. Among these Catalase test, Voges Prokaur (V.P.) test, Arabinose and mannitol (acids) production test, Motility test, Citrate utilization test, Nitrate reduction test were positive and Tyrosin decomposition test was negative.

These tests confirmed that isolated strain was *Bacillus subtilis*.

Histology of normal snails

The posterior half of the snail body is largely composed of digestive and reproductive system. Digestive system canal extends from mouth to anus. Adjacent to digestive loop are gonadal tissues, which lie at the extreme tip of the body.

Digestive system

The digestive system consists of mouth, pharynx and pharyngeal glands, oesophagus, gizzard, intestine and anus. The intestine is lined with a tall, ciliated epithelium, which is supplied with sub0epithelial mucous glands and has an inner layer of longitudinal muscle fibers and an outer muscular layer (Plate Ia).

Hepatopancreas

The hepatopancreas consists of tubules lined by epithelium. The tubules are held together by interstitial cells and whole organ is enclosed in "tunica propria – the epithelial sac". The epithelium consists of secretory and absorptive cells. The absorptive cells are

elongated to oval in shape and are tightly packed. The secretory cells are columnar in shape with large spherical nuclei and dense cytoplasm (Plate Ib).

Gonads

Lymnaea acuminata snails are hermaphroditic. Ovotestis, hermaphroditic duct and spermoviduct are hermaphroditic components. Male part consists of prostrate, vas deferens, penial complex etc., while uterus, vagina and albumin gland make the female parts. The reproductive tract is lined by glandular epithelium with small ciliated cells. The cells of epithelium have spherical form and rounded nuclei (Plate Ic).

Histopathological studies

Histopathological studies were based upon the comparison between the photographs of the tissues of normal and treated snails. The bacteria infected all soft body parts of snails specifically intestine. There were seen discrete lesions in the tissues of treated snails with bacteria in them (Plate IIa,b,c). LT_{50} for normal as well as treated snails were also determined under laboratory conditions (Table I). It was observed that *B. subtilis* had significantly effected ($P<0.001$) the survival of the snails. On the basis of these studies, it was confirmed that *B. subtilis* was suitable to be used as biomolluscicide against *L. accuminata*.

Table I: Determination of LT_{50} and LT for control as well as treated *Lymnaea accuminata*.

	Control	Treated with *B. subtilis*
LT_{50}	290±19.2[a]	98±1.3***
LT	362±9.42	115±1.73***

[a]Mean±S.E.; ***P≤0.001.

Abbreviations used: LT_{50}, Time for 50% mortality (hours); LT, Time for 100% mortality (hours).

DISCUSSION

The freshwater snails act as intermediate hosts for various digenetic trematodes. These digenetic trematodes are organisms of considerable medical and veterinary importance. As snails form an essential and easily vulnerable link in the transmission of trematode infections, their destruction is of considerable value. For this purpose both chemical and biological control methods are employed. The target of chemical control have been achieved by using molluscicides. But the molluscicides have adverse effects on environment as well as on non-target organisms *e.g.*, $CuSO_4$ has been reported to have

lethal effects on fish (Ebele *et al.*, 1990) and mouse (Benthein *et al.*, 1995). Keeping in view such impacts, biological control method has been preferred over chemical control (Paurt de Bach, 1961). Different organisms have been used as biological control agents with varying degree of success. Among them bacteria have been given special consideration against molluscs (Cheng, 1986; Agricultural Genetics Company, 1996). Normally, bacteria belonging to genera *Bacillus, Pseudomonas, Staphylococcus, Aeromonas, Acinotobacter, Micrococcus* and *Citrobacter* have been found to be associated with snails as commensals or pathogens (Cheng, 1986; Watkins and Simkiss, 1990). So some of these bacteria can be used for the biological control of snails. Bacteria belonging to genus *Bacillus* or of special interest as these produce endotoxins, which are toxic to invertebrates, *e.g.*, several strains of *Bacillus thuringiensis* have been found to be effective for mosquito control (Federici, 1995; Smith *et al.*, 1996).

In present study *B. subtilis* was evaluated as biomolluscicide against freshwater snail, *L. acuminata*. The strain was isolated from snail habitats. This bacterial strain did not show β-haemolysis on blood agar Strains showing it could be pathogenic to man and livestock (Cheesbrough, 1993) and thus are unsuitable to use as biomolluscicide.

As microorganisms *B. subtilis* is of special interest, belonging to genus *Bacillus* produce endotoxins, which are toxic to pests. Normally endotoxin proteins are produced during sporulation *i.e.*, when bacteria produce spores (Metlus and Macaluso, 1990; Starzak and Bajpai, 1991). It has been reported that most of the genes coding for endotoxins are present on plasmids (Mahillon *et al.*, 1994). The endotoxin gene is 3rd gene in operon of three genes in which every gene has specific role in the expression of endotoxin gene (Crickmore and Ellar, 1992). There are five conserved regions in the gene and deletion mutation experiments showed that 5th conserved region is necessary for gene expression (Minami *et al.*, 1995). Biopreparations from *Bacillus* endotoxins have been used to control several pests. *Bacillus thuringiensis* and *B. sphaericus* preparation were found highly effective against *Nippostrongylus braziliensis* larvae (Fedianina *et al.*, 1993). In future, genetic engineering of Bacilli, is expected to result in development of more effective (toxic) bacterial strains.

Histology of uninfected digestive system

Digestive system was studied with special reference to intestine as bacteria are mostly associated with the intestine (Watkins and Simkiss, 1990) and endotoxins produced by *Bacillus* spp., bind with specific receptors on the brush border membrane of the villi of the intestine (Ferre *et al.*, 1991; Baur, 1995). Digestive system consists of mouth, pharynx and pharyngeal glands, oesophagus, gizzard and intestine. The intestine is generally lined with a tall ciliated epithelium, which receives sub-epithelial mucous glands and is subtended with an inner layer or longitudinal muscle fibers and an outer circular muscle layer. The intestine opens to exterior through anus.

Our findings about the histology of normal snail tissues are similar to Hyman (1967), Walker (1972), Roland and Garciacorrales (1988), Bush and Maxwell (1988), Boer and Kits (1990) and specifically Tanveer and Samina (1992, 1992a) who worked on *L. acuminata*.

Histopathology induced by bacteria

Histopathological studies were based upon the comparison between the photographs of the tissues of normal and treated snails. Yellow bodies were observed in all soft tissues of treated snails. Histopathological studies revealed that these yellow bodies were discrete lesions containing numerous bacteria (Fig.4) that occur as intracellular parasites particularly in the amoebocytes. Parasitized amoebocytes accumulate in small aggregates with other types of infected cells to form tubercles. Lesions can be seen in all major organs of the body of snails. Bacteria specifically infect the alimentary canal as these have normally been isolated from that region of the snail body (Watkins and Simkiss, 1990). The endotoxins produced from the bacteria bind with the specific receptors on brush – border membrane of the villi of the intestine (Ferre *et al.*, 1991) and produce infection, thus causing the problems in the absorption of food. During the course of experiments, no difference could be found between the normal and treated snails with respect to growth, fecundity or feeding behaviours. The results found were similar to the findings of Dias (1953).

The LT_{50} and LT for both normal and treated snails were determined. Snails treated with *B. subtilis* LT values than snails in control sets indicating that strain was significantly toxic ($P<0.001$) to snails.

On the basis of these studies, it was concluded that bacteria are suitable to be used as biological control agents against snails. This is preliminary work and needs more extensive study in this regard.

Acknowledgement

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WEIGHT-LENGTH AND CONDITION FACTOR RELATIONSHIP OF WILD *CHANNA PUNCTATA* FROM MULTAN

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Abstract: The weight-length and condition factor parameters of wild fresh water *Channa punctata* from a natural pond in Multan have been presented. Log transformed regressions were used to test the allometric growth. It was observed that growth in weight was allometric of the form: $W = -1.86\ L^{2.88}$. The value of slope b = 2.88, is significantly lower than b =3, the weight grows less rapidly as compared to the cube of length. It was concluded that body proportions changed as fish grew in size. Condition - factor showed no significant correlation with increasing length and weight

Key words: Length, weight, condition factor, *Channa punctata*, fish.

INTRODUCTION

The weight–length relationship has mainly been used for two different purposes. Firstly, to describe mathematical relationship between length and weight so that one could be converted into other, secondly to compute the departure from the expected weight for length of individual fish or a group of fish as indications of fatness or degree of well being of fish.

Condition factor or ponderal index is one of the most important parameter, which throws light on the physiological state of the fish in addition to indication of the onset of the sexual maturity. This parameter has been extensively used by Bashirullah and Kadir (1970) Javaid and Akram (1972), Mann (1979), Salam and Janjua (1991), Javaid *et al.* (1992), Salam and Mahmood (1993), Abbas (2000) and reviewed by Weattherly (1972). Ricker (1975), Weatherly and Gill (1987) and Wootton (1990, 1998).

The present study deals with the weight-length and condition factor of a wild, commercially important fish, *Channa punctata.*

MATERIALS AND METHODS

Thirty-seven wild *Channa punctata* from a natural pond in Basti Ratan Wali, Multan were selected for this study during February 2000 to May 2000. The fishes were selected at random for present study. The fishes were killed, blotted dry, weighed to nearest 0.01g using an electronic digital top-pan balance (Chyo, Japan) and their length

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measured to nearest 0.1cm on fish measuring board. Condition factor (K) for each fish was calculated using the formula proposed by Weatherely and Gill (1987) and Wootton (1990, 1998). Weight-length relationship has been calculated by the allometric growth formula used by Huxley (1932), where W (g) is the body weight, a and b are the constants and L (cm) is the length of the fish. Logarithmically transformed, the relationship is represented as:

$$\log W = \log a + b \log L$$

Meek (1903) Le Cren (1951), Kohler (1959), Javaid and Akram (1972), Willis (1989), Atkinson (1989), Salam and Janjua (1991) and Abbas (2000) have applied this technique on a number of species.

Using computer packages Excel and Minitab carried out statistical analysis including regression analysis and calculation of correlation coefficient. Calculation of "t" test and comparison of slopes were done following Zar (1996).

RESULTS

The relationship between wet weight and total length (Fig 1) is exponential having the general form:

$$Y = a X^b$$

or $$W = a L^b$$

The log-log transformed data when plotted against log total length and log wet weight, a linear relationship is obtained having the general form:

$$\log W = \log a + b \log L$$

Table I: The regression parameters of log wet weight (W) on log total length (TL) and of condition factor (K) on total length (L) and wet weight (W).

Regression equation	n	r	r2	S.E(b)	t-value when b=3
Log W = log -1.86+2.88 log L	37	0.9544***	0.911	0.1535	$0.781^{n.s.}$
K = 1.010+0.0010 W	37	$0.2437^{n.s.}$	0.0594	0.0007	P<0.001
K = 0.9578+0.0061 TL	37	$0.221^{n.s.}$	0.0493	0.0045	P<0.001

n.s. = Not significant; *** = P<0.001 (student's "t" test)

Table II: Weight-length parameters and condition of various fish species.

Species	a	b	K	Source
Catla catla	-1.45	2.66	1.16-1.41	Javaid and Akram (1972)
Labeo rohita	-2.04	3.06	0.83-1.32	Salam and Janjua (1991)
Cirrhinus mrigala	-2.03	3.02	–	Salam and Khaliq (1992)
Channa punctata	-1.88	2.9	0.84-1.17	Present study

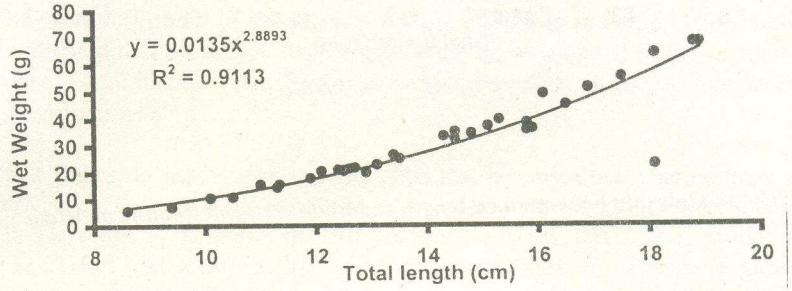

Fig. 1: The relationship between total length and wet weight of *Channa punctata*.

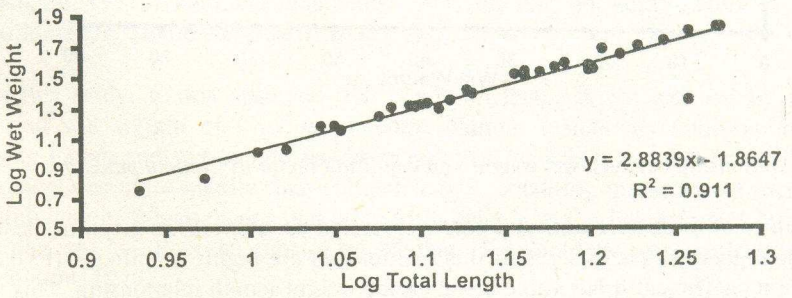

Fig. 2: The relationship between log total length and log wet weight of *Channa punctata*.

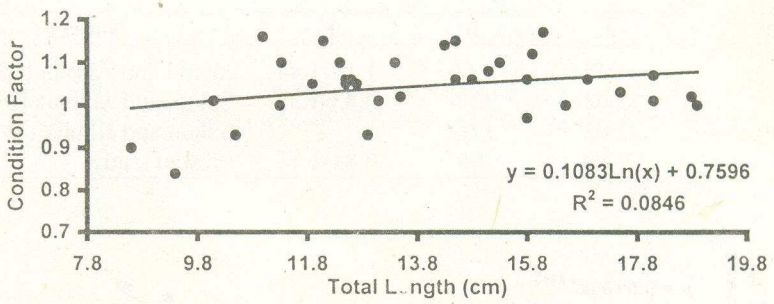

Fig. 3: The relationship between total length condition factor of *Channa punctata.*

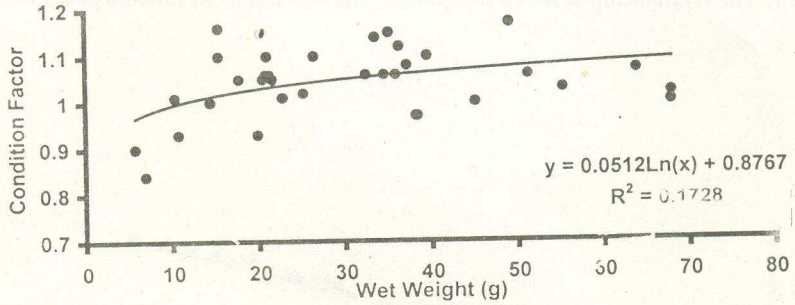

Fig. 4: The relationship between wet weight and condition factor of *Channa punctata.*

The values of these constants and other regression parameters are given in the Table I and II. The regression coefficients of this relationship are highly significant ($P<0.001$). The regression coefficient b has value b = 2.88 for weight-length relationship. This value are less than b =3 (Fig 2). Condition factor K when analyzed against total length and wet weight, it was observed that condition factor remains constant with increasing length and wet weight of *Channa punctata* (Figs. 3-4).

DISCUSSION

In fish, weight is considered to be a function of length. According to Wootton (1990, 1998) if the fish retains the same shape and its specific gravity remain unchanged during lifetime, it is growing isometrically and the value of exponent 'b' would be exactly 3.0 (Ricker, 1975). A value less than 3.0 shows that the fish become lighter for length as it grows an exponent greater than 3.0 indicates that the fish becomes heavier for its length as it increase in size.

The specific gravity of the flesh of fish is known to undergo changes but Le Cren (1951) indicated that the density of the fish might be maintained in the surrounding water by means of swim bladder. The change in weight, therefore, is due to changes in form and not in specific gravity.

Most fishes do not conform to the cube law because they change their shape with growth (Martin, 1949) and the exponent 'b' may have values significantly lower or higher than 3.0. The value of 'b' may vary with feeding (Le Cren, 1951), state of maturity (Frost, 1945), sex (Hile and Jobes, 1940) and furthermore between different populations of a species (Hile, 1936; Jhingran, 1968) indicating taxonomic differences in small populations.

In the present study, *Channa punctata* have the values b = 2.88. This value is significantly less than b = 3 showing that weight of this fish increase less than cube of its length (Table I).

Apart from present study, many workers have reported growth pattern in other fish species (Table 2) indicating that cube law may be held in some cases (Salam and Jnjua, 1991; Khaliq, 1991; Salam and Mahmood, 1993) and may not held in other cases (Javaid and Akram, 1972; Salam and Sharif, 1997).

In this study, it was observed that condition factor is not affected by increasing length and wet weight of *Channa punctata*. Similar results of condition factor with increasing length and wet weight has been reported by Salam and Khaliq (1992). When weight increases more rapidly than cube of length, condition factor would increase with increasing length. Which means that body form change as the fish becomes larger. When weight increase less than the cube of length than 'K' would decrease with growth of the fish (Wootton, 1998).

Condition factor may be influenced by a number of factors (Le Cren, 1951). The factors which affect the value of 'b' in weight-length relationship i.e., age, sex, maturity etc. also influence the value of 'K' except in those which follow the cube law. The factors like food, environment and parasitism influence K directly through growth rate. Seasonal

fluctuations in feeding activity, gonadal development and growth can also bring changes in value of 'K'.

Keeping in view the multiple factors affecting the condition factor, its interpretation is a complicated matter and should be dealt with due care to avoid confusions (Le Cren, 1951).

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AGE AT PUBERTY IN FEMALE DWARF GOAT ON THE BASIS OF HORMONES

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Abstract: A lot of five healthy female Dwarf goat kids were reared at NIAB farm. Blood sampling was started at the age of 75 days. Progesterone, Oestradiol, LH and FSH values were monitored using Radioimmunoassay (RIA) during prepuberal period and oestrous cycle. During prepuberal period, progesterone remained at basal levels of 0.1-0.5 ng/ml. A small rise up to 2 pg/ml was noticed in Oestradiol at the transition from prepuberal to puberal period. Similarly, LH and FSH values were risen up to 2.9 ng/ml and 2.2 ng/ml respectively. Hormonal levels begin to rise at the age of 4-5 months (120-160 days). FSH and Oestradiol started to increase followed by the LH peak. A decrease in the LH peak level after 24 hours followed by FSH and Oestradiol level was noticed. After oestrous phase progesterone level started to rise up to 4-9 ng/ml during the next 15 days. In the next 5 days these levels started to fall to basal values. On the day of oestrous peak values of Oestradiol, LH and FSH ranged from 10-16 pg/ml, 13-23 and 13-22 ng/ml respectively. The overall pattern of release of all these hormones was found similar in all these four animals. So in this lot, the mean age of puberty was found to be 137±31 days with a mean cycle length of 20±1.

Key words: Progesterone, oestradiol, LH, FSH, goat, oestrous cycle, puberty.

INTRODUCTION

Goat is one of the animals domesticated by man a long time ago about 9000-7000 B.C. (Fpstein, 1971; Kamo, 1973) yet the knowledge about its reproductive processes and its physiology is not well understood. Goat is domesticated for the production of meat, milk and skin and it can be easily handled by man. It is the major livestock of the subcontinent (India, Pakistan and Bangladesh). Among all other breeds of goat Dwarf goat is preferred over the others, because it is a non-seasonal breeder. It has a high prolificacy and usually gives birth to twins and triplets, kidding thrice in two years (Srivastava *et al.*, 1968; Khan *et al.*, 1982). These characteristics of Dwarf goat are in contrast to the other breeds and accordingly may exhibit different hormonal mechanisms. The reproductive activity is a physiological process controlled by hypothalamus-pituitary-gonadal interactions through endocrine secretions, receptors at target and feedback mechanism (Tanaka *et al.*, 1992). These processes are initiated with the onset of puberty in the goat kids. Therefore, it is necessary to consider the physiological and endocrinological events involved in the onset of puberty and during

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oestrous cycle. The aim of present work is to study the endocrinological pattern and profile of reproductive hormones (progesterone, oestradiol, LH and FSH) at the onset of puberty and during oestrous cycle and to determine the temporal interrelationship of these hormones. This study gives us the mean age at puberty of Dwarf goat kids.

MATERIALS AND METHODS

A lot of five healthy female Dwarf goat kids (# 451, 456, 457 and 504) were selected from NIAB farm, Faisalabad. One animal did not complete the study due to some casualty. Blood sampling was initiated when the kids were 75 days old, except one kid (504) that was 110 days old. The blood sampling from the jugular vein was conducted with disposable syringes into plain glass tubes twice a week up to the age of 90 days and daily for 150 days. Serum was separated by centrifugation at 2000 revolutions/min and stored in capped tubes at -20°C till hormonal analysis.

Steroid hormones were estimated by single antibody RIA method using kits supplied by IAEA. Whereas protein hormones (LH and FSH) were analyzed by a double antibody competitive binding RIA method with a slight modification in the Kanai and Ishikawa (1988) method. Iodination of LH and FSH was performed by Chloramine-T method at room temperature under fumehood. The activity of iodine used was 0.5 mCi.

RESULTS

Prepuberal period of the Dwarf goat kids

During prepuberal phase, progesterone, oestradiol, LH and FSH remained at basal levels. However, a few days before the onset of puberty, some fluctuations were observed. Progesterone remained at basal (0.1-0.6 ng/ml) level during this period except small irregular fluctuations near puberty. Oestradiol showed tiny pulses, sometimes up to 2.0 pg/ml followed by small rises in LH and FSH levels. After these fluctuations, the animals showed signs of puberty by significant changes in hormone levels indicating first oestrous or the onset of puberal period.

Onset of puberty in Dwarf goat kids

Fig. 1 shows the hormonal patterns of kid # 451, in which first oestrous started at the age of 115 days. Four consecutive oestrous cycles were observed in this kid. The length of each cycle was 20±1 days. First of all a rise in oestradiol level (15.4 pg/ml) was noticed followed by a peak of LH (22.7 ng/ml). This was taken as the day-0 of the oestrous cycle and it was also the oestrous phase of the cycle. Simultaneously a rise in FSH levels (20.0 ng/ml) was obtained. LH peak declined after 24 hours followed by oestradiol and FSH. During this period of 2 or 3 days progesterone remained at basal levels. This phase was

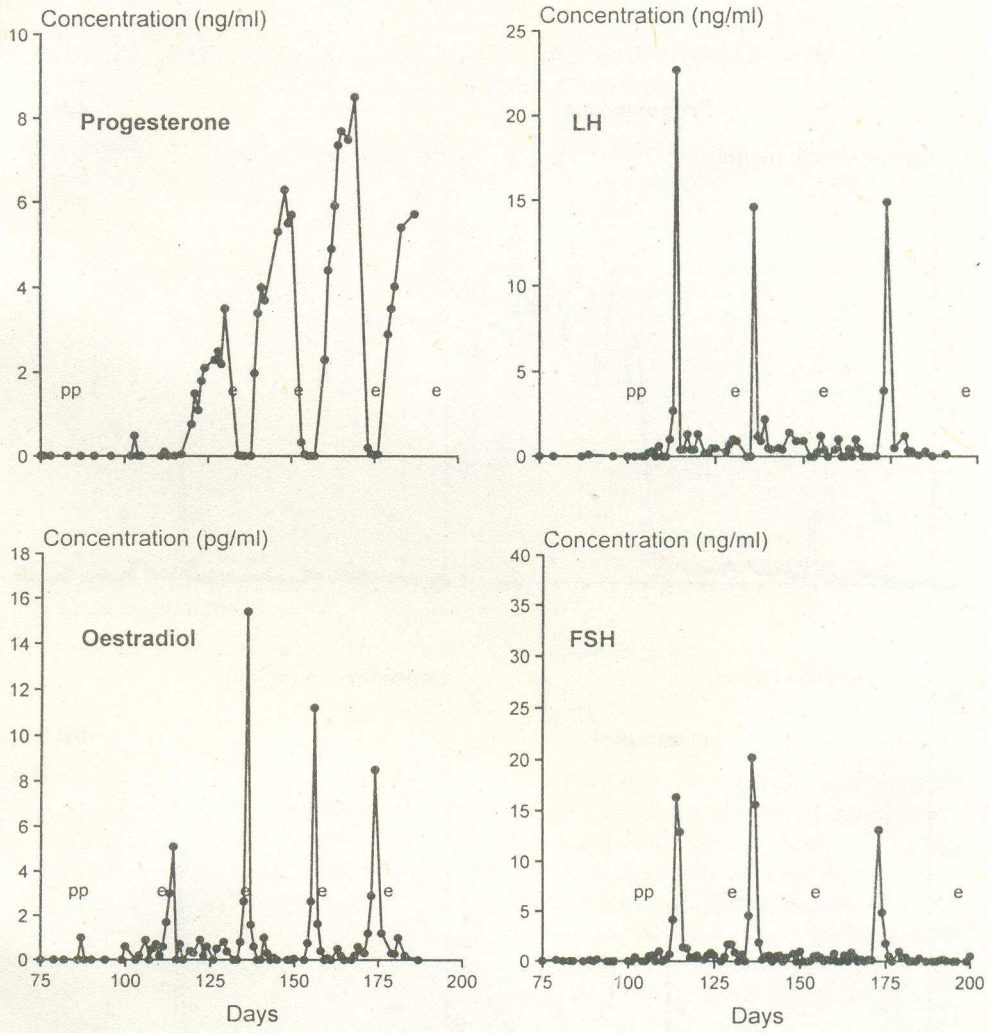

Fig. 1: Serum profile of progesterone, oestradiol, LH and FSH at prepuberal (pb) and puberal (e) phases in kid No. 451.

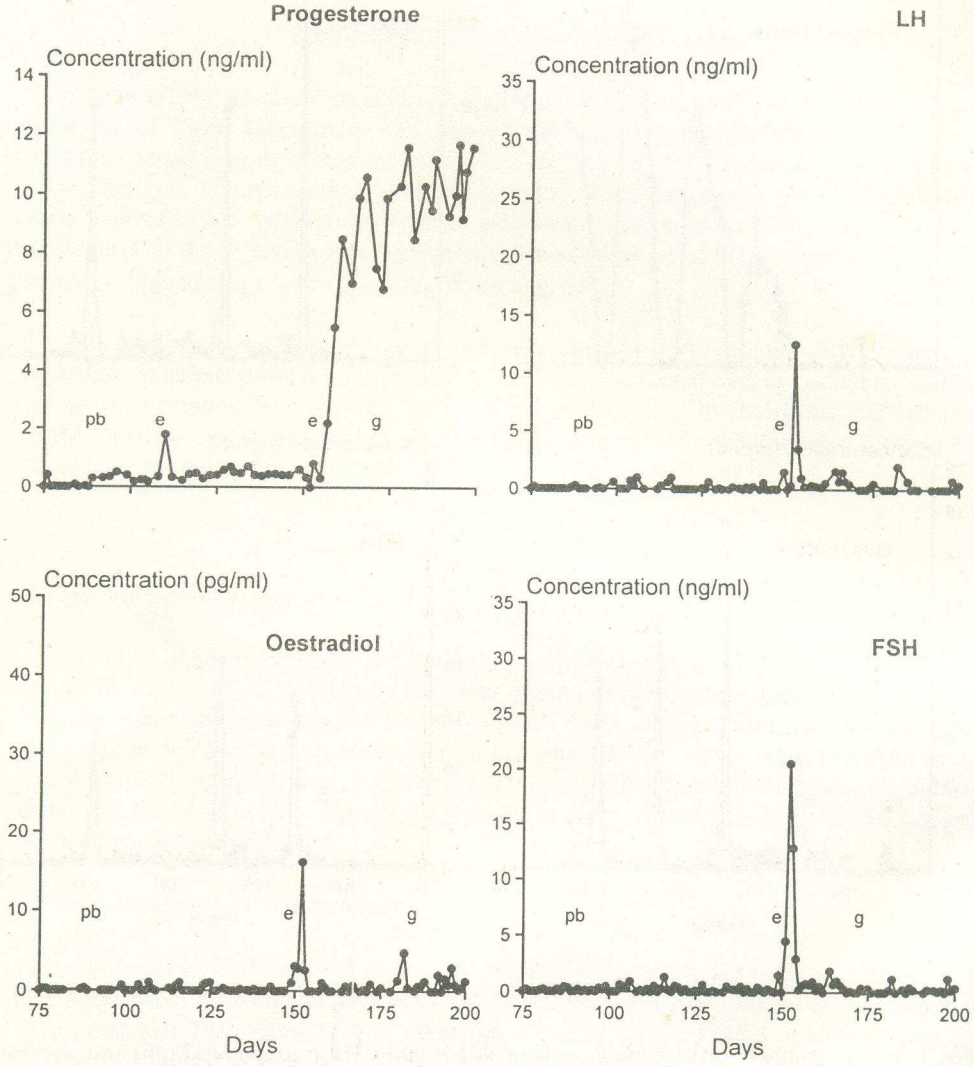

Fig. 2: Serum profile of progesterone, oestradiol, LH and FSH at prepuberal (pb) and puberal (e) phases in kid No. 456.

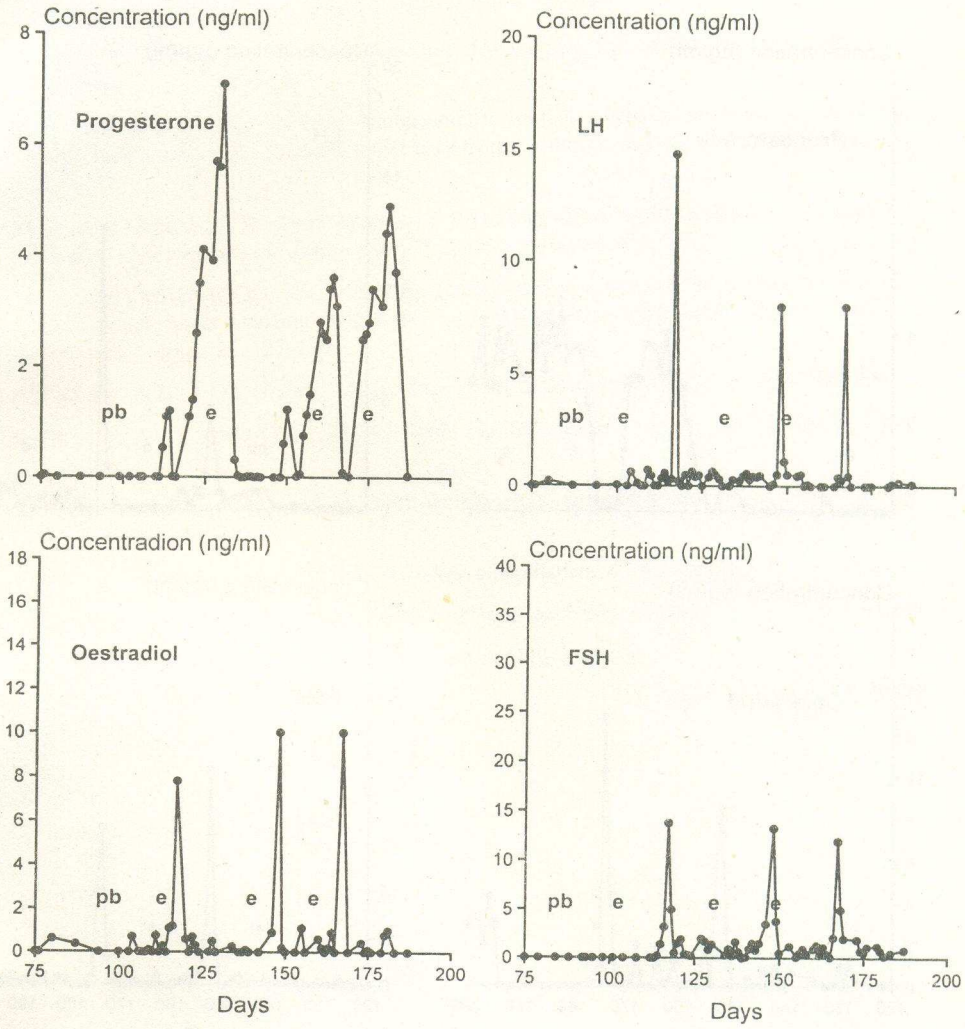

Fig. 3. Serum profile of progesterone, oestradiol, LH and FSH at prepuberal (pb) and puberal (e) phases in kid No. 457.

Fig. 3: Serum profile of progesterone, oestradiol, LH and FSH at prepuberal (pb) and puberal (e) phases in kid No. 457.

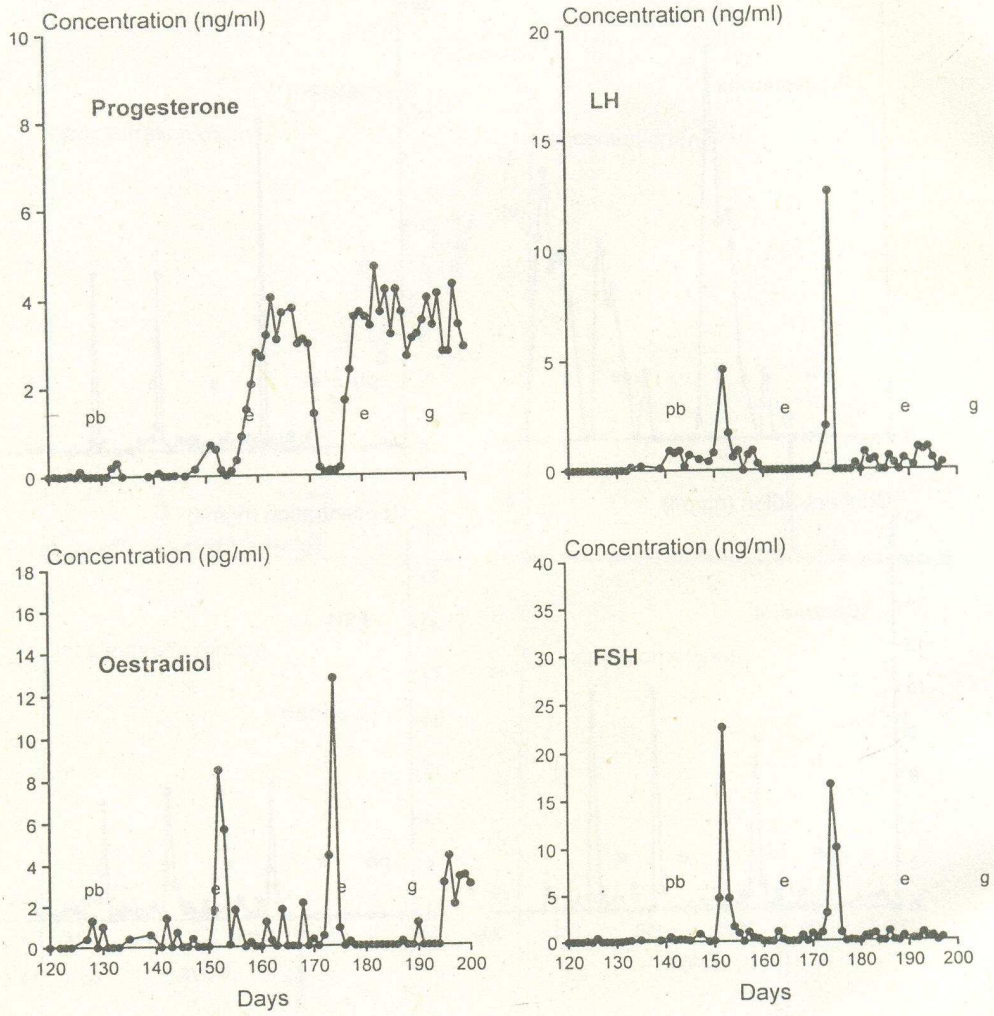

Fig. 4: Serum profile of progesterone, oestradiol, LH and FSH at prepuberal (pb), oestrous cycle (e) and gestation period (g) in goat No. 504.

actually the culmination of oestrous cycle. After this phase progesterone started rising and its value reached up to maximum level (8.5 ng/ml) in the next 10-11 days. Progesterone maintained its maximum level for 4-5 days and then in the next 4 or 5 days, it declined gradually to the basal level.

Puberty started at the age of 120 days in kids # 456 and 457 (Figs.2 & 3). Both these kids showed three oestrous cycles during the study period. After first cycle, a silent period of 15 days was noticed, during which irregular hormonal fluctuations were observed. The pattern of release of hormones and the length of cycle in these kids were same as were in kid # 451. The maximum progesterone level was 7.2 ng/ml and 7.1 ng/ml, the peak of oestradiol, LH and FSH were 16.3 pg/ml, 17.4 ng/ml and 30.0 ng/ml in animal No. 456 and 10.0 pg/ml, 14.9 ng/ml, 13.8 ng/ml in animal No. 457 respectively.

In kid # 504, first oestrous started at the age of 110 days. This kid showed only one oestrous cycle, while during the next cycle, the goat conceived and showed gestation by maintainng the progesterone concentration in the range of 3-5 ng/ml during this period. LH and FSH remained at basal levels during this period while Oestradiol started rising after 25 days of gestation.

DISCUSSION

The hormonal patterns and profiles of progesterone, oestradiol, LH and FSH were followed in kids from the age of 75 days till the assumption of puberty. There were no significant hormonal fluctuations except a few days (10-15) before the onset of puberty. All the four hormones showed tiny irregular peaks a few days before puberty. These findings are similar to that of Gonzalez-Padilla *et al.* (1975) and Kinder *et al.* (1987) in heifers and ewes that progesterone concentration is low during most of the prepuberal period with two rises before puberty. This agrees with the statement of Ojeda *et al.* (1980) that these initial rises in progesterone may sensitize the ovaries for LH as in some postpartum cows. Similarly, a significant increase in oestradiol 17-β levels, 8 days before puberty comparable to normal preovulatory peak in post puberal heifers has been reported by Glencross (1984).

Peters and Ball (1987) found that follicle growth starts soon after birth, as does the production of LH and FSH. These findings support the idea of Ramirez and McCann (1963) that at puberty ovaries and hypothalmo-pituitary axis are fully mature for the sufficient stimulation and secretions of relevant hormones. The length of prepuberal age may be affected by various factors such as inherent character of different breeds, body weight at birth, photoperiod etc.

Age at puberty

During present investigations, the age at puberty in female Dwarf goat was found to

vary between 3-6 months with an average of 4-5 months. Greyling and Niekerk (1990a) reported the puberty age in female Boar goat kids at 5.0-6.5 months and found significantly earlier in kids weaned in breeding season (April) than those in non-breeding season (December).

Oestrous cycle

In this investigation LH and FSH concentration was found low during most of the oestrous cycle and started to increase 1-2 days prior to Oestrous (Rawlings and Cook, 1993). The maximum LH peak that occurred on the day of oestrous was preceded by a gradual rise in the concentration of oestradiol 2-3 days before oestrous (Thomus *et al.*, 1989). Progesterone levels observed were low during oestrous (day-0) and showed a gradual increase and maximum peak in dioestrous within 10 days and dropped during proestrous to the basal levels within 4-5 days before the commencement of metoestrous. Oestrous cycle in the goat is well studied and hormonal profile of LH, FSH, Oestradiol and Progesterone observed during this study were similar to those reported by other workers (Kanai, 1987; Kanai and Ishikawa, 1988; Ryan *et al.*, 1991). However, the high variability of the concentrations found in different reports and in the present studies, the presence of some irregular peaks or occasional drops do not appear to be easily explained in relation to variations in other hormones we have described.

Glencross and Pope (1981) found that oestradiol-17β levels were low in plasma of Taurine cattle for most of the oestrous cycle and rose as the concentration of progesterone began to fall and attained maximum levels after 3-4 days. Probably the drop in progesterone concentration following luteal regression allows the preovulatory follicle to increase its secretion of oestradiol (Karsch *et al.*, 1978). Ilewelyn *et al.* (1987) reported that progesterone concentration started to increase gradually 4 days after oestrous, as the corpus luteum became functional. It reached to maximum level at 11-15 days after oestrous and then declined to basal levels before the next oestrous and ovulation.

Oestrous cycle occurs 1-5 days after the regression of corpus luteum which is brought about by the action of prostaglandins (PGF2α) (Knickerbocker *et al.*, 1988) secreted by the uterus. The duration of each cycle observed in the range of 18-22 days with an average of 20 days is similar to those reported in different breeds of goat (Ali *et al.*, 1973; Ali *et al.*, 1991).

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SHORT COMMUNICATION

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IMPACT OF SOCIOECONOMIC FACTORS IN THE PREVALENCE OF THYROID DISORDERS AT SHAIKH ZAYED HOSPITAL, LAHORE

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Abstract: The impact of socio-economic factors does play a very prominent role in the prevalence of thyroid disorders. A total number of 425 patients were studied in which 105 (24.70%) were males and 320 (75.30%) were females. Male to female ratio found to be 1:3. Of 105 patients, 65 (27.7%) belong to rural areas whereas 40 (21%) belong to urban areas compared to 170 (72.3%) female belong to rural areas and 150 belong to urban areas. We found if proper socio-economic factors are well oriented then it is quite safe and successful in controlling and arresting the phenomena of thyroid disorders.

Key words: Prevalence of thyroid disorders, socioeconomic factors, sexual differences, G.I.T. disorders.

INTRODUCTION

Thyroid disorders are quite common in the semi-developed country like Pakistan. There are many diseases which have very wide prevalence in any poor area. The economic does play a very prominent role in arresting the proliferation of such disorders either it relates to thyroid, G.I., or any other system (Krahn, 1993; Grodin *et al.*, 1993; Kaplan *et al.*, 1993). The restraints of economic relaxation for obtaining the hygienic habitats lead to the avoidance of the catching of the diseases that are very pronable if not controlled.

A questionnaire was developed for taking full history of the patients. This prospective study was carried to determine the diagnostic manifestations in connection with the mode of presentation and outcome of suggestion if any for various thyroid disorders being encountered in these areas.

MATERIALS AND METHODS

Following are the parameters for the proliferation of thyrometabolic disorders:

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1. Unhygienic conditions:
   - Living
   - Sub-living
2. Laboratory tests not available or access to patients.
3. Unawareness to see the doctor.
4. Unauthentic approach to the system.
5. Not timely treatment/negligence.

Evaluation was made by the clinician and the patients were referred to the laboratory for the biochemical analysis. The samples of poor patients were analysed free of charge on Social Welfare Departments request. A questionnaire like proforma was developed and every patient's general history was taken on the proforma and later on an analysis was done.

The patients were examined by a team of doctors specialized in the Endocrinology Department of Medical Division in OPD at Shaikh Zayed Hospital, Lahore and according to the grading of the diseases, subsequent related tests were requested for further analysis.

RESULTS

As it is evident from the Table I, out of total 425 patients, 105 were males (24.70%) and 320 were females (75.30%) with a male to female ratio of 1:3. Of 105 males, 65 (27.7%) belong to rural areas whereas 40 (21%) belong to urban areas. While 170 (72.3%) patients were females belonging to rural areas and 150 (79%) belong to urban areas.

Table I: General distribution of patients living in rural and urban areas

	Patients living in: (n=425)		
	Rural areas	Urban areas	Total
Male	65 (27.7%)	40 (21%)	105 (24.7%)
Female	170 (72.3%)	150 (79%)	320 (75.3%)
Total	235(55.3%)	190 (44.7%)	425 (100%)

Table II shows the percentages of patients living in sub-standard environments like taking unclean and unhygienic drinking water; no proper arrangement of disposing the garbage, lack of proper draining system or its open exposure lead to many health hazards atmosphere involve 29% male and 44% female living in rural areas making the total of 73% whereas 11% male and 16% female living in urban areas making the total percentage to 27%.

The table III demonstrates the percentage of patients who first sought the help from unqualified persons like Pirs, Hakims and lack of other sources forced them (patients) to restrain themselves to seek the proper and timely reference to qualified doctors. As the table III indicate that 81% patients from both rural and urban areas did not seek the help

of qualified doctor/ specialist. Whereas only 19% from the both areas did seek the help of qualified doctors/ specialists for the remedy of their respective problems.

Table II: Percentage of patients sex-wise living sub-standard environments in rural and urban areas

	Rural patients	Urban patients	Total
Male	29	11	40
Female	44	16	60
Total	73	27	100

Table III: Percentage of patients seeking the help of non-doctors as well as of doctors

	Patients (%) referred first to:		
	Other than doctor	Qualified doctor	Total
Male	60	3	63
Female	21	16	37
Total	81	19	100

DISCUSSION

From the point view of thyroid disorders in terms of socio-economic factors, the prevalence, no doubt, has been proliferated due to the incremental sub-standard of a hygienic conditions, ignorance of the patient to whom to refer timely. Lack of economic viability as well as of education, negligence of the patients by thinking that this disorder may be over with the passage of time or if not considering it seriously, may go away by itself. With such misunderstandings, the end result of disorders of the related diseases may become serious to complicate one's life into problems restricting the normal functioning of the patients (Gordan, 1993; Frenk, 1993).

As it is evident in the previous table discussed earlier in the results actively demonstrate that the prevalence of such disorders do depend on the socio-economic factors contributing towards the development of such deterioration if unchecked.

It is, therefore, concluded that if proper socio-economic factors are well oriented then it is quite safe and successful in controlling and arresting the phenomena of thyroid disorders for the better management of such patients.

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