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SERUM PROTEIN FRACTIONS IN HYPERTHYROID WOMEN

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Abstract: Serum samples of healthy women and hyperthyroid patients were obtained alongwith the radioimmunoassay values of triiodothyronine, thyroxine and thyroid stimulating hormone. Samples were diluted in phosphate buffer (pH 7.2) and proteins were denatured by heating with loading dye. SDS-PAGE was employed for studying the protein profile of healthy and hyper thyroid subjects. Gels were photographed and their images were stored for quantification of protein fractions by UVP Gel Base Software Programme that provides the data of molecular weights and percent areas covered by each of the fractions. Data was employed in finding the enhancement or reduction and the appearance or disappearance of particular protein fractions for comparison between healthy and hyperthyroid groups. A total of 21 fractions were detected in normal subjects, all of which were expressed in hyperthyroid group. Most of the fractions in hyperthyroid group were found to be markedly reduced when compared to healthy group. No new fractions were encountered in hyperthyroid compared to healthy group.

Key words: Hyperthyroidism, protein fractions, electrophoresis.

INTRODUCTION

The widespread metabolic role of thyroid hormones, thyroxine and triiodothyronine, commonly called T_4 and T_3, the diverse processes involved in the synthesis, secretion and metabolism of the hormones, and the complex mode of regulation of thyroid function indicate that a great many factors could influence one or more aspects of thyroid hormone economy. Formation of normal quantities of thyroid hormone ultimately depends upon the availability of adequate quantities of exogenous iodine. Although efficient mechanisms exist to conserve iodine in the presence of iodine deficiency, they do not entirely succeed in preventing depletion of iodine stores, ultimately this may lead to insufficient hormone production. The resulting marginal iodine deficiency does, however, predispose to the development of hyperthyroidism upon exposure to sources of additional iodine. The activity of the iodide transport mechanism is influenced by a variety of physiological factors, the most important of which is the level of TSH stimulation. TSH is the major regulator of the morphological and functional state of the thyroid. An increase in both the serum T_4 and T_3 concentrations is the usual pattern in patients with hyperthyroidism. Serum T_4 concentration ranges from values that are only slightly elevated in patients with mild disease to values in excess of 20 μg/dl in the most severe cases. Concentrations of T_3 are almost invariably increased, sometimes to levels that are many times the mean normal

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value. Usually the increase in T_3 concentration is proportionately greater than the increase in serum T_4, so that the T_3/T_4 ratio in serum is almost always elevated (Ingbar, 1985).

The physiologically observable effect of thyroid hormone is due to the new or increased protein synthesis resulting from a new or increased amount of specific mRNA (Rall, 1978). An increase in enzyme activity due to an increase in enzyme protein is the result of a decreased rate of degradation of that protein rather than increased synthesis (Shimke and Doyle, 1970). An increase in the rate of synthesis of serum albumin in man caused by thyroid hormone was clearly demonstrated 20 years ago (Schwartz, 1955; Rothschild *et al.*, 1957; Lewallen *et al.*, 1959). Subsequently, overall protein synthesis was shown to be increased by thyroid hormone and this was considered to be the main effect (Krause and Sokoloff, 1967; Sokoloff and Kaufman, 1961; Tata, 1962; Tata *et al.*, 1963).

Muscle weakness occurs in most patients with hyperthyroidism (thyrotoxic myopathy) and when the hyperthyroidism is severe and prolonged, the myopathy may be severe. The muscle weakness may be due to part of increased protein catabolism (Ganong, 1995). Thyroxine increases the rate of metabolism of all cells, and as a result, indirectly affects protein metabolism. If insufficient carbohydrates and fats are available for energy, thyroxine causes rapid degradation of proteins and uses these for energy. On the other hand, if adequate quantities of carbohydrates and fats are available and excesses of amino acids are also available in the extracellular fluid, thyroxine can actually increase the rate of protein synthesis. Conversely, in growing animals deficiency of thyroxine causes growth to be greatly inhibited because of lack of protein synthesis. In essence, it is believed that thyroxine has little specific direct effect on protein metabolism but does have an important general effect in increasing the rates of both normal anabolic and normal catabolic protein reactions (Guyton, 1991).

Attempts to manipulate various metabolites by endocrine intervention have been made for last several years and protein metabolism in relation to thyroid hormones has been extensively studied. However, the studies regarding electrophoretic protein profile in response to thyroid pathophysiology are meagre and almost non-existent. By keeping in view the importance of proteins in so many physiological phenomenon and the role thyroid hormones play in protein metabolism, the present investigation is carried out to emphasize the effect of thyroid hormone excess on serum protein fractions of female subjects resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Serum samples of healthy women and hyperthyroid patients were obtained from the Institute of Nuclear Medicines and Oncology and Sheikh Zayed Hospital, Lahore along with the radioimmunoassay values of T_3, T_4 and TSH (Fig.1). Samples were diluted in phosphate buffer (pH 7.2) and proteins were denatured by heating with loading dye.

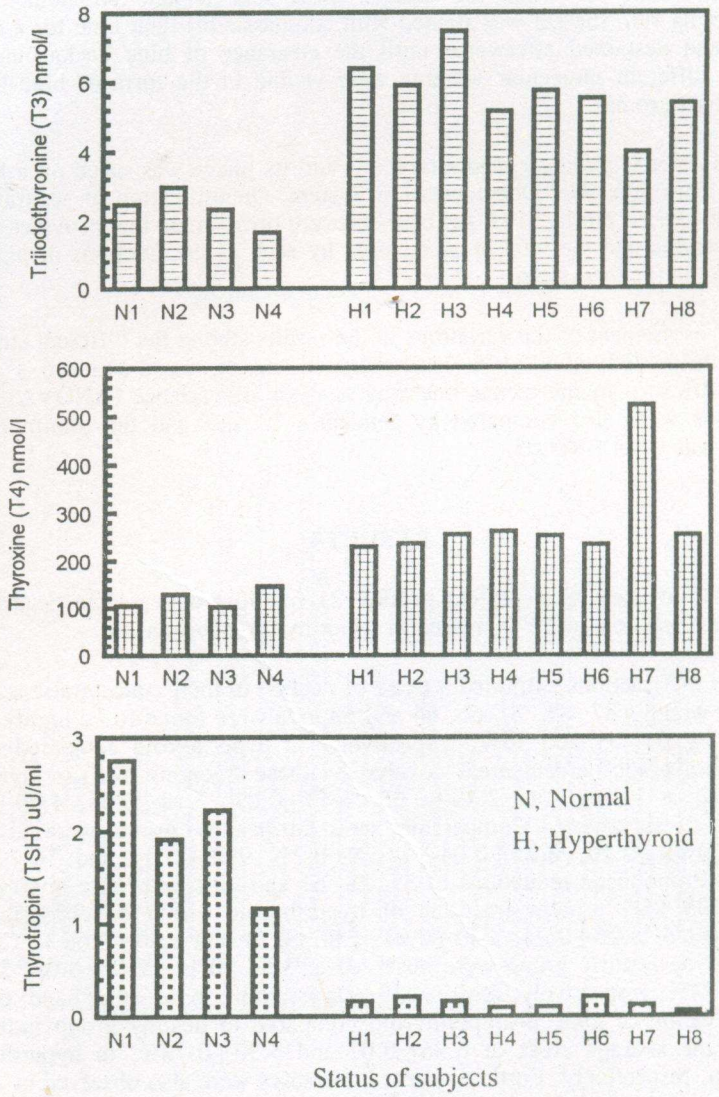

Fig. 1: **Serum hormonal levels of healthy and hyperthyroid women collected from hospitals.**

Polyacrylamide gel (12%) was prepared using the method of Laemmli (1970). Protein size markers and each of the samples were loaded in separate wells and gel was electrophoresed at a current supply of 12 mA and voltage of 150 V, in a cooling chamber maintained at 4°C, for almost three and a half an hour. Following electrophoresing run, the gel was stained with coomassie brilliant blue for a duration of two hours and destained afterwards until the clearance of blue background. Protein fractions of different molecular weights were visible in the form of blue bands on a transparent background.

Stained gel was photographed afterwards and its image was saved on a floppy disk with image store 5000 Gel Documentation System. Quantification of separated protein fractions was carried out by UVP gel base software programme that provides the data of molecular weights and the total areas covered by each of the fractions displayed in the form of peaks in the histogram.

For the assessment of the variations in the results among the different stages within a phase, values of individual goats were averaged and expressed as mean ± SEM. For level of significance in the means one way analysis of variance (ANOVA) was used. Sample means were also compared by Student's "t" test and the minimum level of significance was set at $P<0.05$.

RESULTS

In an overall picture of protein profile, 21 fractions appeared in healthy subjects and all of these fractions were expressed in hyperthyroid subjects.

Most of the fractions exhibited a trend of decline in their concentrations. Fractions of molecular weights 87, 84, 70, 66, 64 and 58 kDa were found to be highly depressed by 38, 51, 49, 51, 51 and 36%, respectively, in hyperthyroid compared to healthy subjects. The average percent areas covered by these fractions in hyperthyroid group were observed at 1.18±0.06, 2.19±0.06, 3.43±0.07, 7.19±0.08, 4.60±0.15 and 6.86±0.06%, respectively. Comparable areas in healthy group were estimated at 1.90±0.12, 4.45±0.10, 6.75±0.04, 14.78±0.22, 9.40±0.34 and 10.70±0.35%, respectively. Pronounced reductions of 37, 38, 51 and 28% were also observed by 56, 52, 43 and 39 kDa protein fractions of hyperthyroid group covering the average respective areas of 2.26±0.04, 3.40±0.04, 1.86±0.11 and 3.36±0.06%. Comparable areas covered by healthy group were observed at 3.58±0.11, 5.48±0.08, 3.83±0.06 and 5.43±0.74%, respectively. A single 35 kDa fraction, on the other hand, displayed a moderate elevation of 29% in hyperthyroid compared to healthy group indicating the coverage of the average areas of 6.86±0.07 and 5.30±0.78%, in hyperthyroid and healthy group, respectively. Further, marked decreases were also observed in 25, 22, 19 and 16 kDa protein fractions covering the average areas of 3.40±0.08, 2.14±0.11, 1.08±0.05 and 1.14±0.07% in hyperthyroid and 5.45±0.04, 3.43±0.06, 2.23±0.20 and 2.83±0.11% in healthy group, respectively. The reductions were found to be 38, 38, 52 and 60%, respectively, in hyperthyroid compared to healthy group. Fractions of molecular weights 100, 95, 48, 33, 28 and 14 kDa, however, did not seem to be affected appreciably (Fig.2).

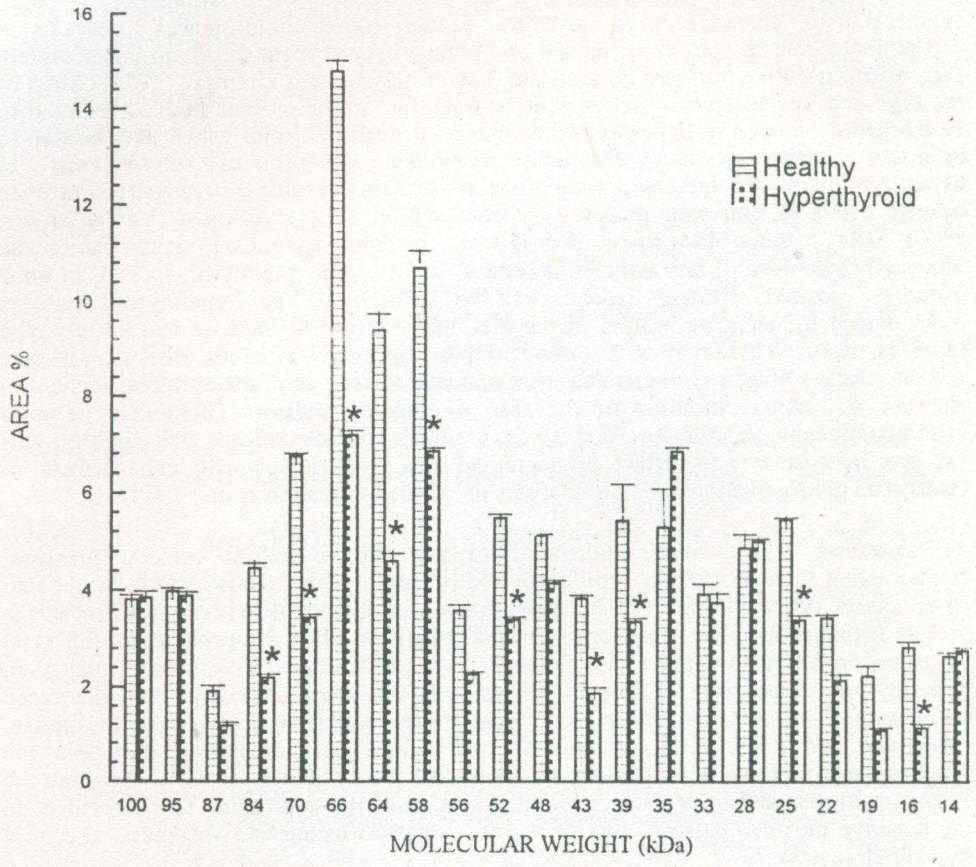

Fig. 2: Average percent area covered by various protein fractions resolved by SDS-PAGE, in healthy and hyperthyroid groups. Values are mean ± SEM. *Significance at $P < 0.05$.

DISCUSSION

The thyroid hormones increase the metabolic activities of all or almost all of the tissues of the body. The basal metabolic rate can increase to as much as 60 to 100% above normal when large quantities of the hormones are secreted. The rate of utilization of foods for energy is greatly accelerated. Although the rate of protein synthesis is increased, at the same time, the rate of protein catabolism is also increased (Guyton, 1991).

Hyperthyroidism, in experimental animals, results in a stimulation of both synthesis and degradation of proteins. Under most circumstances, degradation predominates and in both experimental and human hyperthyroidism, there is net protein catabolism, negative nitrogen balance and loss of muscle mass (Ramsay, 1974). Most of the high and low molecular weight protein fractions, in the present study, are found to be markedly reduced in hyperthyroid compared to healthy group which may be due to increased catabolism and decreased anabolism of proteins in response to hyperthyroidism. An increased catabolism of proteins resulting in negative nitrogen balance occur in untreated diabetes mellitus and in hyperthyroidism (Varley *et al.*, 1980). The increase in protein degradation, in hyperthyroidism, accompanies the increased catabolism of fats and carbohydrates, but it can be minimized. Indeed, in some instances, positive nitrogen balance can be induced, if the hyperthyroid state is accompanied by adequate protein in the diet and a sufficient increase in caloric intake (DuBois, 1936). There may be a loss of protein from the body stores other than muscle and decreased collagen synthesis and increased degradation may, for example, account in part for the typical thinning of the skin in hyperthyroidism. There may be mild hypoalbuminemia and the low density lipoproteins characteristically fall slightly; this fall has been shown to reflect on increased rate of turnover with enhancement of catabolism predominating over stimulations of synthesis (Walton *et al.*, 1965).

Albumin is the main contributor to the plasma colloid osmotic pressure, counteracting the effect of the capillary blood pressure, which tends to force water into tissue spaces (Varley *et al.*, 1980). Most pronounced impact of hyperthyroidism, in the present investigation, has been observed on albumin (66 kDa) and proalbumin (58 kDa) fractions, indicating appreciable reductions of 52% and 36%, respectively, in hyperthyroid compared to normal subjects. It has been reported that decreased concentration of plasma albumin is commonly associated with hyperthyroidism (Mckenzie and Zakarija, 1989). Ingbar (1985) has also commented that both the synthesis and degradation of protein are increased, the later to a greater extent than the former, with the result that there is net degradation of tissue protein. This is evident in the negative nitrogen balance, loss of weight, muscle wasting and weakness, and mild hypoalbuminemia.

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FEEDING PREFERENCES AND DIVERSITY OF TERMITES OF IRAN*

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Abstract: For feeding preferences of termites of Iran, 81 plots of 30 m x 30 m were selected. On the basis of importance value of tree/shrub, five habitats were recognized i.e., *Populus caspica, Tamarix gallica, Morus alba, Salix babylonica* and *Vitis vinfora*. *Populus caspica* habitat harboured *13 species of termites and this habitat was more diverse in its vegetation than other habitats. The values of H, H_{max} and E for termite diversity in P. caspica were 3.4556, 3.7004 and 0.9329 respectively. The species richness was 5.7518. Simpson index being the index of dominance revealed a value of C=0.1783 to which Postelectrotermes pasniensis alone contributes a value of 0.1021. Postelectrotermes pasniensis had more colonies of termites on trees in this habitat as compared to other termite species and its preferred host was P. caspica. In T. gallica habitat only two termite species i.e., Anacanthotermes iranicus and A. vagans* were feeding on trees. *Anacanthotermes vagans* has positive association with *T. gallica* habitat. The value of H, H_{max} and E were 0.9708, 0.9999 and 0.9708, respectively. The species richness was 0.59 and was much less than that of *P. caspica* habitat (5.7518). *Postelectrotermes pasniensis* mostly preferred *P. caspica* and *A. vagans* mostly preferred *T. gallica* and *M. alba*. In *Populus diversifolia* habitat, it is interesting to note that only *P. pasniensis* was recorded, and no other termite species was recorded attacking this tree.

Key words: Feeding, diversity, termites, Iran.

INTRODUCTION

For feeding preferences of termites, eighty-one plots in Iran were surveyed and were classified on the basis of vegetation *i.e.*, trees/shrubs. Tree preferences of termites and their habits of feeding on trees were recorded in different habitats. Lot of work on feeding preferences of termites has been done in different parts of the world (Fougerousse, 1969; Behr *et al.*, 1972; Howick, 1975; Wood and Johnson, 1978; Rugooka and Howick, 1978; Akhtar and Ali, 1979; Akhtar, 1981; Afzal, 1981; Carter, 1981; Collins, 1981; Akhtar and Raja, 1985; Lenz, 1986; Lenz *et al.*, 1987; Waller, 1988; Akhtar and Shahid, 1989). For termites of Iran, this report provides some basic information about the feeding preferences of termites and their diversity in different habitats.

MATERIALS AND METHODS

Different termite affected areas in Iran, *i.e.*, Province of Sistan and Baluchistan, Kerman, Hormuzan, Khurasan, Esfahan, Khuzistan, Fars, Tehran and Mazandaran were

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*Part of Ph.D. Thesis of the first author.

surveyed for the feeding habits and feeding preferences of termites. The plots established for studies in these areas are shown in Fig.1. These plots (81 in total; each 30 m x 30 m) were grouped together on the basis of importance value of the trees.

Vegetation

For vegetation, quadrat of 30 m x 30 m size was used for counting the number of trees/shrubs to work out importance value. Here the importance value is based on the number of trees. As pointed out by Odum (1975) the importance value can be number, biomass, productivity, surface coverage and so on. Habitat was named after a tree or a shrub species having the highest importance value.

Termites

Soldier, workers of the termite species collected from trees from different plots were preserved in 80% alcohol and brought to the laboratory for identification.

The relative frequency of different species of termites was worked out as follows:

$$\frac{\text{Fi of a species}}{\text{Fi of all the species}} \times 100$$

$$\text{Where Fl} = \frac{\text{Point of occurrence of a species in all the quadrats}}{\text{Total number of quadrats}}$$

Species richness was worked out according to Odum (1975).

$$\text{Species richness or variety (dl)} = \frac{S-1}{\log N}$$

where S = number of species and N = number of termite colonies.

Diversity indices were worked out according to Simpson (1949) and Shannon Wiener function. Here the importance value for termites is not based on the number of individuals but on the number of termite colonies/nests recorded from infested trees in different habitats.

i) Simpson index: $(D) = \Sigma(Pi)^2 \times S$

ii) Shannon index $(H) = \Sigma(Pi)\ (\log^2 Pi)$ $i = 1$

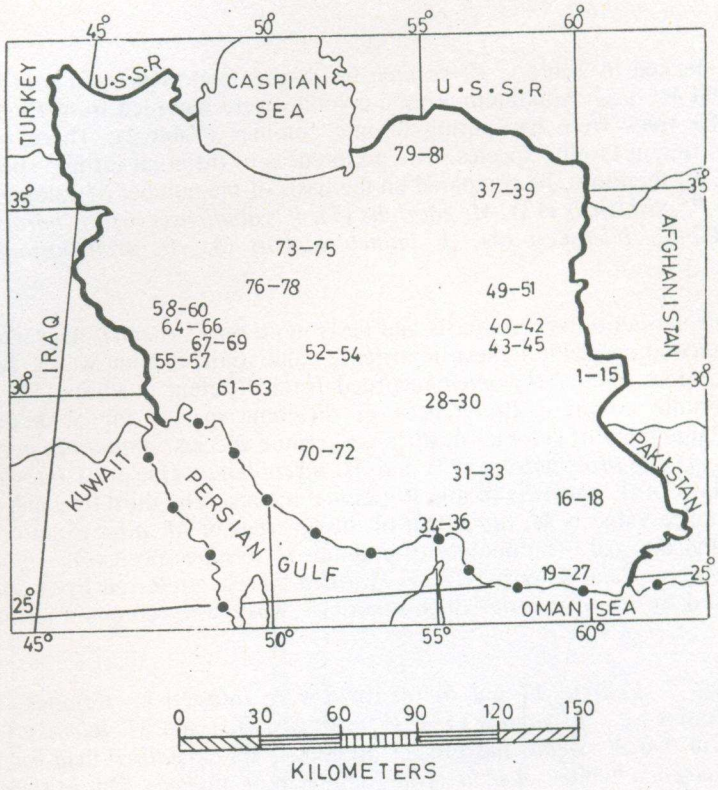

Fig. 1: Map of Iran showing location of sampled pots.

iii) Evenness index $(E) = \frac{H}{\log S}$

H = Shannon index
S = Number of termite species.

RESULTS

Based on importance value (Table 1), five tree habitats were identified. The habitats recognized are: *P. caspica, T. gallica, M. alba, S. babylonica* and *V. vinfora*. Distribution of 16 termite species in these habitats has been studied and is described below:

Populus caspica habitat

As is indicated in Table 1, *P. caspica* has higher importance value (185) than *S. babylonica* and *M. alba*. Maximum termite colonies were recorded from *P. caspica* and 46 out of 185 trees were harbouring termite colonies (Table 2). These 46 colonies belonged to different termite species. The preferences of different termite species for *P. caspica* tree can, therefore, be compared on the basis of the number of colonies (given in parentheses): *P. pasniensis* (13), *M. gabrielis* (7), *P. zabuliensis* (6), *A. paravilis* (6), *P. bidentatus* (4), *M. buettikeri* (4), *A. baluchistanicus* (3), *P. prohybostoma* (2), *H. indicola* (1).

Postelectrotermes pasniensis feeds and nests more on *P. caspica* than other termite species. The second tree with highest importance value in this habitat was *S. babylonica*. Out of the 92 trees of *S. babylonica* recorded from different quadrats, 15 trees were harbouring termite colonies. Preferences of different species for *S. babylonica* is indicated by the number of colonies of different termite species. *Amitermes paravilis* (8), *P. pasniensis* (4), *P. prohybostoma* (2) and *M. mycophagus* (1). *Salix babylonica* was the preferred host of *A. paravilis* in this *P. caspica* habitat. The third tree species having highest importance value is *M. alba*. Out of the 63 trees of *M. alba* examined, only 6 trees were found infested. The number of colonies of termite species *A. vagans* and *P. prohybostoma* were 4 and 2, respectively. *Morus alba* was preferred by *A. vagans* and was not attacked by *P. pasniensis* which otherwise was a very common termite in this habitat.

As regards *T. gallica*, 17 out of 60 trees were infested by termites. Based on number of colonies *i.e.*, *A. vagans* (13), *P. pasniensis* (3) and *M. buettikeri* (1), it is easy to comment that *A. vagans* has greater preference for *T. gallica* than for any other tree in this *P. caspica* habitat. *Acacia seval* comprised of 44 trees. Out of these, 5 were infested and only 2 termite species were nesting on this tree species. *Psammotermes prohybostoma* was represented by 4 colonies and *P. rajasthanicus* by only 1 colony. Thirty-three trees of *P. diversifolia* were recorded in the above mentioned habitat. Out of these, 10 were infested by *P. pasniensis* alone. It is interesting to note that no other termite species attacked *P. diversifolia*.

As regards, *T. diocia*, 5 out of 32 trees were harbouring termites belonging to *A. vagans*. From 31 trees of *Phoenix dactylofolia*, 4 colonies of termites belonging to *M. diversus* were collected. No other termite species attacked this tree, *i.e.*, *P. dactylofolia*. Thirteen trees of *P. alba* were recorded in the *P. caspica* habitat. Out of these, 9 trees were harbouring termites. *Postelectrotermes pasniensis* infested 7 and *P. zabuliensis* infested 2 trees of this species. Out of the 12 trees of *Populus lilita*, only 1 tree was harbouring colony of *P. pasniensis*.

Briefly, 13 termite species were recorded from the *P. caspica* habitat. Out of these, only *P. pasniensis, A. vagans, M. buettikeri, M. gabrielis* have positive association with *P. caspica* habitat (Tables 3 and 4).

Table 1: Habitat types found in the study area of Iran surveyed for termites.

Plot Number	*Populus caspica* 1-27,28-33, 34-36,55-69	*Tamarix gallica* 37-51	*Morus alba* 52-54, 73-78	*Salix baby-lonica* 79-81	*Vitis vinfora* 70-72	Total
Trees/Shrubs						
Populus caspica	185*	-	-	-	-	185
Tamarix gallica	60	98	-	-	-	158
Populus alba	13	-	-	-	-	13
Populus diversifolia	33	-	-	-	-	33
Populus lilita	12	-	-	-	-	12
Tamarix aphylla	26	37	-	-	-	63
Tamarix dioica	32	97	-	-	-	129
Salix babylonica	92	-	-	19	-	111
Phoenix dactylofolia	31	-	-	-	-	31
Morus alba	63	42	46	-	9	160
Acacia seyal	44	-	-	-	-	44
Morus indica	-	46	-	-	-	46
Acer candatum	-	-	-	-	4	4
Acer monspessulanum	-	-	9	-	-	9
Acer cinerascens	-	-	-	11	-	11
Vitis vinfora	-	-	-	-	26	26
Vitis parrifera	-	-	-	-	15	15
Total:	591	320	55	30	54	1050

* Importance value of trees in each habitat.

Species diversity

There were 13 species of termites in *P. caspica* habitat. Diversity of termites (based on number of colonies of different termite species) is indicated in Table 5. Termite diversity (1-D=0.8216) on Simpson Scale is 82% and on Shannon Scale ($H/\log_2 S$) or (H/H_{max}) is 93%. The values of H, H_{max} and E were 3.4556, 3.7004 and 0.9329, respectively. The species richness or variety (dl) was 5.7518 (Table 5). The value of C index of dominance is 0.1783, to which *P. pasniensis* alone contributes a value of 0.1021. This is due to representation of more colonies of *P. pasniensis* on trees in the said plots.

As regards diversity of vegetation (here trees/shrubs) in *P. caspica* habitat, the values of H, H_{max} and E were 3.016, 3.4594, 0.8718, respectively (Table 6). If

compared with diversity of termites in *P. caspica* habitat, there is lesser tree diversity and equitability.

Table 2: Number of trees attacked by termites in different tree habitats.

Trees/ Shrubs	*Populus caspica*		*Tamarix gallica*		*Morus alba*		*Salix babylonica*		*Vitis vinfora*	
	No.of trees	No.of infested trees	No.of trees	No.of infested trees	No.of trees	No.of infested trees	No.of trees	No.of infested trees	No.of trees	No.of infested trees
Populus caspica	185	46	-	-	-	-	-	-	-	-
Tamarix gallica	60	17	98	26	-	-	-	-	-	-
Morus alba	13	9	-	-	-	-	-	-	-	-
Populus diversifolia	33	10	-	-	-	-	-	-	-	-
Populus lilita	12	1	-	-	-	-	-	-	-	-
Tamarix aphylla	26	4	37	4	-	-	-	-	-	-
Tamarix dioica	32	5	97	14	-	-	-	-	-	-
Salix babylonica	92	15	-	-	-	-	19	1	-	-
Phoenix dactylofolia	31	4	-	-	-	-	-	-	-	-
Morus alba	63	6	42	4	46	3	-	-	9	2
Acacia seyal	44	5	-	-	-	-	-	-	-	-
Morus indica	-	-	46	2	-	-	-	-	-	-
Acer candatum	-	-	-	-	-	-	-	-	4	1
Acer monspessulanum	-	-	-	-	9	1	-	-	-	-
Acer cinerascens	-	-	-	-	-	-	11	3	-	-
Vitis vinfora	-	-	-	-	-	-	-	-	26	5
Vitis parrifera	-	-	-	-	-	-	-	-	15	2
Total:	591	122	320	50	55	4	30	4	54	10

Tamarix gallica habitat

As is indicated in Table 1, *T. gallica* and *T. diocia* have importance value (based on the number of trees) 98 and 97, respectively. In this *T. gallica* habitat, maximum (26) colonies were recorded from *T. gallica* (Table 2). The preference of different termite species for *T. gallica* can be compared on the basis of number of colonies (given in parenthesis): *A. iranicus* (20), and *A. vagans* (6). *A. iranicus* has a greater preference for *T. gallica* than that of *A. vagans*.

The number of termite colonies found on *T. diocia* are: *A. iranicus* (10) and *A. vagans* (4). Out of 37 trees of *T. aphylla*, four trees were infested by *A. vagans*. Only 4 out of 42 trees of *M. alba* were infested by termites and only *A. vagans* was recorded feeding on this tree.

Table 3: Distribution of termites in relation to different habitat types in Iran.

Termite species	*Populus caspica*	*Tamarix gallica*	*Morus alba*	*Salix babylonica*	*Vitis vinfora*	Total
Postelectrotermes pasniensis	++	-	-	-	-	1
P. zabuliensis, n.sp.	+	-	-	-	-	1
P. bidentatus, n.sp.	+	-	-	-	-	1
Anacanthotermes vagans	++	++	+	-	-	3
A. gurganiensis, n.sp.	-	-	-	+	-	1
A. iranicus, n.sp.	-	+	-	-	-	1
Psammotermes prohybostoma, n.sp.	+	-	-	-	-	1
P. rajasthanicus	+	-	-	-	-	1
Heterotermes indicola	+	-	-	-	-	1
Microtermes mycophagus	+	-	-	-	-	1
Microcerotermes diversus	+	-	-	-	+	2
M. buettikeri	++	-	++	-	-	2
M. gabrielis	++	-	-	-	-	1
Amitermes paravilis, n.sp.	+	-	-	-	-	1
A. baluchistanicus	+	-	-	-	-	1
A. belli	-	-	+	-	-	1
Total:	13	2	3	1	1	20

+ present, - absent, ++ positive association
For method of calculation see Table 4.

Fourty-six trees of *M. indica* were recorded in *T. gallica* habitat. Out of these only 2 trees were infested with *A. vagans*.

Briefly speaking only 2 termite species *i.e.*, *A. iranicus* and *A. vagans* were feeding on trees in *T. gallica* habitat. Only *A. vagans* has got positive association with *T. gallica* habitat (Tables 3 and 4).

Species diversity

There were 2 species of termites in *T. gallica* habitat. Diversity of termites is indicated in Table 7. Termite diversity on Sampson Scale is 48% and on Shannon Scale 97%. This is expected because more even or equitable distribution of number of colonies among species will increase diversity measured by Shannon-Wiener function. The values of H, H_{max} and E were 0.9708, 0.9999 and 0.9708, respectively. The species richness index = 0.5917 (Table 7).

Table 4: **Association between *Populus caspica* habitat and termite species.**

Termite species	Observed value			
	Present	Not present	Total (A)	Association
Postelectrotermes pasniensis	10	41	51	Positive
P. zabuliensis, n.sp.	3	48	51	Negative
P. bidentatus, n.sp.	2	49	51	Negative
Anacanthotermes vagans	10	41	51	Positive
Psammotermes prohybostoma, n.sp.	3	48	51	Negative
P. rajasthanicus	1	50	51	Negative
Heterotermes indicola	1	50	51	Negative
Microtermes mycophagus	1	50	51	Negative
Microcerotermes diversus	2	40	51	Negative
M. buettikeri	5	46	51	Positive
M. gabrielis	6	45	51	Positive
Amitermes paravilis, n.sp.	3	48	51	Negative
A. baluchistanicus	2	49	51	Negative
Total (B):	49	614	663 (C)	
Expected value =	3.76	47.23		

* Calculated by comparing observed value (number of plots having a particular termite species) and expected value. Expected value was determined by multiplying marginal totals (A & B) and dividing the product by grand total (C). Positive association: Observed value > Expected value. Negative association: Observed value < Expected value. Independent: Observed value = Expected value.

As regards diversity of vegetation in *T. gallica* habitats, the value of H, H_{max} and E were 2.1912, 2.3219 and 0.9437, respectively (Table 8). If compared with diversity of termites in *T. gallica* habitat, there is greater tree diversity but lower equitability.

Morus alba habitat

As is indicated in Table 1, *M. alba* has higher importance value than *Acer monspessulanum*. More termite colonies were recorded from *M. alba* and 3 out of 46 trees were infested by termites (Table 2). Preference of different species can be compared on the basis of number of colonies (which are given in parentheses): *M. buettikeri* (2), *A. vagans* (1) and *A. belli* (1). Only *M. buettikeri* has positive association with it.

Table 5: Proportional abundance of termites and their diversity indices in *Populus caspica* habitat.

Termite species	No. of colonies	Average frequency	% of total	Proportional abundance (P*i*)	P*i*2xS*	P*i* log2** (P*i*)xS
Postelectrotermes pasniensis	39	0.7647	31.96	0.3196	0.1021	0.5259
Anacanthotermes vagans	26	0.5098	21.31	0.2131	0.04541	0.4752
Amitermes paravilis, n.sp.	14	0.2745	11.47	0.1147	0.01315	0.3583
Psammotermes prohybostoma, n.sp.	9	0.1764	7.37	0.07377	0.005442	0.27736
Postelectrotermes zabuliensis, n.sp.	8	0.1568	6.55	0.06557	0.004299	0.25769
Microcerotermes gabrielis	7	0.1372	5.73	0.05737	0.003291	0.2365
M. buettikeri	5	0.09803	4.10	0.04098	0.001679	0.18887
Postelectrotermes bidentatus, n.sp.	4	0.07843	3.27	0.03278	0.001074	0.16163
Microcerotermes diversus	4	0.07843	3.27	0.03278	0.001074	0.16163
Amitermes baluchistanicus	3	0.05882	2.45	0.02459	0.0006046	0.13145
Heterotermes indicola	1	0.01960	0.82	0.008196	0.00006717	0.05679
Microtermes mycophagus	1	0.01960	0.82	0.008196	0.00006717	0.05679
Psammotermes rajasthanicus	1	0.01960	0.82	0.008196	0.00006717	0.05679
Total:	122	2.39191			D=0.17832	H=3.45225

*: Simpson index; **: Shannon-Wiener function

E = 0.9329
dl = 5.7518

Species diversity

There were 3 species of termites in *M. alba* habitats. The value of H, H_{max} and E were 1.4997, 1.58449 and 0.9464, respectively. The species richness index is 3.32 (Table 9).

As regards diversity of vegetation in *M. alba* habitat, there are only 2 species of trees and value of H, H_{max} and E are 0.6428, 0.9999 and 0.6428, respectively (Table 10). The species richness index is 0.5747. The value of C (index of dominance) is 0.7260 to which *M. alba* contributes a value of 0.6993. This is due to presence of more trees of *M. alba* in the said plots.

Table 6: Proportional abundance of tree and their diversity indices in *Populus caspica* habitat.

Tree species	No. of trees	Average frequency	% of total	Proportional abundance (P*i*)	P*i*2xS*	P*i* log2** (P*i*)xS
Populus caspica	185	3.6274	31.3028	0.3130	0.09796	0.5245
Salix babylonica	92	1.8039	15.5668	0.1556	0.02421	0.4170
Morus alba	63	1.2352	10.6598	0.10659	0.01136	0.3442
Tamarix gallica	60	1.1764	10.1522	0.1015	0.01030	0.3349
Acacia seval	44	0.8627	7.4450	0.07445	0.005542	0.2790
Populus diversifolia	33	0.6470	5.5837	0.05583	0.003116	0.2324
Tamarix dioica	32	0.6274	5.4145	0.05414	0.002931	0.2277
Phoenix dactylofolia	31	0.6078	5.2453	0.05245	0.002751	0.2230
Tamarix aphylla	26	0.5098	4.3993	0.04399	0.001935	0.1982
Populus alba	13	0.2549	2.1996	0.02199	0.000483	0.1210
Populus lilita	12	0.2352	2.0304	0.02030	0.000412	0.1141
Total:	591	11.5877			D=0.161000	H=3.016

*: Simpson index
**: Shannon-Wiener function

E = 0.8718
dI = 3.608

Table 7: Proportional abundance of termites and their diversity indices in *Tamarix gallica* habitat.

Termite species	No. of colonies	Average frequency	% of total	Proportional abundance (P*i*)	P*i*2xS*	P*i* log2** (P*i*)xS
Anacanthotermes iranicus, n.sp.	30	2	60	0.6	0.36	0.4421
Anacanthotermes vagans	20	1.3333	40	0.4	0.16	0.5287
Total:	50	3.3333			D=0.52	H=0.9708

*: Simpson index
**: Shannon-Wiener function

E = 0.9708
dI = 0.5917

Salix babylonica habitat

As is shown in Table 1, *S. babylonica* has higher importance value than *Acer cinerascens*, only one species of termites that is *A. gurganiensis* was collected from this

habitat. Three colonies were collected from *A. cinerascens* and only one from *S. babylonica* (Table 2).

Table 8: Proportional abundance of tree and their diversity indices in *Tamarix gallica* habitat.

Tree species	No. of trees	Average frequency	% of total	Proportional abundance (P*i*)	P*i*2xS*	P*i* log2** (P*i*)xS
Tamarix gallica	98	6.5333	30.625	0.30625	0.09378	0.5228
T. dioica	97	6.4666	30.3125	0.303125	0.09188	0.5219
Morus indica	46	3.0666	14.375	0.14375	0.02066	0.4022
M. alba	42	2.80	13.125	0.13125	0.01722	0.3845
Tamarix aphylla	37	2.4666	11.5625	0.115625	0.01336	0.3598
Total:	320	21.3331			D=0.2369	H=-2.1912

*: Simpson index
**: Shannon-Wiener function

E = 0.9437
dl = 1.5967

Table 9: Proportional abundance of termites and their diversity indices in *Morus alba* habitat.

Termite species	No. of colonies	Average frequency	% of total	Proportional abundance (P*i*)	P*i*2xS*	P*i* log2** (P*i*)xS
Microcerotermes buettikeri	2	0.2222	50	0.5	0.25	0.4999
Anacanthotermes vagans	1	0.1111	25	0.25	0.0625	0.4999
Amitermes belli	1	0.1111	25	0.25	0.0625	0.4999
Total:	4	0.4444			D=0.375	H=1.4997

*: Simpson index
**: Shannon-Wiener function

E = 0.9464
dl = 3.32

Vitis vinfora habitat

In *V. vinfora* habitat again only one species of termites that is *M. diversus* was present. The preference of this termite species can be understood by the occurrence of termite colonies on different species of trees *i.e.*, *V. vinfora* (5), *V. perrifera* (2), *M. alba* (2) and *Acer candatum* (1) (Table 2).

Table 10: Proportional abundance of tree and their diversity indices in *Morus alba* habitat.

Tree species	No. of trees	Average frequency	% of total	Proportional abundance (P*i*)	P*i*2xS*	P*i* $\log_2$** (P*i*)xS
Morus alba	46	5.1111	83.6363	0.8363	0.6993	0.2156
Acer monspessulanum	9	1	16.3636	0.1636	0.02676	0.4272
Total:	55	6.1111			D=0.7260	H=0.6428

*: Simpson index
**: Shannon-Wiener function

E = 0.6428
dl = 0.5747

Feeding habits

Postelectrotermes pasniensis: The species was recorded excavating irregular galleries inside infested portion of trees. There was no external indication of the presence of the termite on the infested trees. No galleries were present on the bark of the tree. This species has positive association with *P. caspica* habitat and *P. caspica* was its preferred host.

Postelectrotermes bidentatus: Its feeding pattern and nature of damage to infested trees was similar to that of *P. pasniensis*.

Postelectrotermes zabuliensis: Its feeding pattern was almost similar to that of *P. pasniensis*. It makes irregular galleries in the infested portion of the tree and galleries on the surface of the bark were not seen on any infested trees. It makes its nest inside the stump of the tree. Its host was *P. caspica*, but does not indicate positive association with *P. caspica* habitat.

Anacanthotermes vagans: It was feeding on trees by making galleries on the bark of the trees. Foraging galleries on the adjoining ground were also present. Pieces of grasses were recovered from nest. This species makes small lumps of mound type structure consisting of loose soil particles in Province of Sistan and Baluchistan but makes mounds in Province of Khorasan.

Anacanthotermes gurganiensis: This species was recorded only from 2 trees (*i.e.*, *A. cinerascens* and *S. babylonica*). The former was its preferred host in the type locality. As regards the nature of damage on these trees, there was no external indication of the presence of the termite in the infested trees. The species excavates galleries inside the trees.

Anacanthotermes iranicus: As regards its feeding pattern it was almost similar to that of *A. vagans*. The nature of damage involved making of mud sheeting and galleries on trees. Pieces of grasses were also recorded from their nests in soil.

Psammotermes rajasthanicus: This species was collected only once feeding on *A. seyal*. The nature of galleries was similar to that of *P. prohybostoma*.

Psammotermes prohybostoma: This species was foraging by making clayee galleries in the soil and surface of the trees. The consumed portion of stump was not replaced by mud.

Heterotermes indicola: This species was collected only once feeding on *P. caspica*. This species was foraging by constructing galleries on the bark of the trees and branches.

Microtermes mycophagus: This fungus growing termite was collected only once from *S. babylonica*, feeding under clayee galleries. The damaged and consumed portion of the tree was replaced by the clay by this species.

DISCUSSION

Gay *et al.* (1954) carried out studies on standard laboratory colonies of termites for evaluating the resistance of timber, timber preservation and other materials to termite attack. Their comparative tests of matched timber specimens in standard laboratory colonies of *N. exitiosus, C. lacteus* and *C. acinaciformis* showed that the tree species of termites have different food preferences and rates of attacks, so that results obtained from tests with only one species do not necessarily apply to other species and may, in fact, be misleading.

Sen-Sarma and Chatterjee (1965) tested natural resistance of various species of Indian and exotic woods to different species of termites under laboratory conditions. They reported that termites avoided *Cedrus deodara*, and resistance appears to be due to certain chemical substances which act as repellent to termites. Akhtar (1980) reported that forced feeding of *Bifiditermes beesoni* on *Cedrus deodara* caused diarrhoea in this termite which failed to produce normal fecal pellets. Sen-Sarma and Chatterjee (1965) also reported that woods of *Albizzia odoratissima, Dalbergia latifolia, Prosopis spicigera, Swictenia macrophylla, Tectona grandis* and *Xylia xylocapra* revealed the presence of some toxic chemical substances because they caused high mortality in test termites.

MacMahan (1966) worked on *Cryptotermes brevis* (Walker) and reared them in different termitaries made of 15 kinds of wood veneer and reported that this termite preferred some wood over others. Poplar and maple both were ranked as more preferred woods.

Smythe and Carter (1969) showed that samples from different trees of loblolly pine and bald cypress showed a marked variation in susceptibility to termite attack.

Nel *et al.* (1970) studied food preferences of laboratory colonies of the Harvester termite, *Hodotermes mossambicus* (Hagen) and reported that colonies deteriorated

rapidly when fed exclusively on green *Themeda triandra*, and also confirmed poisonous quality of *Chrysocoma tenuifolia* towards *H. mossambicus*.

Minnick *et al.* (1972) studied feeding preferences of the drywood termite *Cryptotermes brevis* and reported that only pine was consumed in significantly less quantities both by volume and weight as compared to balsa western redcedar. Beal *et al.* (1974) studied force feeding response of *Reticulitermes flavipes* (Kollar) and *Coptotermes formosanus* Shiraki exposed to blocks of 97 species of tropical woods. Bultman *et al.* (1977) evaluated natural resistance of some tropical African woods to *Coptotermes formosanus* Shiraki and reported that 19 out of the 42 woods were highly damaged.

Gentry and Whitford (1982) used point-quarter technique to estimate the abundance of *Reticulitermes* in dead wood in four southeastern coastal habitats in the USA. The four habitats chosen were: lowland hardwood forest dominated by oak (*Quercus* spp.), sweet gum (*Liquidambar styraciflua*) and poplar (*Liriodendron tulipfera*); turkey oak (*Quercus nigra*) woodland, and burned and unburned pine (*Pinus* spp.) plantations. Two burned pine plantation had the highest estimated termite density, 13×10^6/ha and 2.2×10^6/ha, respectively; and the turkey oak woodland had an estimated density of 61.9×10^3/ha.

Kayani *et al.* (1985) carried out some studies on abundance of termites in various plant communities of Eastern Baluchistan, Pakistan. On the basis of termite nest density and extent of termite infested area it was concluded that out of the 19 plant communities four have serious termite infestation problem (nest density 7124/ha).

Thirugnanasuntharan (1987) studied feeding and survival of *Glyptotermes dilatatus* and *Postelectrotermes militaris* on five regions of tea stems. The trunk region of the tea plant showed the highest percentage of termite survival. *Postelectrotermes militaris* lived better than *G. dilatatus* in tea stems. *Postelectrotermes militaris* showed some wood preference in the tropical woods tested; while pine was more susceptible than beech. El-Sherif *et al.* (1987) determined relative susceptibility of 11 species of wood to attack by *Psammotermes hybostoma* by measuring the amount of wood consumed, from samples for two successive years. They reported that *Albizia lebbek* was consumed least by this termite.

Supriana (1988) compared resistance and repellency of 28 tropical timbers to *Cryptotermes cyanocephalus* and *Coptotermes curvignathus*. They also reported that the presence of other species in the tests probably affected the feeding behaviour patterns.

Braithwaite *et al.* (1988) described structure of termite communities in the Australian tropics. They described the distribution of 50 species of termites across five habitat types in Kakadu National Park. They further reported that open forests are richest in species and monsoon forests are species poor.

The present studies describe the distribution of 16 species of termites in five tree habitats: *P. caspica, T. gallica, M. alba, S. babylonica* and *V. vinfora*. *Populus caspica*

habitat has higher importance value and more rich in termite species than *S. babylonica* and *M. alba* habitats.

Delaplane and Lafage (1990) have reported on feeding excavations on wood block by *Coptotermes formosanus*, *Reticulitermes flaviceps* and *R. virginicus*. Feeding rates was greatest in *C. formosanus* followed by *R. flaviceps* and then *R. virginicus*.

Agbogba (1990) reported that the termite population and vegetation was sampled along seven 17 km transects covering 34 ha in the Lac de Guiers region of Senegal. *Psammotermes hybostoma* builds thick layers of earth on trees and attacks both the bark and the wood under the earth. *Microcerotermes* sp. builds tunnels up the trunks and penetrates trees via galleries bored into the wood. *Psammotermes hybostoma* was the dominant species. Termite damage was greatest in areas with sparse tree cover. Of 31 tree species recorded, 11 had been attacked. Damage to *Balanites aegyptica* by *Psammotermes hybostoma* was the most severe (33-100%) and sometimes led to the death of the tree; however, damage to *B. aegyptica* diminished where the diversity of tree species increased. *Acacia* was relatively undamaged. It is noted that *P. hybostoma* is a species which had previously been thought to feed only on dead vegetable matter.

Present report based on the importance value, revealed that maximum termite species (13) were recorded in *P. caspica* habitat. Different species had different tree preferences, *Postelectrotermes* fed and nested more on *P. caspica* and *P. diversifolia* than other tree species. The values of H, H_{max} and E were 3.4556, 3.7004 and 0.9329, respectively for termite species diversity in *P. caspica* habitat. Wood *et al.* (1982) have also reported values of termite species diversity for a riparian forest in the Southern Guinea with H, H_{max} and E for plots 39 and 40 as 3.367, 4.475, 0.752 and 3.333, 4.367 and 0.763, respectively.

Delaplane *et al.* (1991) studied number of workers, mass of individual workers, and % of soldiers of foraging group from *Coptotermes formosanus* colonies living in Cypress tree at the Calcasieus River lake charles, Lousiana (USA).

Duncan and Hewitt (1991) observed foraging behaviour of *Hodotermes mossambicus* and noticed division of labour among the workers. The major workers mainly cut plant material and the minor workers mainly transported it. Comparison between the dimensions and mass of the forage items and commercial baits showed that the baits were generally shorter, heavier and wider than the natural food.

Present studies in Iran are the first of its kind. Nobody has reported on species diversity or species richness. *Populus caspica* habitat which had greater number of tree species was more rich in termite species (dl=5.7518) compared to *T. gallica* habitat (dl=0.59). Presently it is concluded that termite species richness is to a great extent related to tree species richness.

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CERVUS TRIPLIDENS LYDEKKER FROM TYPE LOCALITY DHOK PATHAN, CHAKWAL DISTRICT, PUNJAB, PAKISTAN

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Abstract: A well preserved part of a skull with M^{1-3} of both sides and portions of palate and frontals is described from Dhok Pathan (Middle Siwaliks), Chakwal district, Punjab, Pakistan. The median basal pillar of the molars is an important feature of the species *Cervus triplidens* Lydekker. It is quite prominent in all the teeth and all show an advanced stage of wear in the specimen under study.

Key words: *Cervus triplidens*, palate, frontals, Middle Siwaliks, median basal pillar.

INTRODUCTION

The cervids (Mammalia, Artiodactyla, Cervidae) are characterized by the presence of antlers and prominent lacrymal depressions anterior to the eyes that are occupied by the pre-orbital glands in the living deer. Many species of the Siwalik cervids have been reported from the Middle and Upper Siwaliks (Lydekker, 1876, 1884; Brown, 1926; Colbert, 1935; Azzaroli, 1954). Among these species *Cervus triplidens* Lydekker, *C. simplicidens* Lydekker, *C. sivalensis* Lydekker, *C. punjabiensis* Brown and *C. colberti* Azzaroli, are considered valid. The earlier identifications of these species were based on mandibulary and maxillary fragments, isolated upper and lower molars and antlers fragments.

Recently Arif and Shah (1991) established a new species of cervids, *Cervus rewati* from the Upper Siwalik beds near Rewat, Rawalpindi district, Punjab, Pakistan. This new cervid is based on a right mandibular ramus bearing P_3-M_3. The diagnostic features of *C. rewati* are the small size of the teeth, presence of median basal pillars and well developed anterior folds on the molars.

The material under study is a part of a skull with M^{1-3} of both sides and portions of palate and frontals. This specimen is referred to the species *Cervus triplidens* Lydekker. The measurements are given in millimeters.

Systematic Account

Order Artiodactyla Owen

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Suborder	Ruminantia Scopoli
Infraorder	Pecora Linnaeus
Superfamily	Cervoidea Simpson
Family	Cervidae Gray
Subfamily	Cervinae Baird
Genus	*Cervus* Linnaeus
Species	*Cervus triplidens* Lydekker

Holotype

G.S.I. (Geological Survey of India) No. B 204, a right maxilla with M^{2-3}.

Locality

Punjab.

Horizon

"Higher pliocene of Sub-Himalaya" Lydekker (1883).

Diagnosis

Molars hypsodont, with a large median basal pillar and rugose enamel.

Material studied

P.U.P.C. (Punjab University Paleontological Collection) No. 84/118, with M^{1-3}.

Horizon

Middle Siwaliks.

Locality

Dhok Pathan, Chakwal district, Punjab, Pakistan.

Description (Fig. 1)

The specimen under description is a part of a skull with M^{1-3} of both sides and portions of palate and frontals. The width of palate is 72 mm between the first molars, 75 mm between the second molars and 68 mm between the last molars. The height of the skull above M^3 is 90 mm and it slightly slopes anteriorly, as is the general rule.

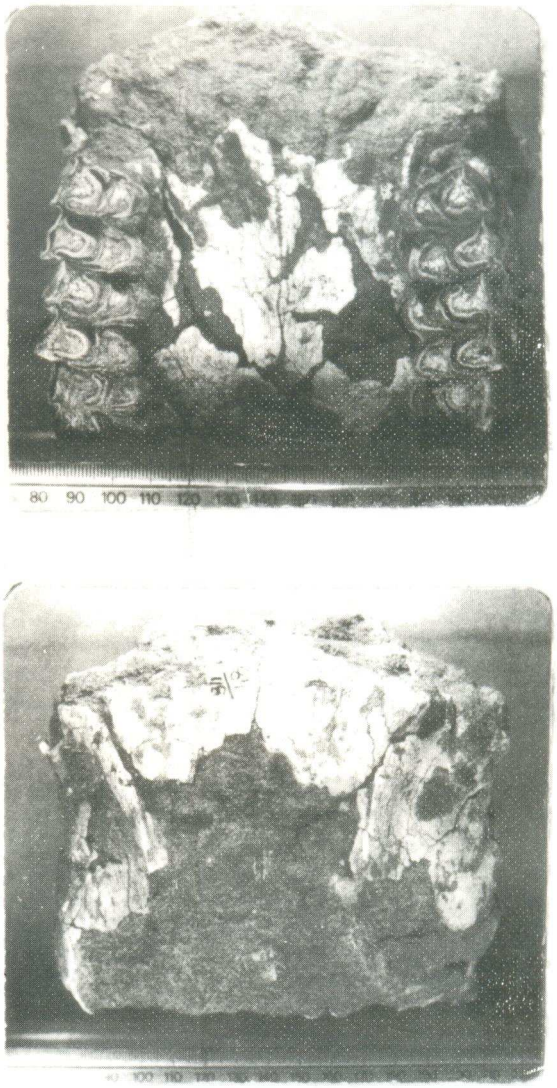

Fig. 1: *Cervus triplidens* Lydekker, a damaged skull (P.U.P.C. No. 84/118) from Dhok Pathan, Chakwal district, Punjab, Pakistan. A. Palatal view, B. Dorsal view.

The median basal pillar of the molars is an important character of this species. It is quite distinct in all the molars and all show an advanced stage of wear in the specimen under study.

DISCUSSION

The material under study is a part of a skull with M^{1-3} of both sides and portions of palate and frontals. The width of palate is 72 mm between the first molars, 75 mm between the second molars and 68 mm between the last molars. This is because, as usual, the tooth row is crescentic, as is also seen in Am. Mus. (American Museum of Natural History) No. 19792 described by Colbert (1935). This feature is not visible in the type specimen as the latter consists of two molars of one side only. The height of the skull above M^3 is 90 mm and it slightly slopes anteriorly, as is the general rule.

The skull under study is of an old individual, while the one described by Colbert (1935) is less so and the type specimen is of a young individual. Lydekker (1876) says, "The specimens figured consist of the second and third molars of the right maxilla; the first of these is slightly worn, while the second is merely a germ, as yet untouched by wear". From his diagram it is evident that the vertical surfaces of opposite sides were not parallel. Thus, with wear the length and width ratio of the crown surface must change.

The Am. Mus. specimen No. 19792 is of an older individual and the specimen under study is the oldest one. Crown surface of M^3 is perfectly squarish in the type specimen and the one described by Colbert (1935), while in the specimen under study the width is very slightly less than the length. This meagre difference can have no taxonomic value, it must be considered an individual difference concerned very probably with age of the individual.

The crown view of the teeth of the type specimen shows two pairs of crescents, which are the modified cusps. The transverse dimension of each cusp is very thin at the apex but gradually becomes thick towards the root. This is evident from Am. Mus. No. 19792 and the specimen under study. In the former the crescents are thick in the middle and thin toward the tips. With age cusps merge with each other. This feature shows its beginning in Am. Mus. No. 19792 and an advanced stage in the specimen under study. Anteriorly and posteriorly these cusps merge with each other. M^1, which is always the oldest molar, shows the maximum merger.

A median basal pillar of the molars is an important feature of this species. It is quite prominent in all the teeth and all show an advanced stage of wear in the specimen under study. In the type specimen these pillars are very well developed. However, they do not reach the tips of the crowns and are, therefore, unworn. In Am. Mus. No. 19792 they are well worn in M^{1-2} but not in M^3. In M^1 of the latter this pillar shows the maximum wear so that it has merged with the anterointernal cusp (protocone). The same feature is evident in the specimen under study. According to Lydekker (1883), the species *Cervus triplidens* has a large median basal pillar to the molars. Another feature,

mentioned by early workers and shown by the present specimen, is the rugosity of the enamel. Matthew (1929) regards the fossils as inadequate of no correlation value.

Table 1: Comparative measurements (mm) of the teeth of *Cervus triplidens* Lydekker

Specimen Nos.	Length and Width		
	M^1	M^2	M^3
P.U.P.C. No. 84/118	22 x 26	26 x 30	30 x 27
Am. Mus. No. 19792	20 x 24	24 x 26.5	26 x 26
G.S.I. No. B 204		20 x 23.7	25 x 25

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A STUDY ON METAL RESISTANCE AND LEAD DETOXIFICATION EFFICIENCY OF A GRAM POSITIVE BACTERIUM ISOLATED FROM EFFLUENTS OF TANNING INDUSTRY

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Abstract: A Gram positive lead resistant bacterial isolate was got form effluents of tanning industry. Maximum lead resistance of the isolate was determined. The strain CMBL17 tolerated maximum lead concentration at 2 mg/mL of the medium. Resistance of the isolate against cadmium, and chromium was also checked. Optimum growth conditions *i.e.*, incubation temperature, pH and inoculum size were noted for the growth of the isolate. The isolate was used for processing of lead in the medium. The bacterium showed fairly high efficiency in processing of lead present in liquid medium. Location of genes for lead processing was determined by detection of plasmids, curing of plasmids and determination of lead resistance after curing.

Key words: Bioremediation, lead resistant bacteria, industrial effluents, metal resistant bacteria.

INTRODUCTION

Lead gets entry into the environment as a result of a number of agricultural and industrial practices. Unfortunately it is a contaminant of soil, water and air. Contamination of soil by lead takes place as a consequence of the use of lead arsenate insecticides. High levels of lead in waste waters may originate from combustion of lead containing gasoline, coal burning or metal smelting (Trajanovska *et al.*, 1997). The practice of mining of lead for nuclear and metal industry and leaching from natural deposits are other sources of contamination. Certain transition metals like copper, cobalt, nickel and zinc, in trace amounts are essential for cellular metabolism. These usually act as cofactors of enzymes or part of certain complex organic molecules. However, higher concentrations of these metals becomes toxic for the cell. Other heavy metals like lead, cadmium, mercury, chromium and silver have no known beneficial functions inside the eukaryotic or bacterial cells. Rather these are toxic even at very low concentrations (Brown, 1992; Gadd, 1992).

A large number of bacteria have been isolated from metal contaminated sites receiving effluents of industrial wastes and from soil samples contaminated by battery manufacturing operations (Manovski *et al.*, 1992; Haq *et al.*, 1997). Environmental pollutants pose a high risk not only to human beings and other animals but also to bacterial populations in the area. However, the adaptive responses of bacteria are usually

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prompt and studied extensively. Various studies have indicated that bacteria which survive, and indeed, flourish in such environments have developed or acquired genetic systems which help bacteria resist and usually detoxify the metal ions in the environment. The genes coding cadmium, zinc, cobalt, chromium, copper, arsenic and nickel have been predominantly located on plasmids (Mergeay *et al.*, 1985; Chen *et al.*, 1986; Nucifora *et al.*, 1989; Cervantes *et al.*, 1990; Cooksey *et al.*, 1990; Nies *et al.*, 1990; Nies, 1992; Silver and Walderhaug, 1992; Liesegang *et al.*, 1993; Nies *et al.*, 1993; Williams *et al.*, 1993; Stoppel and Schlegel, 1995).

MATERIALS AND METHODS

Samples collection and isolation of lead resistant bacteria

Water samples were collected from effluents released by tanning industry. The samples were collected in sterile screw capped glass bottles, brought to the laboratory and stored at room temperature before spreading on plates. For selection of lead resistance bacteria LB agar plates with 1 mg/mL lead were used. Lead acetate was used as source of Pb^{2+}. Lead acetate solution and LB agar medium were autoclaved separately and allowed to cool down. When the temperature of the two solutions was slightly less than 60°C, the solutions were mixed and poured into plates. Industrial effluent (100 μl) was spread on the plates and the growth of colonies was observed after 24 hours. Colonies were picked and streaked for purification and for determination of maximum resistance of the strains against lead.

Resistance against cadmium and chromium

Resistance of the bacterial isolate against cadmium and chromium was checked by taking various concentration of chromium ($K_2Cr_2O_7$) and cadmium ($CdCl_2$), starting from 10 μL/mL of each metal ion in the medium. Minimum inhibitory concentration was recorded when the colonies failed to appear within 48 hours.

Estimation of lead in the medium

In order to estimate the amount of lead in the medium, dithizone method (E. Merck) was used. Reagent 1 (R1) was prepared by dissolving 10 mL of hydrazinium hydroxide in 70 mL of 1N HCl, then adding 20 g of NaCl and making the volume up to 100 mL with distilled water. Reagent 2 (R2) was prepared by dissolving 20 g potassium hydrogen carbonate, 5 g potassium cyanide, 5 g potassium sodium tartarate and 25 mL ammonium solution in 100 mL of distilled water. Dithizone solution was prepared by dissolving 15 mg of dithizone in 1000 mL of chloroform. Five mL of LB broth with lead concentration of 1 mg/mL was taken in test tubes. The tubes were inoculated with fresh bacterial cultures. After incubation for 24 hours 1 mL culture was taken from the tube aseptically. The culture was diluted to 25 times volume with distilled water. This mixture was taken in a separating funnel and 2.5 mL of R1 were added followed by addition of 2.5 mL of R2. After that 12.5 mL of dithizone solution was added. The mixture was shaken for 5 minutes and the pressure developed by shaking and reaction was released by removing the stopper or valve. The mixture was allowed to stand for a

few minutes. Two layers were formed. The lower layer containing chloroform was collected in a glass bottle and OD of the solution was taken at 515 nm against a blank which was prepared through a similar procedure by taking 25 mL distilled water without any culture. The same procedure was adopted to estimate lead at 0, 48 and 72 hours. Control of the lead processing was run using the medium without inoculation incubated at same conditions as that of the culture. Bacterial processing of lead was assessed by estimating the amount of lead in the medium after various time intervals. All the readings were taken in triplicate for statistical analysis.

Amount of lead was calculated by the following formula:

$$G = \frac{M \times 95.4 \times \text{dilution factor}}{a}$$

where G = amount of lead in mg/L, a = amount of water used in mL, M = absorbance.

Isolation of plasmid

A single colony of various lead resistant strains was selected and grown in LB liquid medium for plasmid isolation. The procedure adopted for isolation of plasmids was as described by Holmes (1984). Plasmid DNA isolated from the bacterial isolates was run on agarose gel and visualized under UV illuminator after staining with ethidium bromide. The isolation experiment was repeated three times to ascertain the presence of the plasmids.

Curing of the plasmid

Curing of the plasmid present in the strain was done by using ethidium bromide. The strain was grown in the medium containing ethidium at concentrations; 50, 100 and 150 μg/mL of the medium. After the growth, the culture was spread on LB agar plates. The colonies appearing on the plates were tooth picked on to the LB agar plates and the plates containing lead at a concentration of 1 mg/mL (both the plates had a grid for location of colonies. The colonies appearing on agar plates but not appearing on plates containing lead were taken as indication of the cells having the plasmid cured.

RESULTS

Isolation of lead resistant bacterial strain

For isolation of lead resistant bacterial strain, LB plates containing lead at 1 mg/mL concentration were used. Colonies appeared after 24 hours. A Gram positive bacterial isolate was selected for lead resistance and lead processing ability.

Metal and antibiotic resistance of the isolate

The bacterial isolate was grown on increasing concentrations of lead. The minimum inhibitory concentration of lead for the strains was 2 mg/mL. Metal resistance of the isolate against cadmium and chromium was also checked. The isolate showed resistance against these metals at a concentration of 70 mg/mL and 80 mg/mL respectively. The sensitivity of the isolate against various antibiotics was checked. The isolate showed resistance against septran and ceptazimide at a concentration of 30 μg/mL while it showed sensitivity against ampicillin, augmentin, amoxycillin, cefazolin, doxycyclin, enoxocin, erythromycine, gentamicin and minocyclinE.

Identification of the isolate

Gram staining of the bacterial culture showed Gram positive rods. The isolate formed circular white colonies with entire margins on LB agar plates. The strain showed positive activity for catalase, oxidase and Voges-Proskauer test and it was negative for coagulase test, starch utilization test, McConkey test, citrate utilization test and nitrate reduction test.

Optimum growth conditions and growth curve

The growth curve of the isolate was determined. It showed a lag phase of four hours after inoculation followed by an exponential phase of 10 hours and then a decline phase (Fig.1). The optimum pH for the growth of the bacterial strain was 7.0, temperature 37°C and inoculum size 20 μl/5 mL of the medium (Figs.2a,b,c).

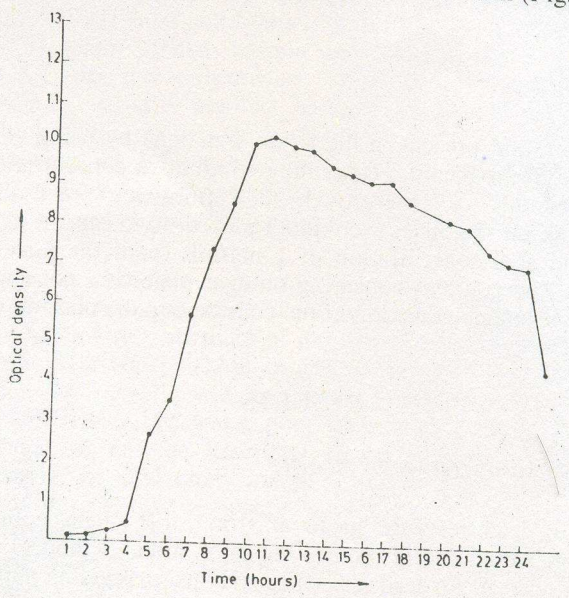

Fig. 1: Twenty four hours growth pattern of CMBL-17.

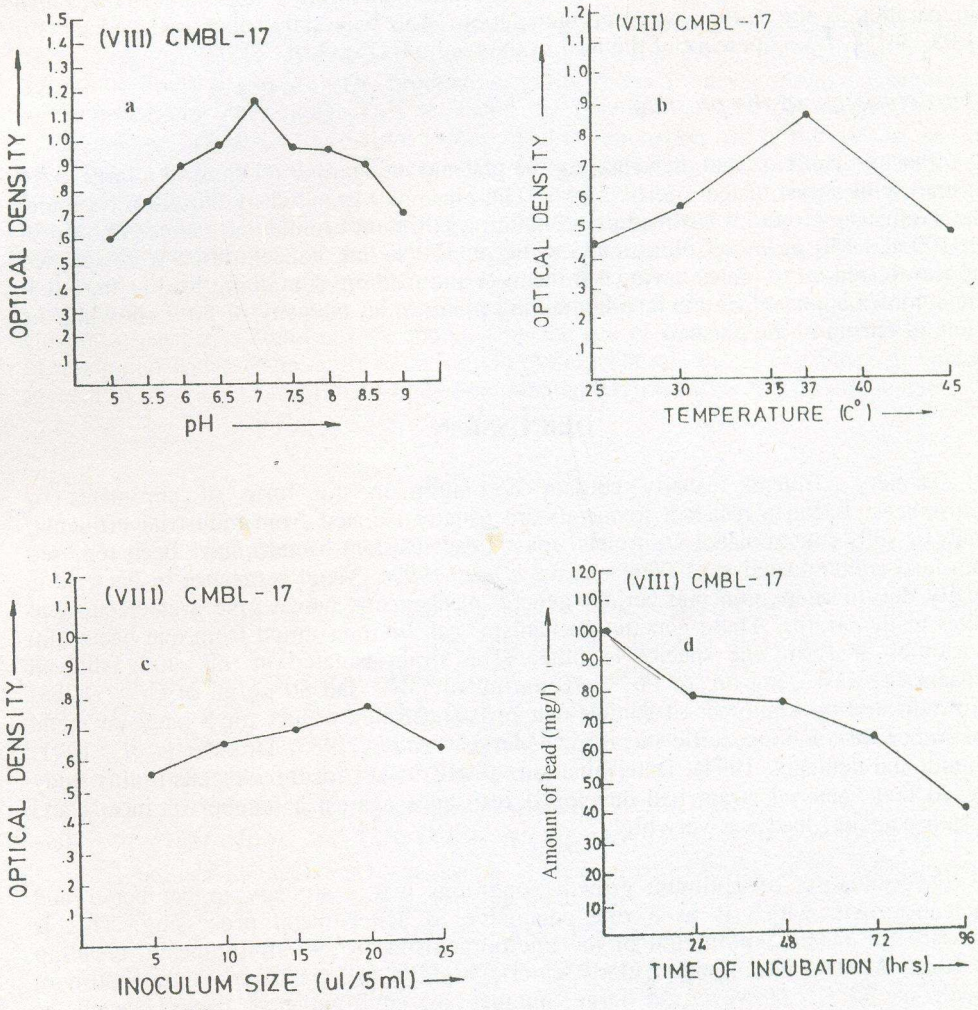

Fig. 2: Effect of: a, pH; b, temperature; c, inoculum size on the growth of bacterial isolates; and d, effect of bacterial activity on lead decontamination in the culture.

Processing of lead by the bacterial strain

A culture of the isolate was prepared in LB medium containing lead at a concentration of 1 mg/mL. The estimation of the amount of lead was done at 0, 24, 48, 72 and 96 hours of incubation. A control (medium containing 1 mg/mL lead) was also run parallel to the culture without inoculation. The bacterial isolate reduced 21.39, 23.05, 34.54, 60.63 percent of the lead in the medium (Fig.2d).

Characteristics of the plasmid

Plasmid isolation and then running the plasmid on agarose gel showed a large sized plasmid with an estimated size of 23 kb. The plasmid was cured by ethidium bromide. The strain was grown in LB medium containing ethidium bromide at a concentration of 50, 100 and 150 μg/mL. Colonies appearing on 150 μg/mL concentration were selected and transferred on to plates having LB medium and medium containing lead (1 mg/mL). The colonies appeared on the lead containing plates at an intensity of 50% showing the extent of curing of the plasmid.

DISCUSSION

Tannery effluents usually contain chromium in the form of chromates or dichromates. Bacteria resistant to metals are usually isolated from industrial effluents, ponds or soils contaminated with metal ions. Lead resistant bacteria have been reported from lead contaminated sites (Trajanovska *et al.*, 1997). Metal resistance in bacteria is usually due to adaptation and certain genetic mechanisms which give greater survival values to the strains. These genetic mechanisms can be transmitted from one bacterium to another or from one species to other. The strain isolated in this study showed resistance against 2 mg/mL of Pb^{2+}, 70 μg/mL of Cd^{2+} and 80 μg/ml of Cr^{6+}. It has been indicated by a number of studies that resistance often occurs for a range of metal ions rather than for a specific metal ion (Mergeay *et al.*, 1985; Dressler *et al.*, 1991; Schmidt and Schlegel, 1994). Determination of MIC values for three metals in this study showed that bacterial strain had developed resistance against a number of metals and resistance against lead was very high.

Determination of optimum growth conditions was a strategy to get better and efficient growth which is as a rule conducive to better metal processing. This is necessary for better exploitation of the microorganisms in the environmental clean-up operation. Many bacteria, particularly enteric bacteria and pathogens show optimum growth at 37°C. However, a large number of environmental bacteria such as *Pseudomonas* show optimum growth at 30°C. Many of the metal resistant bacteria can resist a number of heavy metal ions present in the environment (Collard *et al.*, 1994) possibly due to possession of common mechanisms of resistance. There are various external environmental conditions for better xenobiotic detoxification. In this regard, another important condition is the pH of the medium. Microorganisms are metal accumulator, and their ability is controlled by external pH (Morley and Gadd, 1995). They causes a change in valence or ionic state of metal which leads to effect the growth

of the microorganisms (Francis, 1990). Moreover, one or more types of processing become rate limiting at extreme temperatures (Innis and Ingraham, 1978).

The strain isolated in this study showed a considerable capability of processing lead. It reduced 21.39, 23.05, 34.54, 60.63 percent of the lead in the medium in 24, 48, 72 and 96 hours of growth. The processing ability showed that the strain can be used in metal processing operations. This indicated the potential of lead detoxification in the environmental clean-up operations. One of the conclusion from the results is that ethidium bromide proved an efficient agent for curing of plasmids. The presence of the plasmid in the strain indicated that the gene for processing of lead might be present on the plasmid. The failure of the growth of the isolate on lead containing medium after curing procedure indicated that the gene for processing of lead was possibly plasmid coded as it has been discovered in other studies that metal resistance and metal processing genes are usually plasmid borne. Another explanation would be that the chromosomal genes could also be mutated by the use of ethidium bromide, as ethidium bromide can cause frame shift mutations in genes. The antibiotic resistance of bacteria present in the industrial effluents and contaminated environments is well documented.

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ROOT ASSOCIATED MODERATELY HALOPHILIC AND ALKALIPHILIC BACTERIA FROM *CONVOLVULUS ARVENSIS*

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Abstract: Seven isolates from the histoplane (Cs-1, Cs-2, Cs-3, Cs-4) and rhizoplane (RCs-1, RCs-2, RCs-3) of *Convolvulus arvensis*, growing in saline area were obtained. They were gram-negative motile rods (except Cs-2 which was gram negative motile cocci) and could tolerate 2.0-3.0M NaCl in the medium. Optimal salt concentration for their growth was 0.5M (Cs-2, Cs-3, RCs-1, RCs-3), 1.0M (Cs-4, RCs-3) and 1.5M (Cs-1), hence all of them were moderately halophilic bacteria. Strains Cs-4 and RCs-2 shared characters with genus *Halomonas*, while others belonged to group gram-negative facultative anaerobic rods (except Cs-2). Tolerance level to salts such as $NaHCO_3$, Na_2SO_4, $MgSO_4$, KNO_3 and KCl varies for different strains. Depending upon the strain temperature for maximum growth of these bacteria was 28°C (Cs-4, RCs-2), 32°C (RCs-1, RCs-3), 37°C (Cs-2, Cs-3) or 42°C (Cs-1). They exhibited wide pH range with optimal pH 8 (except for Cs-2 where it was 9) in the presence of 1M NaCl, thus belonging to moderately alkaliphilic bacteria. However their pH optima varied from 5-10 in the absence of NaCl. These strains confer resistance to kanamycin in the presence of 1M NaCl but not in its absence. Whereas resistance behaviour to ampicilline was different for different strains. They also exhibited multiple resistance to different metallic salts. Apart from Cs-2, other strains harbor plasmid/s.

Key words: Histoplane, rhizoplane, moderately alkaliphilic, salt-tolerant bacteria.

INTRODUCTION

Among the abiotic stresses that limit crop productivity, salinity ranks as the most detrimental. Organisms specially bacteria which can resist saline stress are being intensively investigated but the molecular basis of microbial resistance to salinity are not fully understood. Two major approaches by which microorganisms respond to raised external salinity are the accumulation of intracellular compatible solutes (osmoprotectants) and modification of membrane composition and function (Russell, 1989; Csonka, 1989; Wohlfarth *et al.*, 1990; Severin *et al.*, 1992; Russell, 1992). Two distinct classes of osmoprotectants exist, those which act as genuine osmolytes such as glycine betaine, proline or glutamate and those which act as chemical mediators *e.g.* ectoine (Talibart *et al.*, 1994). It is imperative to comprehend the mechanism/s utilize by bacteria to withstand the osmotic and ionic stresses imposed by high salinity. Bacterial population from the natural habitat provide a rich source for the understanding of osmoregulation. Bacteria growing in saline habitat fall in two categories; halotolerant, which do not require NaCl for growth but can grow under saline conditions and halophiles, which must have NaCl for growth. Halophiles can be classified into three groups on the basis of their response to NaCl. Slightly halophilic bacteria, yield optimum growth at 0.2-0.5M NaCl, moderately halophilic bacteria grow

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best in 0.5-2.5M NaCl and extremely halophilic bacteria prefer 2.5-5.2M NaCl for their optimal growth (Kushner, 1978). In this connection we are isolating and characterizing the salt tolerant/moderately halophilic bacteria from different sources *viz*., soil, plant's histoplane, rhizoplane and rhizosphere (Sherwani and Hasnain, 1990a,b; Hasnain and Taskeen, 1989; Yasmin and Hasnain, 1993a,b). Here we are describing the isolation and characterization of moderately halophilic bacteria from the rhizoplane and histoplane of *Convolvulus arvensis*.

MATERIALS AND METHODS

Isolates were obtained from the histoplane and rhizoplane of *Convolvulus arvensis*, growing in the saline patches around Lahore, by the method of Yasmin and Hasnain (1993a). Only those isolates which could bear 1M or above concentrations of NaCl in the growth medium were selected. Purified isolates were characterized following Gerhardt *et al*. (1994). Some biochemical tests were also accomplished by using Q.T.S-20 and CO strips (DESTO Laboratories, Karachi, Pakistan).

Resistance profile of these strains for NaCl (0.5-3.0M) and some other salts involve in salinity such as $NaHCO_3$ (0.1-0.5M), Na_2SO_4 (0.1-1.0M), $MgSO_4$, KNO_3 and KCl (0.1-2.0M) were studied. Resistances to $NaHCO_3$. Na_2SO_4, $MgSO_4$, KNO_3 and KCl were examined both in the presence and absence of 1M NaCl. Following Yasmin and Hasnain (1993b), bacterial growth curves, temperature (25°, 28°, 32°, 37°, 42°C) and pH ranges (5, 6, 7, 8, 9, 10, 11) of these isolates were accomplished both in the presence and absence of NaCl. Resistance pattern of these isolates to antibiotics kanamycin (Km, 10-50 $\mu g\ ml^{-1}$), ampicillin (Ap, 100-500 $\mu g\ ml^{-1}$), tetracycline (Tc, 5-25 $\mu g\ ml^{-1}$), streptomycin (Sm, 100-500 $\mu g\ ml^{-1}$), chloramphenicol (Cm, 5 $\mu g\ ml^{-1}$) as well as metallic salts of Ni ($NiSO_4$), Sn ($SnCl_2$), Ba ($BaCl_2$), Pb [$Pb(NO_3)_2$], Cr ($CrCl_3$), Mn ($MnSO_4$), Zn ($ZnSO_4$), Fe (Fe_2SO_4), Mo (Na_2MoO_4), Co ($CoCl_2$) and Cu ($CuSO_4$) were also determined. Media (L-agar or L-broth) was supplemented with the respective antibiotics and metallic salts and after inoculation/ streaking and incubation at 37°C for 24 hours, growth was checked or OD was monitored at 600 nm, as the case may be.

Bacteria were screened for the presence of plasmid/s by the gel electrophoresis of total cell lysate (Thomas, 1984).

RESULTS

Isolates were obtained from the histoplane (Cs-1, Cs-2, Cs-3, Cs-4) and rhizoplane (RCs-1, RCs-2, RCs-3) of *Convolvulus arvensis* growing in the saline area around Lahore. All the isolates, both from rhizoplane and histoplane of the plant, had circular, entire (except Cs-3) and convex colonies with different shades of yellow except for Cs-1 and Cs-2, where colonies were dirty white and white, respectively. Isolates Cs-4 and RCs-2 were strictly aerobic while rest of them were facultative anaerobes. All isolates were gram-negative motile rods except Cs-2 which was found to be gram-negative motile cocci. Isolates Cs-4, and RCs-2 could be affiliated with genus *Halomonas*, while

Cs-1, Cs-3, RCs-1, RCs-3 were identified as the members of group facultatively anaerobic gram-negative rods (Holt *et al.*, 1994). Affinities of Cs-2 (gram-negative facultative anaerobic cocci) remained uncertain. Excluding Cs-1 and RCs-1 for few tests, they showed similar biochemical attributes (Table 1).

Table 1: Biochemical characterization of isolates from *Convolvulus arvensis*.

Name of Test	Strains						
	Cs-1	Cs-2	Cs-3	Cs-4	RCs-1	RCs-2	RCs-3
Gram staining	-	-	-	-	-	-	-
Oxidation fermentation	+	+	+	-	+	-	+
Cytochrome oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Denitrification	-	-	-	-	-	-	-
Voges Proskaur	-	+	-	-	-	-	-
Methyl red	-	+	-	-	-	-	-
Phenylalanine deaminase	+	+	+	+	+	+	+
ONPG	+	-	-	-	+	-	-
Sodium citrate	-	-	-	-	-	-	-
Sodium malonate	-	-	-	-	-	-	-
Lysine decarboxylase	-	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-
Urea hydrolysis	-	-	-	-	-	-	-
Tryptophane deaminase	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-
Acetoin	+	-	-	-	-	-	-
Gelatin hydrolysis	+	+	+	+	+	+	+
Acid from glucose	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+
Acid from maltose	-	-	-	-	-	-	-
Acid from sucrose	-	-	-	-	-	-	-
Acid from manitol	-	-	-	-	-	-	-
Acid from arabinose	+	+	+	+	+	+	+
Acid from rhamnose	-	-	-	-	-	-	-
Acid from sorbitol	-	-	-	-	-	-	-
Acid from inositol	-	-	-	-	-	-	-
Starch hydrolysis	+	-	-	-	-	-	-
Pigment production	-	-	-	-	-	-	-

+ = Positive reaction; - = Negative reaction

Apart from RCs-1, they could bear upto 2.5M NaCl in the medium (Fig.1). These strains showed their best growth in the salt medium between 0.5-1.5M NaCl, hence belong to moderately halophilic bacteria. As regards the resistance profile against other

salts, pertaining to saline habitat, Cs-1 could not tolerate even low level of $NaHCO_3$ (0.1M) in the medium. Whereas other strains showed variable behaviour to this salt. Cs-2, Cs-3 and RCs-1 could grow at low level of this salt in the absence of NaCl but in the presence of NaCl these strains showed quite sensitive behaviour (Fig.2a, 2b). Cs-4 could grow, both in the presence and absence of NaCl, relatively in wide range of $NaHCO_3$. Whereas RCs-2 and RCs-3 showed better growth response in this salt in the presence of NaCl (Fig.2b). Cs-2, Cs-3, Cs-4, RCs-1, RCs-2 and RCs-3 showed comparatively better growth response in the presence of $NaHCO_3$ in NaCl free media reflecting the halophilic nature of these isolates. These strains also showed relatively better growth response (Fig.2a, 2b) in the presence of Na_2SO_4. All strains could tolerate Na_2SO_4 in NaCl supplemented as well as NaCl free medium. Generally these strains gave comparatively better growth yield at low concentrations of Na_2SO_4 in the presence of NaCl, but at higher concentrations in the absence of 1M NaCl (Fig.2a, 2b). They could also tolerate $MgSO_4$, KNO_3 and KCl from 0.1-2.0M both in the presence and absence of 1M NaCl. In general growth of these strains was better in these salt media when supplemented with NaCl. But some strains such as Cs-2, RCs-1, RCs-2 (in case of KCl), Cs-1, Cs-2, RCs-1 (in case of KNO_3) and Cs-2, RCs-2, RCs-3 (in case of $MgSO_4$) yield better growth at some concentrations in the absence of NaCl.

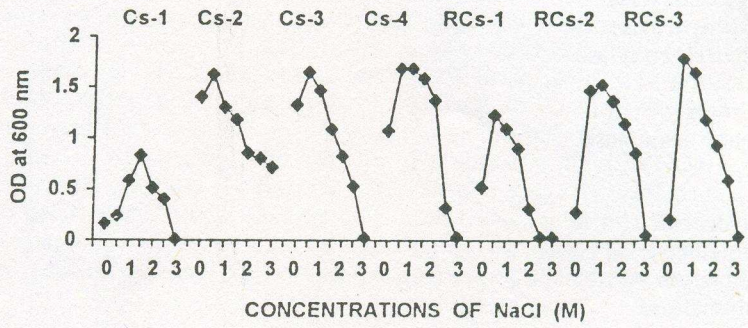

Fig. 1: **Growth responses of moderately halophilic bacterial strains, obtained from *Convolvulus arvensis*, in the presence of different concentrations of NaCl after 24 hours of growth at 37°C.**

Growth pattern of these strains revealed that they require 2 to 6 hour of lag period and stationary phase could be achieved between 18-24 hours of incubation at 37°C (Fig.3). These isolates could grow under wide range of temperatures (25°-42°C) (Fig.4) with different optima. The optimum temperature for the growth of Cs-1 was 42°C, whereas Cs-2 and Cs-3 gave best growth at 37°C. Not much difference in the bacterial growth yield of Cs-4, RCs-1, RCs-2 and RCs-3 was observed over the range of 28°-37°C, hence optimal temperature for these strains ranged from 28°-37°C. Excluding Cs-2 and Cs-3, all strains could grow over wide pH range both in the presence and absence

Table 2: **Resistance of isolates against 100 μg ml^{-1} salts of heavy metal in the presence and absence of 1M NaCl.**

Metallic salts/NaCl conc.		Strains						
		Cs-1	Cs-2	Cs-3	Cs-4	RCs-1	RCs-2	RCs-3
Nickle sulphate	0M	++	++	++	++	++	++	++
	1M	++	++	++	++	++	++	++
Stannous chloride	0M	++	++	w+	++	-	-	-
	1M	++	++	++	++	+	+	-
Barium chloride	0M	-	++	++	-	-	++	++
	1M	++	++	++	++	++	++	++
Lead nitrate	0M	+	++	++	++	++	++	++
	1M	++	++	++	++	++	++	++
Chromium oxide	0M	+	+	++	+	++	-	++
	1M	-	+	++	+	++	-	++
Manganese sulphate	0M	++	++	++	++	++	-	++
	1M	++	++	++	++	++	++	++
Zinc sulphate	0M	-	++	++	-	++	-	-
	1M	-	-	++	-	++	++	++
Ferrous sulphate	0M	++	++	++	w+	++	++	++
	1M	++	++	++	++	++	++	++
Sodium molybdate	0M	++	++	++	++	++	++	++
	1M	++	++	++	++	++	++	++
Cobalt chloride	0M	++	++	++	++	++	++	++
	1M	++	++	++	++	++	++	++
Copper sulphate	0M	++	++	++	-	++	++	++
	1M	++	++	++	++	++	++	++

of 1M NaCl. Cs-2 and Cs-3 had a bit narrow pH range in the NaCl supplemented medium. In the presence of 1M NaCl optimal pH for all strains was 8 (except for Cs-2 where it was 9), whereas in the absence of NaCl it was 5 (Cs-1), 6 (RCs-3), 7 (Cs-3), 8 (Cs-4, RCs-2), 9 (Cs-2) and 10 (RCs-1) for different strains (Fig.5).

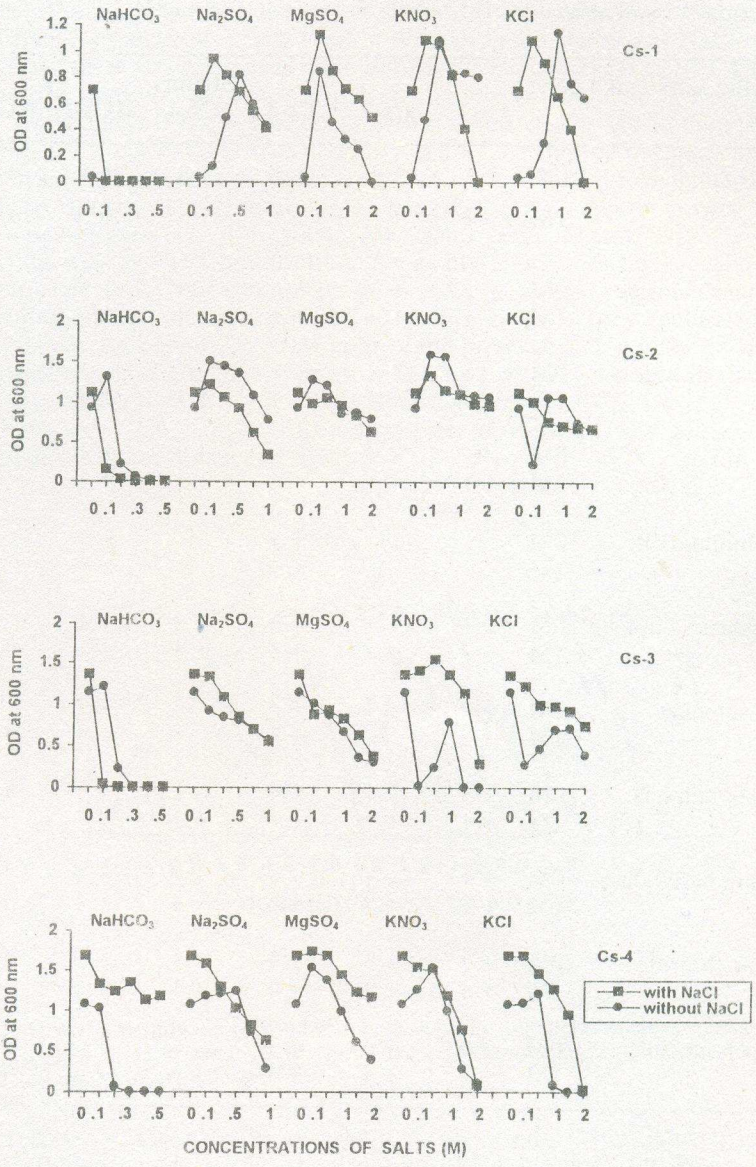

Fig. 2a: Effect of $NaHCO_3$, Na_2SO_4, $MgSO_4$, KNO_3 and KCl (both in the presence and absence of 1M NaCl) on the growth of moderately halophilic bacterial strains obtained from *Convolvulus arvensis*, at 37°C after 24 hours incubation.

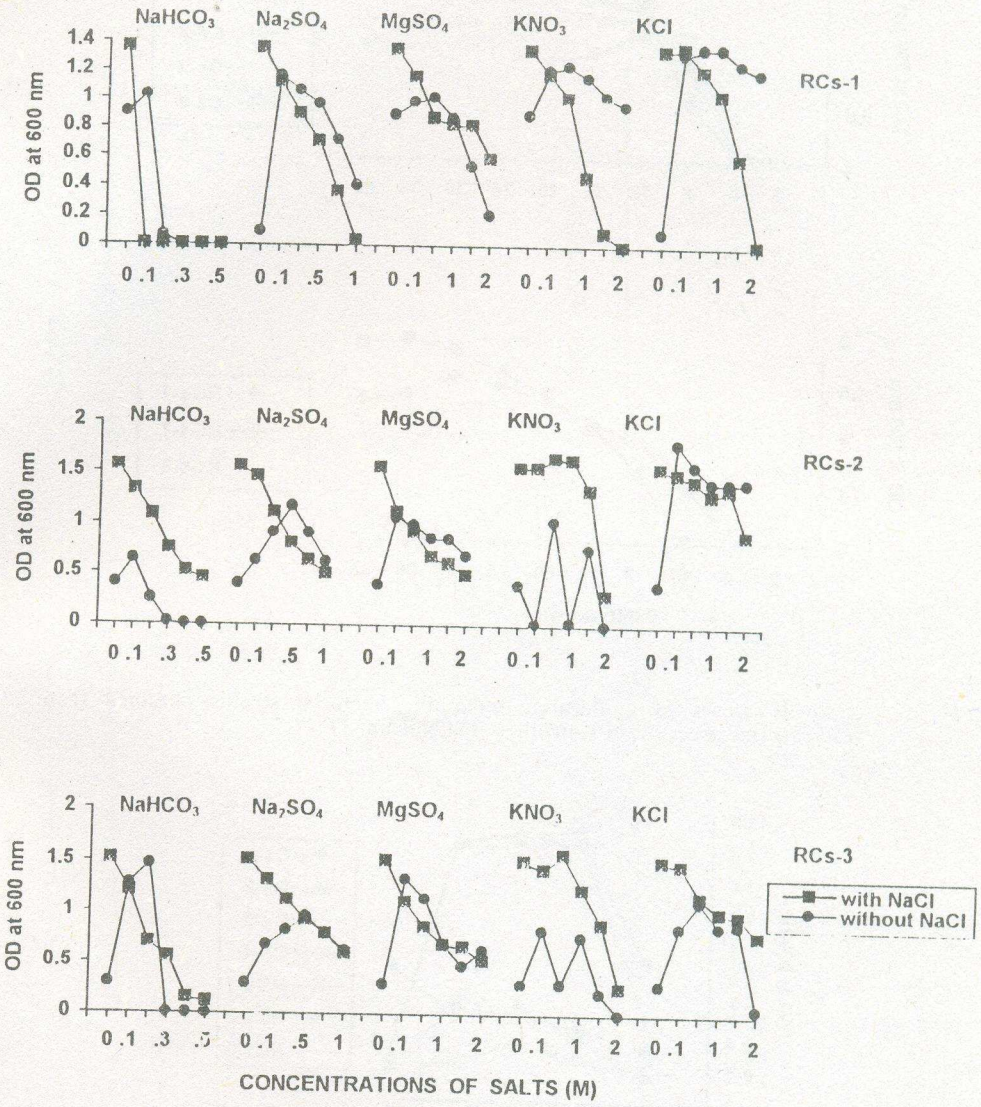

Fig. 2b: Effect of $NaHCO_3$, Na_2SO_4, $MgSO_4$, KNO_3 and KCl (both in the presence and absence of 1M NaCl) on the growth of moderately halophilic bacterial strains obtained from *Convolvulus arvensis*, at 37°C after 24 hours incubation.

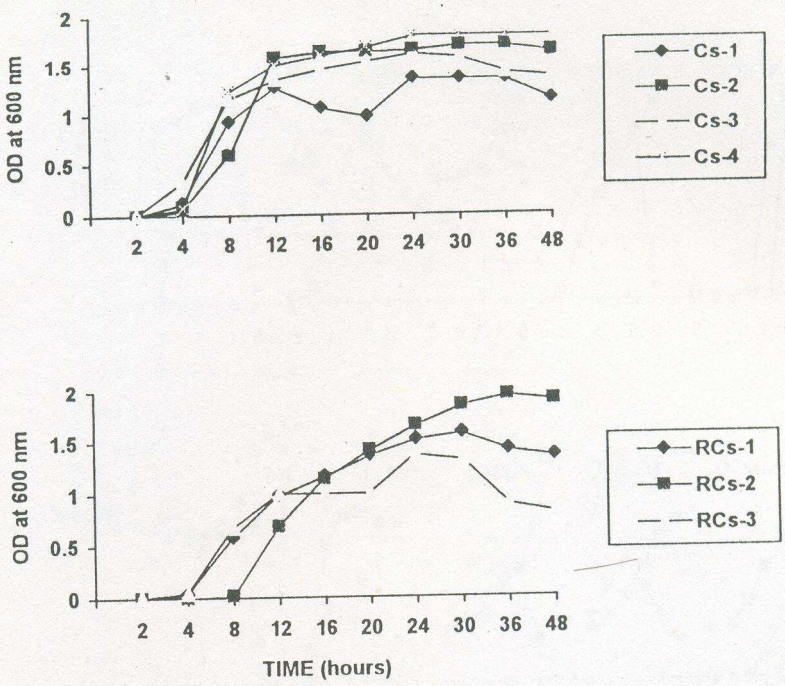

Fig. 3: Growth curves of moderately halophilic bacterial strains obtained from *Convolvulus arvensis*, in L-broth + 1M NaCl at 37°C

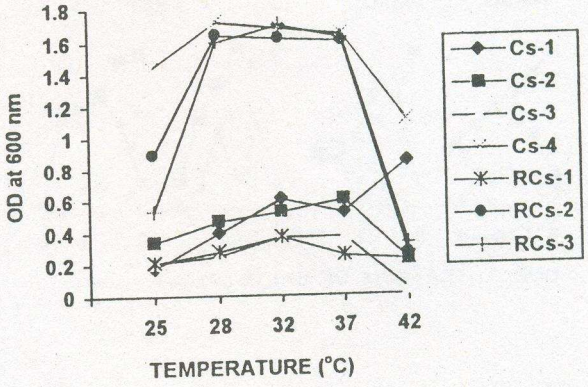

Fig. 4: Effect of different temperatures on the growth of moderately halophilic bacterial strains obtained from *Convolvulus arvensis*.

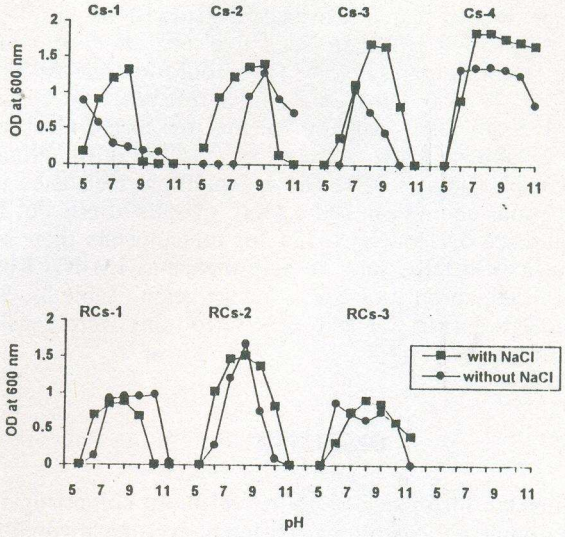

Fig. 5: Effect of pH (in simple as well as 1M NaCl supplemented L-broth) on the growth of moderately halophilic bacterial strains, obtained from *Convolvulus arvensis*, at 37°C after 24 hours of growth.

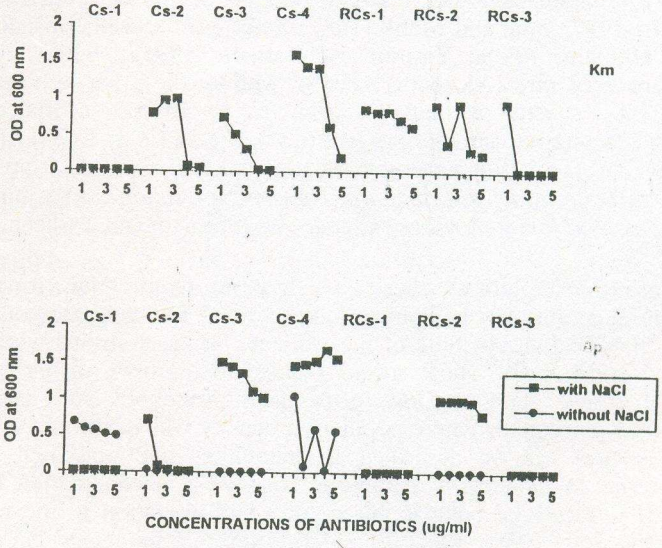

Fig. 6: Resistance of moderately halophilic bacterial strains, obtained from *Convolvulus arvensis*, against different concentrations of kanamycin at 37°C after 24 hours of growth.

Excluding Cs-1 all isolates shared resistance to Km (10-50 μg ml^{-1}) in the presence of 1M NaCl, but were sensitive to Km in NaCl free medium. For ampicillin resistance behaviour of different strains varied (Fig.6). Cs-1 could tolerate Ap (100-500 μg ml^{-1}) in the absence of salt, Cs-2, Cs-3 and RCs-2 confer resistance to Ap in the presence of 1M NaCl, while Cs-4 could resist Ap both in the presence and absence of salt, but resistance was better exhibited in the presence of NaCl (Fig.6). None of them could resist to Tc, Sm and Cm. These isolates showed multiple resistance to salts of heavy metals (Table 2). All strains could bear 100 μg ml^{-1} of salts of Ni, Pb, Fe, Mo, Co both in the presence and absence of 1M NaCl, but for other metals their behaviour varied, they conferred resistance to metallic salts in the presence of 1M NaCl but were sensitive to some metallic salts in the absence of NaCl or vice versa (Table 2). Some strains *i.e.*, Cs-1, Cs-4 (for Zn), RCs-2 (for Cr) and RCs-3 (for Sn) were sensitive both in the presence and absence of NaCl.

DISCUSSION

Bacteria inhabiting the histoplane of roots and those colonizing in the rhizoplane encounter diverse environmental conditions. Different ecological conditions might affect bacterial population, their growth behaviour, biochemical and genetic attributes. Hence seven bacterial isolates were obtained from the histoplane (Cs-1, Cs-2, Cs-3, Cs-4) and rhizoplane (RCs-1, RCs-2, RCs-3), of *Convolvulus arvensis* from saline locality and were studied. Isolates belonging to two sources were slightly different from one another. All of them were gram-negative rods (except Cs-2 from histoplane). Many workers have reported that root associated salt tolerant bacteria are usually gram-negative rods (Reinhold *et al.*, 1987; Bilal and Malik, 1987; Zafer *et al.*, 1988; Hasnain *et al.*, 1989; Sherwani and Hasnain, 1990a; Yasmin and Hasnain, 1993a), while cocci and gram-positive rods are very rare (Alexender, 1985). Mainly these bacteria belonged to two main groups *i.e.*, strictly aerobic bacteria (Cs-4, RCs-2, affiliated with genus *Halomonas*) and facultative aerobic bacteria (Cs-1, Cs-2, Cs-3, RCs-1, RCs-3). Gram-negative aerobic and facultative anaerobic bacteria are also reported from the rhizoplane and histoplane of *Desmostachya bipinnata, Heleochloa schnoites* (Yasmin and Hasnain, 1993a) and *Leptochloa fusca, Atriplex rhogodoides* (Hasnain and Taskeen, 1989).

In juxtapose to halotolerant bacteria, which do not require NaCl for growth but can grow under saline conditions, halophiles require NaCl for their growth. To determine the halophilic/ halotolerant attribute of the isolates, bacterial strains were grown in the presence of 0.5-3.0M NaCl. These strains yielded best growth in the range 0.5-1.5M NaCl. Bacterial growth yield was low in the medium without NaCl and was declined above 1.0M NaCl (except for where population density was maximum at 1.5M). Hence the bacterial isolates can be described as halophiles and classified as moderately halophilic bacteria. In moderately halophilic eubacteria haloadaptation is required via accumulation of intracellular compataible solutes and alteration in the membrane lipid composition (Csonka, 1989; Russell, 1989). Aerobic moderate halophiles usually cumulate amino acids (*e.g.*, glutamate and proline) and related compounds such as glycine betaine as their major compatible solutes, when grown on complex media (Imhoff, 1986; Wohlfarth *et al.*, 1990; Severin *et al.*, 1992). On contrary, when cultures are grown on defined media the major compataible solutes are commonly the

tetra-hydropyrimidines, ectoine and hydroxy ectoine, which are made by *de novo* biosynthesis process (Bernard *et al.*, 1993). Talibert *et al.* (1994) reported that ectoine is not involved in reiteration of osmotic balance of cells, rather its role is in triggering the synthesis of endogenous osmolytes. The most commonly observed salinity-dependent change in lipid composition of this group of eubacteria is an increase in the proportion of anionic phospholipids (Russell, 1992; Kuchta and Russell, 1994). At higher concentration of NaCl (2.0-3.0M NaCl), growth rate of the halophilic bacteria decreased and cell yield was reduced to 90%. Drastic decrease in cell population at higher osmolarity is also reported by Barnard *et al.* (1993). They attributed abrupt reduction in cell density to decreased ectoine content in the cells. *de novo* ectoine synthesis depends on the external osmolarity and from 1.0-2.0M NaCl the ectoine content decreased, which caused decrease in self-osmoprotection of bacteria.

In saline environment salts other than NaCl are also present and different cations and anions such as Ca^{++}, Mg^{++}, K^{+}, SO_4^{-}, HCO_3^{-}, NO_3^{-} are also important. Hence bacteria were also exposed to the medium containing $NaHCO_3$, Na_2SO_4, $MgSO_4$, KNO_3 and KCl. Their individual effects as well as combined effects with 1M NaCl revealed that $NaHCO_3^{-}$ was the most toxic salt and potassium salts were least toxic (Fig.2a, 2b), but the resistance pattern of individual isolates differed. Some isolates were more sensitive to single salt as compared to when that salt was present with 1M NaCl, whereas others showed the opposite behaviour. This may be due to the involvement of more than one osmoregulatory mechanisms (Yasmin and Hasnain, 1993b). Requirement of Na^{+} for halophilic microorganisms is indispensable, but it may be replaced with Mg^{++} or Ca^{++}, although Na^{+} with either Mg^{++} and Ca^{++} is necessary for the most rapid growth (Sheikh, 1993), but Bernard *et al.* (1993) observed that replacement of NaCl with other ionic salts did not change the growth pattern of bacteria. Among the sodium salts Cl^{-} was preferred by all strains and with HCO_3^{-} growth was relatively inhibited. Na^{+} and Cl^{-} might be helping in keeping the osmotic balance of the cell as compared with other cations and anions. We have also observed similar responses of Pseudomonad strains to different salts (Yasmin and Hasnain, 1993b). In mesophilic halophilic bacteria Na^{+} is needed for a number of complex and crucial cellular function such as permease systems involved in uptake of exogenous substrates (Fein and Macleod, 1975), preservation of solutes within the cells (Wong *et al.*, 1969) and the perpetuation of cell wall integrity (Forsberg *et al.*, 1970). In mesophilic halophic Na^{+} has also been found to stimulate the activities of amylase (Onishi, 1972) and cytochrome oxidase (Kushwaha *et al.*, 1977). Whether Na^{+} have any of these functions in the strains described here has to be investigated. All strains could grow in the presence of high level of salts of K and Mg in the absence of NaCl. Hence the requirement for Na^{+} could be satisfied by K^{+} or Mg^{++}, indicating that requirement for Na^{+} is an osmotic function. Shieh (1993) reported a halophilic thermophilic bacterium in which requirement for Na^{+} could be replaced by Mg^{++} or Ca^{++}.

All the isolates reported here were mesophilic in nature with different optima (Fig.4). Generally halophilic and halo-tolerant bacteria are mesophilic in nature (Sheih, 1993; Yasmin and Hasnain, 1993a, b). They have a wide pH-range and might be placed in alkaliphilic bacteria, as they could grow well at pH 10/11, with optimum pH 8 or 9 in the presence of NaCl (Fig.5). Growth of most isolates was better in the pH medium supplemented with NaCl as compared to the medium when NaCl was absent. An

increase in Na^+ concentration allowed the cells to grow at alkaline pHout (Nakamura *et al.*, 1992). When pH of medium was checked at the end of experiment, a rise in the pH of the medium initially adjusted to pH 6-8 and a decrease in the pH of highly alkaline media was recorded. Previously moderately halophilic gram-negative rods which prefer alkaline pH have been described (Del-Moral *et al.*, 1988; Yasmin and Hasnain, 1993a, b). Ni *et al.* (1994) described a moderately halophilic and alkaliphilic methanogen, grew over a wide pH range from 6.8-9.0. Cells suspended in medium with a pH above 8.2 reversed their transmembrane pH gradient by making their cytosol more acidic then the medium.

Antibiotic profile of the mesophilic halophilic strains revealed that they confer resistance to ampicillin and kanamycin, but resistance pattern for these antibiotics was different for different strains (Fig.6). They also showed multiple resistances to a number of heavy metal salts (Table 2). Some strains could resist some metals only in the presence of 1M NaCl or vice versa. Cd toxicity was reported to be decreased (Onishi *et al.*, 1984), while Zn toxicity increased (Babich and Stozky, 1978) with the increase in NaCl level, but our observation for Zn was of two types, Cs-2 (from histolane) was resistant to Zn in the absence of 1M NaCl, while RCs-2, RCs-3 (both from rhizoplane) confer resistance to Zn in the presence of 1M NaCl. Hence bacterial isolates from different sources behaved differently. Many workers reported moderately halophilic/ salt tolerant bacteria having multiple resistances to antibiotics and heavy metals (Nieto *et al.*, 1989; Del-Moral *et al.*, 1988; Hasnain and Yasmin, 1991; Yasmin and Hasnain, 1993a, b). In many cases these resistances were plasmid encoded, in present case isolates harbor plasmid, whether these resistances are plasmid borne is still to be determined.

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A NOTE ON THE FISH FAUNA OF CHASHMA LAKE, PAKISTAN, WITH THE RECORD OF *RACOMA LABIATA* McCLELLAND (PISCES : CYPRINIDAE)

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Abstract: Some fishes were sent by the junior author from Chashma Lake, Mianwali District. In addition to the fishes already known from the Chashma Lake, there was a large specimen of *Recoma labiata* McClelland belonging to the subfamily Schizothoracinae, family Cyprinidae of the teleostean fishes. This specimen is 445 mm in total length and weighs 860 grams. This species has been recorded from Kalabagh in the sub-mountainous Indus Region about 80 km upstream. It is normally found in the hill streams from 300 m upto about 4500 m above sea level. In the Punjab Plain, it was previously reported from the upper Jhelum canal near Chechian (Gujrat District) and the Trimmu Lake of river Chenab (Jhang District). The present specimen is the third specimen of *R. labiata* collected from the Punjab Plain. It is the new record for species, genus and subfamily from the Chashma Lake and is being recorded from the river Indus downstream of Jinnah Barrage for the first time.

INTRODUCTION

The Chashma Lake is formed as a result of the storage of water of rivers Indus and Kurram by the Chashma Barrage (32.28 N, 71.20 E) at Chashma in Mianwali District. The maximum water area of the Barrage is about 36000 hecters. Its gross storage capacity is 0.497 (MAF). Its bed elevation is 188 m from S.P.D.

The fish fauna of the lake was studied by Naik (1985), Mirza and Abubakr (1988) and Hussain and Mirza (1996). The total number of species recorded by these authors was 57 belonging to 17 families and 8 orders. No High Asian species was reported.

During December, 1997, the following three species of fish were presented by contractor to the junior author:

Tor putitora macrolepis (Heckel, 1838).
Labeo dero diplostomus (Heckel, 1838).
Recoma labiata McClelland, 1842.

Among these, *R. labiata* belongs to the snow-carp subfamily Schizothoracinae (Family Cyprinidae) and hence is a new record for the species, genus and subfamily from the Chashma Lake. The other two species are already known from this lake.

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RESULTS AND DISCUSSION

The general features of the specimen of *R. labiata* fall within the range of variations of this species as described by Mirza and Awan (1979). However, the lower lip of the present specimen is poorly developed. This is probably due to the physical conditions of the lake as there is no need for a highly developed lower lip for the attachment to the substratum. The lower lip is clearly trilobed, which is a characteristic of this species.

The size of the fish is large: about 445 mm in total length and 860 grams in weight. It is a bottom feeder. The gut was long, highly coiled and full of algal matter. It was 114 cm which is 2.56 times of the total length of the fish.

Racoma labiata is normally found in the hilly areas upto about 4500 m above sea level in Tibet (Zhang *et al.*, 1995). Its presence in the Chashma Lake is interesting. It has been previously recorded from the river Indus near Kalabagh about 214 m in elevation, and more than 80 km upstream from Chashma by Mirza and Jan (1993). It is widely distributed in the Northern Areas and Azad Kashmir. In NWFP, it has been recorded from the Chitral, Swat, Kabul, Kurram rivers and the Indus itself. It has also been reported from northeastern Baluchistan in the headwaters of the rivers Zhob, Bolan and Nari. In the Punjab, it has been collected from the Indus near Attock Khurd, from the river Haro near its confluence with the Indus at Gharyala (Attock District), from the upper Jhelum canal near Chechian (Gujrat District), and from the river Chenab near Trimmu (District Jhang) which is about 150 m above sea level. This is the lowest elevation for any member of Schizothoracinae. But this is not the normal range of the species. The fishes collected from Trimmu and Chashma lakes are, most probably, stray specimens washed down during the floods. The lower lip of the specimen collected from Trimmu Lake was also poorly developed quite similar to that of the present specimen. Due to the poorly developed lower lip, these fishes could not resist the fast water current in upper parts of the rivers and thus were washed down.

The lower lip of the mahseer specimen from the same locality is also poorly developed, just like that of *R. labiata* mentioned above. The similarity of the trilobed labial fold in *R. labiata* and *T. putitora* is remarkable and is a good example of convergent evolution. The lips in both species are highly variable depending upon the habitat, force of the water current and nature of substratum.

The Trimmu specimen was collected on 23rd March, 1980 about one and a half km upstream from Trimmu by a fisherman, Mr. Allahyar. It was 36 cm in total length (Hassan, 1982). It has not been collected since then from Trimmu (Afzal *et al.*, 1995). *R. labiata* has been recorded from the rivers Kabul and Helmand in Afghanistan also (Coad, 1981).

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EFFECT OF ACTELLIC ON OVIPOSITION IN *CALLOSOBRUCHUS CHINENSIS* (BRUCHIDAE : COLEOPTERA)

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Abstract: The toxic effect of Acetellic was tested against adults of *Callosobruchus chinensis*. LD_{50} for Acetellic was found to be 0.5559 ppm while 0.9 ppm caused 100% mortality after 48 hours. No treatment resulted in no mortality. There was a significant reduction in number of eggs between the treated and the control insects. The number of the eggs deposited decreased with the increase in the concentration of the insecticide.

INTRODUCTION

A great loss to world production of cereal grains, pulses and oil seeds is caused by insects, rodents and rot. If the crops could be protected from their ravages then it would not only be possible to store them for long periods without deterioration but also to meet the supply of most food needs of famine areas in the world (Cotton, 1963).

There are various types of contamination of stored grains and their sources. Many stored grain destructive pests reside inside the grain or pulses in their larval or grub stage. Inside the grain they devour the endosperm and leave their excreta inside them. These types of infested kernels or pulses are unfit for the human usage. Keeping this in view, the prevention of insect infestation of grain is of great importance, not only for the farmers but also for the users of these products (Cotton, 1963).

The most popular method to control these pests is the use of chemicals which ranges from chlorinated hydrocarbons, organophosphates and carbamates to pyrethroids. All insecticides have been used one after the other. Organophosphorus compounds contain one or more phosphorous atom chemically bound either directly to the carbon atoms of organic radical or indirectly through nitrogen, oxygen or sulfer (Gunther and Jeppson, 1960). They show a fast effect on the insects and their rate of accumulation in animals is comparatively less than chlorinated insecticides (Sun, 1968). The organophosphates are classified as stomach and systemic poisons, some as contact poisons, while others as fumigants (Hassall, 1983).

In the present study the toxic effect of Acetellic was assessed against *C. chinensis*.

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MATERIALS AND METHODS

Collection and maintenance of insects

To obtain the adults of *Callosobruchus chinensis* old infested grains were collected from local sources quality stores and shops selling provisions). These insects were separated and kept in sterilized glass jars (3" x 6") containing gram (*Cicer arietinum*), whole grain moong (*Vigna radiata*) and masoor (*Lence esculentum*) pulses at 28±20 and 65-70% relative humidity. The grains having unhatched eggs were daily separated and kept in fresh sterilized rearing jars. The newly emerged adults from these eggs were also transferred daily to fresh rearing bottles of similar dimension containing different pulses. The adults of the same age raised from this stock in the laboratory were used for experimental purposes.

Insecticide used and its treatment

In the present study Actellic which is an organophosphate and an ICI product was selected for use. It is marketed as liquid grain protectant and was obtained in commercial formulation of 50 EC from Grain Management Cell, Punjab Food Department.

Filter paper method

The bases of the Petri dishes (3" diameter) were fitted with filter paper and marked with a lead pencil for different doses. The dilutions (0.5 ml) were applied with the help of a pipette, starting from the periphery to the centre in a continuous circle for even distribution. The filter paper was placed on three common pins and was left to dry. Afterwards these were placed in Petri dishes to which 5 grams of grains were also added so that the eggs could be laid on them. Five pairs of newly emerged beetles were kept in each dish. Three replicates for each dose were set up. Mortality was noted after 48 hours and LD_{50} was determined by probit analysis (Finney, 1952; Busvine, 1971).

RESULTS

Effect of Actellic on mortality

The objective of the determination of LD_{50} was to measure the effectiveness of the insecticides against the adults of *C. chinensis* under laboratory conditions. The LD_{50} of Actellic in ppm determined by probit analysis was calculated to be 0.5559 ppm after 48 hours.

At 0.4 ppm, the mortality was 3.3%, 53.3% and 100% respectively after 2, 4 and 9 days, while 0.5 ppm caused 18.3%, 66.6% and 100% mortality after 2, 4 and 7 days, respectively. Treatment with 0.6 ppm resulted in 80% mortality after 2 days and 100% after 3 days. With 0.8 ppm, mortality was 86.6% after 2 days and 100% after 3 days, while with 0.9 ppm 100% mortality occurred within 2 days (Table I).

Table I: **Percent mortality at differennt doses of Actellic against adults of *C. chinensis*.**

Dose (ppm)	Actellic treatment (days)								
	1	2	3	4	5	6	7	8	9
0.4	0	3.3	25	53.3	58.3	80	85	95	100
0.5	10	18.3	45	66.6	78.3	95	100		
0.6	15	80	100						
0.8	36.6	86.6	100						
0.9	38.3	100							

n = 100 in all cases.

Effect of Actellic on oviposition

The rate of egg laying was 25-30.6 eggs per female in the untreated controls. The acetone treated controls laid 26-30 eggs per female. The treatment with Actellic inhibited oviposition and the number of the eggs laid per female decreased with the increase in concentration of the insecticide.

The rate of egg-laying was 23.16, 20.9, 19, 16.8 and 14 eggs per female at 0.4, 0.5, 0.6, 0.8 and 0.9 ppm dose respectively. The rate of egg laying was reduced from 23 egg per female to 14 eggs per female, as the dose was increased from 0.4 ppm to 0.9 ppm. The total number of egg laid by five females is given in Table II.

Table II: **Oviposition of *C. chinensis* at different doses of Actellic.**

	0.4 ppm	0.5 ppm	0.6 ppm	0.8 ppm	0.9 ppm
Untreated control	150.33 ±20.60	128.00 ±3.348	134.00 ±5.74	125.30 ±10.76	139.66 ±3.604
Treated control	146.66 ±15.80	150.6 ±16.99	131.00 ±18.08	131.00 ±21.478	139.00 ±16.287
Treated	115.8 ±14.67	104.5 ±3.06	98.00 ±25.25	84.1 ±1.964	74.8 ±5.948

L.S.D. = 26.1215

DISCUSSION

As insects are the major cause of damage and loss to the stored products various forms of preventive measures are designed to keep the pest population below the economic level. All kinds of gram, pulses, etc., are devoured and rendered useless by these hungry weevils (Metcalf and Flint, 1967). Food grains are commonly protected by contact insecticides or fumigation. A simple method of killing the weevils and preventing the destruction caused by them is to treat them chemically with organophosphates or pyrethroids. The effectiveness of different insecticides varies with the pest species and their developmental stages (Yana, 1966; Champ *et al.*, 1969; Samson and Parker, 1989). Hence pesticides have specific mode of action and toxicity (Corbett, 1974; Matsumura, 1975).

In the present study Actellic which is an organophosphate and a fast acting insecticide with both a contact and a fumigant action was used (Worthington and Walker, 1987). During the present work exposure of the adults of *C. chinensis* to different doses of Actellic revealed the LD_{50} as 0.55 ppm, after 48 hours. By increasing the dose mortality also increased and at 0.9 ppm 100% mortality occurred after only 48 hours. This type of fast effect has been shown by other organophosphate compounds and reported by various workers like Worthington and Walker (1987).

Various workers have revealed that after topical application *in vivo*, the concentration of the insecticides at the primary site of action may be influenced by a large number of factors including penetration, distribution, selective accumulation in insect tissues, biotransformation and elimination (O'Brien, 1967; Narasashi, 1971; Brooks, 1976). During the present work the mode of application of the insecticide had a 2 fold action *i.e.*, penetration through the cuticle and via mouth, so it worked both as a contact and a stomach poison.

However, todate there is no generally accepted opinion on how the insecticides arrive at the target site. The conflicting view points are that on one hand, penetration through the cuticle and subsequent transport by the haemolymph occurs and on the other hand, that there is lateral transport in the cuticular wax layer and tracheal lining following by penetration through the glial cells into the central nervous system (Geroltt, 1970). All this finally results into the mortality and subsequent decrease in the population abundance of the pest under consideration.

Orr and Downer (1982) have reported that primary site of action of most of the insecticides is the nervous system, however, in addition, many physiological and biochemical effects of insecticides have been described in tissues outside the nervous system. These extraneuronal effects have been attributed to insecticide induced release of hormones or neurohormones from neuroendocrine tissues and consequent perturbation of normal physiological functions. When treated with Actellic ovipositional activity was found to be disturbed. There appeared a significant reduction in ovipositional activity as the dose was subsequently increased from 0.4 ppm to 0.9 ppm.

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ON BIOLOGY OF HOUBARA BUSTARD (*CHLAMYDOTIS UNDULATA MACQUEENII*) IN BALOCHISTAN, PAKISTAN: BREEDING

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Abstract: Spotting of 7 nests and 10 broods in Balochistan (Pakistan) suggests a regular breeding of 50-100 hens over some 9,000 km^2. Breeding potentials are high for Rakshan (Bisemah and Nag), medium for northern Chagai and Kharan, and low for south-western and central Kharan. Average clutch (2.0 ± 0.2) and brood (1.9 ± 0.2) size suggests normal breeding potentials. Egg laying starts from late February and lasts till late April, while chicks appear between late February and mid May. Egg laying is restricted to stony deserts located in mild hilly undulates at >600 m above sea level. Shrub (6-11%) and herbal (5-19%) cover is low and all the species are green and sprouting. *Zygophyllum* sp., *Anabasis* sp., *Astragalus hyrcanus-ziziphus* sp. and *Cocculus* sp. communities have been identified. *Lepus capensis, Vulpus* sp., *Gazella bennetti, Felis* sp., *Canis lupus, C. aureus* (mammals), *Alaemon alaudipes, Pterocles* sp., *Cursorius cursor, Burhinus oedienemus, Ammoperdix griseogularis, Ammomanes deserti, Alauda arvensis, Galerida cristata* and a number of raptors (birds) are widely distributed. Insects (beetles, ants, grasshoppers) and reptiles (lizards, snakes) are common. Grazing stress and human disturbance are high. Male starts displaying at its own, selecting a raised surface. Nest represents a scrap on the ground and is placed on the ridge away from shrubs. Only hens incubate the eggs and protect chicks. Hens frequently leave nest on spotting approaching predator.

Key words: Grounds, potential, chronology, ecology, behaviour.

INTRODUCTION

Despite, speculations on presence of breeding of the Asian race of Houbara (*Chlamydotis undulata macqueenii*) in southern coastal Balochistan (Pakistan), *i.e.*, Makran (Ali and Ripley, 1983, original version appearing in 1969; Siddiqi, 1972) and collection of an egg from Maslakh (20 km in north-west of Quetta; Anonymous, 1972), it was not regarded as a regular activity for the area. Persistent reports of hunters and prominants from central highland Balochistan during our tour (Mian and Surahio, 1983) and responses to a questionnaire forced us to suggest that some 50-100 Houbara do lay eggs in these tracts (Mian, 1983, 1984). This was supported by subsequent collection of an egg from Yakmuch (western Chagai; Karim and Hasan, 1983) and a report suggesting that Dubai Wildlife Centre received 3 eggs from this area (Kermani, 1983; IG Forests, Pakistan; personal communication). The presence of regular breeding was later adequately confirmed by sighting of eggs, chicks and adults (Mian, 1985). This paper attempts at presenting some more supportive data

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on breeding of Houbara from different tracts of Balochistan, with some preliminary information on its breeding biology.

MATERIALS AND METHODS

Possible breeding grounds of Houbara in Balochistan (Yakmuch, Nokchah, Padag, Pul Chutao, Inam Bostan, Dak Plain, Jalwar, Sarwan, Urmagai, Bisemah, Rakshan, Patao, Maslakh) were visited between end March and mid April during 1984 and 1985 under the guidance of pre-contacted guides. Breeding was ascertained through direct observations on adult birds, eggs and chicks (aided by binoculars 8x40) and indirectly through spotting fresh foot tracks, faecal materials and/or feathers. All the guides were strictly instructed not to collect eggs or chicks. They were only required to communicate sighting of eggs or chicks to be confirmed by the author

A tour of potential breeding tracts (Chagai, Kharan, Bisemah, Rakshan) was conducted during March-May 1986, through field guides to collect some preliminary ecological data. Vegetative communities were established and named on visual dominance of the species (Braun-Blanquet, 1932). Physical habitat type, general topography, associated plant species, general shrub cover and phenology were recorded directly, while altitudes were adopted from Champion *et al.* (1965). Sighting records on general fauna were maintained and relative abundance of dominant species was judged through frequency of their encounter. Record on number of grazing livestock, distribution of cultivations and human settlements, and level of human activity was also maintained for different tracts.

Nests were located with the help of local guides. Number of eggs, general location and presence of markers around the nest was recorded. Activity of adult bird was judged through foot prints present around the nest. A search was maintained to record male display and courtship during different tours of Houbara wintering tracts.

RESULTS

Breeding tracts and potentials

Information available till date (Table 1; Fig. 1; partly reported in Main, 1985) suggests that regular egg laying was present in Inam Bostan plains (north-eastern Chagai), Gonnakoh (north-west Chagai), Dalloh (north-western Kharan), Bedi (north central Kharan), Bisemah (north-eastern Khuzdar), Rakshan and Nag (north Punjgur). There were some indications of breeding in Kissan (south-eastern Kharan), Maslakh (Pishin), Qamar-uddin Karaz (Zhob) and south-eastern coastal tracts of Gwadar, but these could not be adequately confirmed. Chicks and adults have been spotted over a wider-range. These were present in Padag, Pul Chutao, Nokchah (north central Chagai), Gurrak Rakh (north Kharan) and Ranawar (north west Kharan), apart from all the tracts where the eggs were spotted.

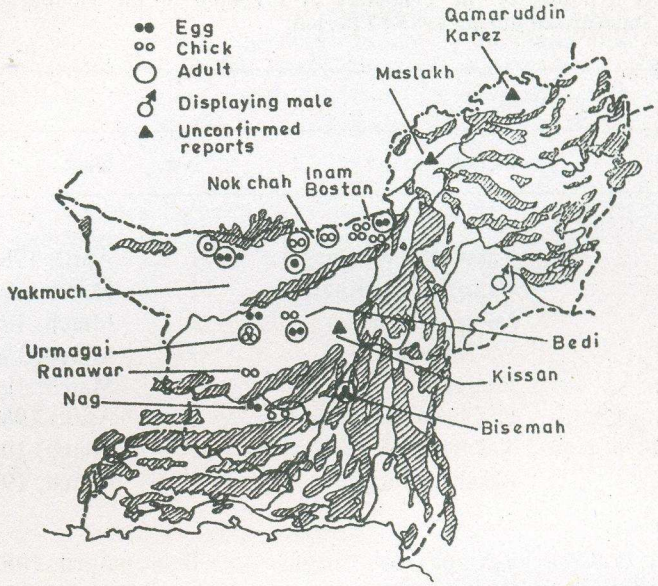

Fig. 1: Line sketch of Balochistan, indicating approximate areas, from where positive indications of breeding of Houbara have been collected during 1983-85 period.

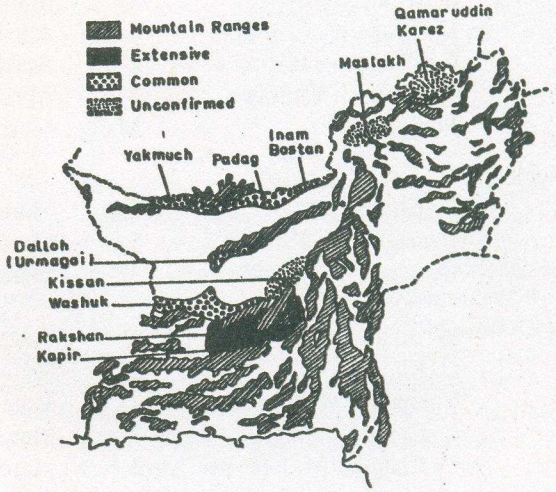

Fig. 2: Tentative distribution of the breeding population of Houbara in Balochistan.

Table 1: A synopsis of the evidences of breeding of the Houbara collected from Balochistan during 1983-87 period.

	Area		
Specific	General	No.	Date
Eggs			
Gonnakoh	Yakmuch, W. Chagai	1	April, 1983**
Dalloh	Urmagai, W. Kharan	2	March, 1983#
Bedi	Jawar, C. Kharan	2*	March, 1983#
Bisemah	W. Khuzdar	3*	March, 1985
Rakshan	Punjgur	2*	March, 1985
Inam Bostan	E. Chagai	2*	April, 1986
Gonnakoh	Yakmuch, W. Chagai	2*	March, 1986
Nag	Rakshan, Punjgur	2	March, 1986
Chicks			
Gonnakoh	Yakmuch, W. Chagai	1*	April, 1983#
Padag	C. Chagai	2	April, 1983#
Dalloh	Urmagai, W. Kharan	3*	March, 1983#
Jalwar	C. Kharan	2	April, 1983#
Padag	C. Chagai	1*	March, 1984#
Nokchah	C. Chagai	2*	March, 1984#
Gurrak Rakh	C. Kharan	2	March, 1984#
Inam Bostan	E. Chagai	2	April, 1984#
Inam Bostan	E. Chagai	2	April, 1985
Ranawar	Washuk, S. Kharan	2	April, 1985
Nag	Punjgur	Many	March-May, 1986,1987
Adult breeding flock			
Jalwar	C. Kharan	2@	March, 1984#
Dalloh	Urmagai, W. Kharan	5	March, 1984#
Dalloh	Urmagai, W. Kharan	3?	March, 1984#
Urmagai (western valleys)	W. Kharan	1	March, 1984#
Gurrak Rukh	C. Kharan	1?	March, 1984#
Gonnakoh	Yakmuch, W. Chagai	4?	March, 1984#
Pul Chatao	C. Chagai	2	March, 1984#
Nokchah	C. Chagai	2	March, 1984#

* eggs/chicks associated with hen. ** as reported by Karim and Hasan, 1983. # Mian, 1985. @ sufficient indications of 6 foraging birds. ? not confirmed. $ persisted throughout summers.

Data on 7 clutches, available from the area, suggest that majority (5) had 2 eggs, while 3 and 1 (incomplete clutch ?) eggs were present in one clutch each. The average clutch size is calculated to be 2.0±0.2 (mean±standard error of mean) eggs/hen. The information available on 10 broods, with 10-30 days old chicks, suggest an average brood size of 1.9±0.2, majority (7) having 2 chicks (3 chicks in 1, and 1 chick in 2 broods).

The eggs were recorded between early March and late April. The chicks were recorded between late March and end May or early June.

Ecological studies

Physical habitat

Table 2 presents a synopsis of the ecological characters of different potential breeding grounds of Houbara. The table suggests that the breeding was present in the areas located at 600-1,000 m above sea level.

Most of the tracts shared a common character of being stony deserts. The soil was sandy showing different degrees of stabilisation. A layer of loose stones formed a cover, varying from an almost complete cover of grey stones, in relatively stabilised tracts, to very scattered stones, in more sandy tracts. Egg laying was more frequent in stabilised tracts, while some egg laying was recorded in depressions, present between sand dunes, having stabilised silty soil with very widely dispersed stones. No egg laying was recorded in sand dunes. Chicks and adults were, however, present in sandy tracts.

All the valleys, having confirmed egg laying, were open, having very mild to mild hilly undulates, with slops gradually ascending towards one of the nearby mountain or hill. Water courses were dispersed between undulates.

Phytohabitat

Four vegetative communities were distributed in Houbara breeding tracts in Balochistan (Table 2). In Chagai and parts of Kharan, *Anabasis* sp. community was recognised. Dominant species was associated with *Calligonum comosum, Pennisetum dichotomum* and *Rhazya stricta* in different combinations. A few other species also gave scattered appearance. In southern tracts (eastern Kharan, Washuk and Rakshan), breeding was associated with *Zygophyllum* spp. community. Dominant species was generally associated with a number of species. The most close associates included *P. dichotomum* and *R. stricta*, though *Artemisia maritima* and *Withania coagulans* were also widely distributed in different stands. *Astragalus hyrcanus-Ziziphus* sp. community was present in Dalloh valley (Urmagai), which was composed of many species sharing the general vegetative cover, including *P. dichtomum* and *R. strict*. In Bisemah, *Coccuhus* sp. community was identified, where the dominant species was associated with 4 (*P. dichtomum, R. stricta, Alhagi camelorum, Peganum harmela*) species, each sharing a low general vegetative cover.

Table 2: A synopsis of the ecological data on different breeding tracts of the Houbara in Balochistan.

Area	Physical Features			Vegetative Features		
	Altitude (m)	Habitat Type	Topography	Community	Shrub Cover (%)	Potentials*
Rakshan	1,000	Stony deserts	Mild Hilly undulates	*Zygophyllum* sp.	9.78	A1
West Chagai	900	"	"	*Anabasis* sp.	5.65-9.29	A2
East Chagai	700	"	Middle undulates	"	8.55	A3
Washuk	700	"	Flat plains, mild undulates	*Zygophyllum* sp.	16.22	B1
North Kharan	600	"	Hilly valleys, mild undulates	*Astragalus hyrcanus-Ziziphus* sp.	9.77	B2
Bisemah Centre	1,000	"	Hilly undulates	*Cocculus* sp.	2.09	B3
Kharan	700	Sandy depressions	Hard flat plains with sandy undulates around	*Anabasis* sp.	11.29	C1
East Kharan Centre	700	Stony deserts	Hilly undulates	*Zygophyllum* sp.	9.44	C2
Chagai	800	"	"	"	6.55	C2

Breeding potentials: A high, B confirmed, C weak positive evidences; 1 high, 2 medium, 3 low.

Apart from the perennials, which provide the major part of the shrub or community cover, there was a rich growth of spring ephemerals which provide a good herbal cover (5-19%) during breeding season. All the shrubs were green bearing profusely sprouting foliage. Zygophyllum and Cocculus were on flowering or fruiting stage. The average shrub cover ranges between 2 and 16% though for the major parts it remains between 6 and 11%. The shrub cover was mainly present in depressions and water courses, while the undulates/ridges had very scattered and isolated distribution of bushes/shrubs, yielding a very low cover. The herbal cover was, however, higher on the ridges than in depressions. The shrub or herb cover did not follow a regular pattern with increasiing breeding potentials of the species.

Fauna

Mammal fauna was dominated by cape hare (*Lepus capensis*) and fox (*Vulpes* sp.), which were still present as good populations. Indian gazelle (*Gazella bennetti*) and cats (*Felis* sp.) gave rare appearance. There were indications of presence of wolf (*Canis lupus*) and jackal (*C. aureus*) but were very rare. Some desert rodents were also present as small populations.

Dominant species of birds included hoopoe lark (*Alaemon alaudipes*), sandgrouses (*Pterocles* sp.), cream coloured courser (*Cursorius cursor*), stone curlew (*Burhinus oedicnemus*), seesee partridge (*Ammoperdix griseogularis*) and larks (*Ammomanes deserti*, *Alauda arvensis*, *Galerida cristata*). All these species breed in the valleys and were widely distributed as scattered individuals, but as significant populations. Amongst raptors, eagles, vultures, kestrels and owls have been frequently recorded, hovering over the breeding tracts.

Lizards (including monitor ?) were frequently observed, while indirect evidences suggested the presence of snakes.

Amongst insects, beetles (family Tenebrionidae), large black ants (Formicidae) and *grasshoppers* (*order Orthoptera*) were frequent, while butterflies gave a rare appearance.

The majority of the nests examined under present study were located in the areas having high to very high population of the grazing livestock. There was a fairly high level of human activity associated with grazing and fallow land cultivation in all the tracts having significant egg laying. The nomadic camps were also scattered in all the valleys.

Behaviour

A male was observed performing courtship display for four consecutive days in Sibi during early February. The bird selected an embankment (about 1 m high) erected around the cultivated wheat field and displayed regularly in the morning and the evening. No female was seen near, though a flock of birds used to regularly visit the area for night foraging. Male used to go for a brief casual grazing in the cultivation at intervals.

A nest was observed in deeper parts of Bisemah valley. It represented slight depression on bare ground, produced by scraping away of the stones. It was placed on a ridge at a distance of 3-4 m from and in the centre of a triangular disposition of three small bushes of *Alhagi camelorum*. Nest indicated a successful hatch of both the eggs. Foot prints of the adult bird (female ?) indicated its frequent movement from/to the nest. All the other nests examined shared this general description with minor variation in number, position, size and species composition of the shrubs. A general impression created on seeing the location of the nests consistently suggested that one of the bushes around the nest was taller than the others. One of the guides reported that the incubating hen left the nest, when he was still at a considerable distance. All the presently collected reports suggested that the young chicks were either alone or associated with the hen

only. The chicks have never been seen with the male and/or with a male-female pair of adults.

DISCUSSION

Potential Areas

The presently collected information suggest that Houbara regularly breeds in highland valleys of Rakshan (extending from Bisemah to some 30 km west of Nag), northern Chagai (a belt of different widths extending from Inam Bostan to Gonnakoh), north-western Kharan (Urmagai and associated valleys). The breeding potentials are very high for Rakshan and high for Chagai and Kharan. Breeding could be confirmed for south-western (Washuk) and central Kharan. Sporadic breeding may occur in south-eastern Kharan (Kissan valley), northern highlands (Maslakh and Qamar uddin Karaz) and coastal tracts (Fig. 2). None of the previous reports has indicated the presence of breeding in major parts of the presently reported tracts (Ali and Ripley, 1983; Siddiqi, 1972; Anonymous, 1972). The areas exploited for chick rearing are more widely distributed and include the associated tracts, especially those falling along the spring migratory routes of the species/race.

Breeding Potentials

The present results suggest that majority of the females in the area lay 2 eggs, though some may lay 3 or 1, with an average clutch size of 2.0 ± 0.2 eggs/hen. This partly goes against Anonymous (1986) suggesting that the majority of the hens lay 3 eggs in the area. The average clutch size is close to that recorded for Central Asia (2.4: Alekseev, 1980) and slightly lower than that for Israel (2.8: Lavee, 1988). This suggests that this population is not remarkably different in its breeding potentials from the populations in the other established breeding grounds.

A larger brood size (1.9 ± 0.2 chicks/brood) can thought be an artefact attributable to the loss of entire brood or clutch (Lavee, 1988), yet it goes in conformity with the general observations of Anonymous (1986), suggesting that the females in the areas successfully hatch the major part of the clutch and chicks have a better survival rate.

Breeding Chronology

Sighting of eggs in early March and chicks towards end March suggests that nesting starts during mid February and egg laying towards end February. Fledging of chicks starts towards mid March. The presently available evidences suggest that egg laying extend up to April and chicks can appear till mid May. This breeding schedule seems partly supported by the previous reports suggesting presence of mature eggs in ovary of a female is hot during February from Sindh (Roberts, 1985) and records on breeding chronology from the Gulf States (eggs collected in March: Dickson, 1949; Platt, 1985). Breeding appears to initiate earlier in Balochistan than that suggested for breeding grounds of the Central Asia, where the species lays eggs in early to mid April

(Ponomareva, 1979) extending up to late April (Alekseev, 1980) and chicks appearing in May. Such an adjustment can be partly explained on the expected difference in temperature and associated biotic conditions.

Status of breeding population

Number of eggs and chicks recorded in different breeding tracts, suggest that an estimated population of some 50-100 hens regularly lay eggs in the areas under Balochistan. This confirms our early report (Mian, 1984). These estimates also stand supported by the estimates of P. Paillat (Leader, Pakistan Egg Collection Team, National Wildlife Research Centre, Taif, Saudi Arabia, 1987; personal communication) suggesting that some 60 hens laid eggs in Rakshan valley during 1987. Houbara breeds over some 9,000 km^2 (northern Chagai=4,000; Rakshan valley = 3,750; Kharan = 1,250) of Balochistan giving an estimated population density of a breeding hen/90-180 km^2 (around 0.01 hen/km^2). The available estimates on population of some most favourable breeding tracts of Houbara in Central Asia, suggest a density of about 1 hen/25 km^2 (Ponomareva, 1979, some 10,000 breeding hens distributed over 250,000 km^2 of ideal breeding grounds in Kyzylkum). This suggests that the areas under Balochistan do constitute significant breeding tracts of this bustard species holding reasonable density of the breeding population.

Breeding ecology

The present results allow us to develop following generalizations on possible control of ecological factors on breeding activities of Houbara:

Altitude

Egg laying occurs at altitudes above 600 m. Higher altitude ensures favourable temperature for the brooding adults and eggs. A temperature range of 17-20°C has been reported during spring (the breeding period of Houbara) for Canary Islands (Collar and Goriup, 1983; Collins, 1984). It appears that this race is adapted to breed at lower temperatures as compared with other two races of Houbara (Cramp and Simmons, 1980) or other species of bustards distributed in Indo-Pakistan subcontinent (Great Indian bustard: Ali and Rahmani, 1982; Manakadan, 1985). A comparatively mild temperature in coastal tracts (Gwadar) may also be within tolerable limits of this race.

Physical habitat

The egg laying is restricted to the stony deserts. Topographically these are slop plains having mild undulations. Grey stones of varying sizes are scattered over loose sandy or relatively stabilised soil. Though egg laying has been recorded in bare silty plains having few scattered stones, yet it is probably not a regular breeding habitat of the race/species. The conditions of physical habitat, suggested under present study, agree in the general description of the breeding grounds provided by Ponomareva (1979) and Alekseev (1980).

Vegetative cover

Present results suggest an absolute shrub cover of 6-11% plus a significant growth of the spring ephemerals, for the major parts of the tracts under breeding population. This indicates that the egg laying occurs in the tracts having adequate vegetative cover to afford protection and food to the incubating hen and newly hatched birds. Quantitative estimates on vegetative cover are not available from other parts of Houbara breeding range. However, reported distribution of egg laying in the areas with sufficient shrub cover and a good growth of ephemerals, and absence of nests in completely bare tracts, may provide some indirect support to our present findings (Ponomareva, 1979; Alekseev, 1980). These findings also get some support from Haddane (1985) suggesting that drought (and consequent limitation of vegetation) limits the egg laying potentials of this species in Morocco.

Presence of nest on the ridges, representing almost bare tracts with very scattered small shrubs (major part of the vegetation in depressions), may suggest that vegetative cover is not required for camouflaging of the brooding female. The species appears to depend upon its own camouflaging armoury. Such a position of the nest possibly saves the species from predation of eggs by the animals, which mainly concentrate in patches having higher vegetative cover.

Vegetative communities

The major part of the breeding occurs in *Anabasis* sp. and *Zygophyllum* sp. communities, though some scattered nests have been observed in *Astragalus hyrcanus-Ziziphus* sp. and *Cocculus* sp. communities. Dominant species in different communities are though associated with different other plant species, yet *Pennisetum dichotomum* and *Rhazya stricta* are present in all the stands. It is difficult to suggest a specific significance of these species in the breeding biology of Houbara and can only be regarded as a chance association.

Grazing stress

The presence of breeding in the areas under very high to high livestock grazing stress and in association with fallow land cultivations suggests that Houbara in the area has adopted to breed in fairly disturbed tracts. The breeding tracts, being located in the hilly valleys and at higher altitudes, having moderate temperature and higher availability of surface water, also attract nomads along with their livestock. These findings go against the previous reports suggesting that nesting occurs in very desolated areas and that grazing causes disturbance to the nesting Houbara (Ponomareva, 1979; Alekseev, 1980) or Great indian Bustard (Ali and Rahmani, 1982). Present observations are in conformity with similar reports from Israel (Lavee, 1985, 1988), where Houbara has adapted to breed in tracts having high disturbances from grazing livestock and associated human activity.

Fauna

The fauna is Palearctic in character as may be expected under the geographical location of such tracts. Houbara shares almost same set of animal species in its breeding grounds of the Central Asia (Ponomareva, 1979, 1985; Alekseev, 1980).

Our results suggest a considerable similarity between faunal associates of the wintering and nesting Houbara. Mammals are largely residents and hence a pronounced seasonal variation in their distribution is not expected. Major part of the avifauna is winter visitor. However, under favourable temperatures the dominant bird species persist in the area to lay eggs. A number of species of raptors also breed in the associated hills/mountains (Ticehurst, 1926-27) and hence there is a significant increase in their populations in these areas. The limited available data on the relative abundance suggest an increase in the population of all the insects and a higher activity of reptiles under suitable temperatures. The general insect population and reptile activity increase under optimal temperatures (Ali and Rahmani, 1982; Manakadan, 1985). Higher population of insects can increase the availability of food, while those of raptors and reptile can increase adult, chick and/or egg predation.

Breeding behaviour

Courtship display

Displaying males selects a prominent place, where other birds (females) are most likely to visit. The period of display coincides with the optimal foraging activity. This goes in conformity with many previous reports on Houbara and other birds. Such a behaviour increases the chances of finding the mate. However, absence of females near the displaying males suggests that the pair bond is weak, confirming the reports of Collins (1984) and Gal (1980). This also suggests that males start displaying at their own and are not conditioned to the presence of female/females.

Nest

The nesting site is very ingeniously selected. Laying eggs on bare ground, away from the shrubs and having growth of ephemerals, in the stones, having camouflaging background and avoiding depressions or dried water courses are all adaptive characters. This saves the adults, eggs and/or chicks from detection by predators as also suits the alert nature of adult Houbara, allowing an early detection of predator. The shrubs, water courses and depression prove vulnerable points for detection of the nest by carnivores and reptiles. The ephemerals around nests provide food within easy access of the incubating female and reduce the chances of leaving the eggs unattended.

Significance of a prominent bush near the nest can not be anticipated with the present level of information. However, it may work as a marker to guide the females to the nest. Such markers have special value in desert steppes, which are remarkably similar looking.

Incubation/progeny rearing

Present results support the previous suggestion that only females incubate the eggs (Lavee, 1985) and protect/rear the chicks (Collins, 1984). The females frequently leave the nest at suitable part of the day for foraging and/or when staying on the nest is considered unsafe for the hen or the nest.

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HAEMOCYTES OF COMMON LAND SNAIL FROM LAHORE, PAKISTAN

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Abstract: Two basic types of haemocytes *i.e.*, granulocytes and hyalinocyte were present in the blood of snail, *Bensonies jacquemonti*. Most of the haemocytes observed (97.2%) were granulocytes. Their diameter varies from 18-36 μm. Ectoplasm was not very distinct. The endoplasm have granules of various sizes. The pseudopodia are either filopodia or lobopodia. Each granulocyte forms 2-17 filopodia which are upto 12-62 μm long. Terminal or basal webs showed dichotomous branching or branches on one side. Granulocytes can merge with each other or with hyalinocytes thus loosing their cytoplasmic identity and form large aggregates of cells. The nucleus of granulocytes was rounded to oval, kidney-shaped or lobulated, 9-12 μm in size. Hyalinocytes (2.8%) were rounded cells, without pseudopodia, have diameter 13-42 μm. They differed from granulocytes in the presence of few granules, in having hyaline ectoplasm. The nucleus of hyalinocytes was rounded to oval or somewhat oblong with diameter 9-12 μm. A few spent granulocytes and hyalinocytes were also present. Some haemocytes (2-3%) of *B. jacquemonti* were found binucleated.

Key words: Blood cells, Bensonies jacquemonti, granulocyte, hyalinocyte.

INTRODUCTION

Considering the defensive role of molluscan blood cells against parasites (Cheney, 1971), different parasitologists and malacologists have paid particular attention to them (Cheng, 1975; Sminia, 1981; Bayne, 1983; Ottaviani, 1983). Each cell is delimited by a unit membrane and a compact nucleus developed by a double-membraned nuclear envelop. In large granulocytes nucleolema complex surrounds the nucleolus (Cheng and Rifkin, 1970). The cells have granular or agranular cytoplasm besides a variety of organelles and inclusion, seen under electron microscope (Jeong and Heyneman, 1976; Ruddel, 1971; Feng *et al.*, 1971; Sminia and Barendsen, 1980). These cells cannot be compared with vertebrate erythrocytes functionally or morphologically (Sminia, 1981).

Depending upon the classification system one to four different cell types have been reported in the molluscan blood (Cheng and Guida, 1980a,b; Sminia, 1981; Dikkeboom *et al.*, 1984; Tanveer, 1989, 1990, 1991) and named as leukocytes, lymphocytes, haemocytes, amoebocytes, granulocytes, macrophages and hyalinocytes etc. In Pakistan freshwater snails are of considerable medical and veterinary importance and found throughout the year except a short period in extreme cold and hot (Tanveer and Khan, 1989). Although the work on various aspects of haemocytes of freshwater snails have

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been worked out by Tanveer (1989, 1990, 1991) and Tanveer *et al.* (1995) but terrestrial snails has never been given consideration as far as their blood cell morphology is concerned. It was therefore, considered desirable to undertake such findings in common garden snail *Bensonies jacquemonti* Austin-Goodwin with a view to provide basic information regarding their structure which contribute in the understanding of phagocytosis.

MATERIALS AND METHODS

The snails collected from Jinnah Garden, Lahore, were maintained in the laboratory in large earthen pots half filled with humus soil at temperature 25.0±2.0°C. The snails used in this study measured 1.75±0.33mm - 2.45±0.65mm in shell width. The blood samples were taken out by inserting a micropippette directly into the heart region after removing a part of shell above this region (Guida and Cheng, 1980). The haemolymph was placed on glass slides and left undisturbed for 30 minutes at room temperature. These blood cells were fixed in 1% glutaraldehyde in Sorensen's buffer (pH 7.4) at 5°C and stained with lead haematoxylin and basic fuchsin following Guida and Cheng (1980) and microphotographs were made at an enlargement of X1000.

RESULTS AND DISCUSSION

Two basic types of cells were noted, (1) the granulocytes, (2) the hyalinocytes and several developmental stages or the spent granulocytes and hyalinocytes.

Granulocytes

Granulocytes were 97.2% of the total blood cells examined. These are typical amoeboid cells with highly variable shape depending on the activity, they may appear from rounded to elongated. The ectoplasm is not distinctly separate from the endoplasm. The cytoplasm has some highly pronounced characteristics. Firstly, it contains certain granular structures (Figs.1,2) which provide the basis for the name given to these cells. The cells have a tendency to spread on contact with a solid substratum and the degree of this spreading depends somewhat on the time (for which they are left undisturbed), temperature and the medium in which the cells are kept. It has been observed that in normal saline or in calcium rich media the cells have tendency to become rounded and to withdraw the pseudopodia. However, they spread more quickly in warmer temperatures and in the normal serum. Secondly, they react by throwing out pseudopodia, which may be of various types and shapes. Thirdly, they have remarkable ability to merge with each other (Figs.3,4) so much so that they loose their cytoplasmic identity but the nuclei always remain separate and merging cell can separate subsequently along with their nuclei. This merging may be an extension of their tendency to phagocytose foreign materials. This seems to suggest that in their reaction to solid object, the cells are not able to identify self from nonself and they react in the same manner to another cell of their own type (Fig.4) as they would to a foreign solid object.

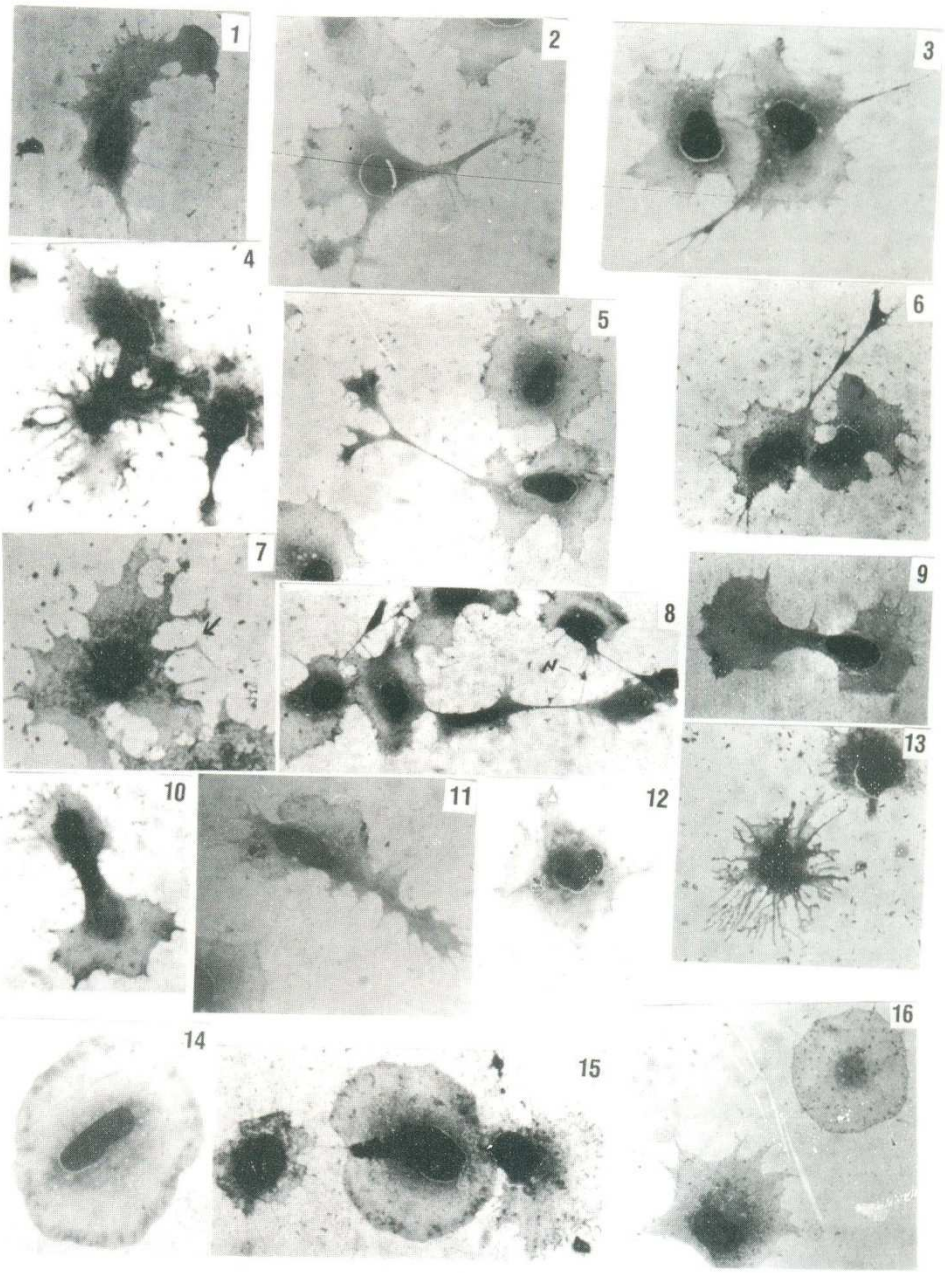
The diameter (μm) of granulocytes and their nucleus measures 26.15 ± 14.28 and 10.45 ± 1.66 respectively. Their respective area $(\mu m)^2$ measures 910.28 ± 325.44 and 101.14 ± 23.21. The cell to nucleus ratio comes to 6:1 (Table 1). It was further observed that 2-3% of the blood cells have double nucleus. The diameter (μm) and area of these cells measured 32.48 ± 19.22 and 9.6 ± 2.03 respectively. Their respective area was measured 1841.34 ± 629.12 and 95.21 ± 28.33, along with 7:1 and 65:1 cell to nucleus ratio.

As can be seen from the measurements given above the size is highly variable and cannot have any taxonomic value, except when the maximum size is considered.

Pseudopodia of granulocytes

All granulocytes of *B. jacquemonti* form pseudopodia which in almost all cases seem to be a reaction to the presence of solid objects (Fig.8) in the vicinity of the granulocytes. This property seems to be lodged in the cytoplasm itself, because a pseudopodium along its course will react exactly in the same manner to the presence of another solid particle with the result that the pseudopodia can behave in various ways. They may branch dichotomously (Fig.5) or form terminal webs (Fig.6) or basal webs (Fig.13). They may bend (Fig.7) as a reaction to strong stimulus from the lateral side. But the pseudopodia wherever and whenever in contact with solid substratum retain property of spreading (Fig.8). The formation of pseudopodium is not restricted to a foreign object only, they will be formed as a reaction to the presence of another homo-haemocyte that ultimately resulted in the formation of large aggregates of cells due to the fusion of cytoplasm as has also been noted by Cheng and Guida (1980), in *Bulinus truncatus rohlfsi* and Tanveer (1989, 1990, 1991), in pulmonate, planorbid and prosobranch snails.

In some cells there was a basal ectoplasmic web (Fig.13). The pseudopodia are generally in the form of filopodia which radiate from the cell and may have a narrow core extending to some distance. The maximum length in pspeudopodia observed in this study was 62 μm beyond the margin of the cell body. In several cases the pseudopodia are seen to be dichotomously branched or sometimes the branches may be present on one side only (Fig.3). This obviously depends on the presence of solid particles in that direction as can be seen in Fig.8, showing a solid particle (marked N). Cheng *et al.* (1979) have stated that fine filopodia radiate along the glass substrate and that the cytoplasmic granules may extend along certain pseudopodia. But it is difficult to answer why a pseudopodium will not be formed towards a solid object which is not in the plane of the glass substratum. They are also of the view that the free terminal of all filopodia end in bulbs, this does not seem to be true in all the cases in the granulocytes of *B. jacquemonti*. The maximum size of the pseudopodia in their study is smaller than the pseudopodia of *B. jacquemonti*. In a similar study by Cheng and Guida (1980) the maximum length of pseudopodia is 35 μm and some of the pseudopodia are lobopodia which is also the case in the present study (Figs.9,10). However, the pseudopodia in the present study do not necessarily have a tappered construction as stated by Cheng and Guida (1980a). Ectoplasmic terminal webs and dichotomous branching have also been demonstrated in *B. truncatus rohlfsi*. Sminia (1981) stated that the pseudopodia have a hyaline appearance, this would be dependent on the type of staining, but should also be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expected because the pseudopodia are largely of ectoplasmic structure. Cheng and Guida (1980a) stated that the lobopodia of the granulocytes of *B. truncatus rohlfsi* are of different shapes *i.e.*, rounded bulges, triangular funnels and broad peaks such variations have also been recorded in the present study.

The granulocytes under study occasionally produced 1-40 pseudopodia each. Similar multifilopodial blood cell was also reported by Cheng and Guida (1980) in the stained preparations of *B. truncatus rohlfsi*. They suggested that it was a pathological condition. However, in the present investigation it cannot be said positively particularly in view of the fact that only a few cells were found in the specimens, which otherwise had normal granulocytes and hyalinocytes.

Nucleus of the granulocytes

The nucleus of the granulocytes is variable and it was rounded to oval kidney-shaped or lobulated (Figs.11,12). Sminia (1972) and Tanveer (1989, 1990, 1991) also reported the similar observations. In addition to these many other shapes have also been found in the present study as reported by Jeong and Heyneman (1976). Cheng and Auld (1977) found that nucleus of *B. glabrata* granulocyte are typically subovoid. Whereas Sminia (1977) found in *Lymnaea stagnalis* that the nuclei may be rounded, kidney-shaped or lobulated. The nuclear chromatin according to Cheng and Guida (1980a) was in the form of the interrupted strand or condensed thick strands depending upon the size of the amoebocyte. It appears that when the haemocytes spreads on the substratum the nucleus also spreads with it and it has been proved by Cheng and Guida (1980a) that one or two nucleoli were present in each nucleus.

Cellular inclusions

The vacuolated nature observed in the present finding has been reported by Sminia (1972) along with extensive golgi apparatus as demonstrated by ultrastructure. Carter and Bogitsh (1975) demonstrated lysosome like structure and golgi apparatus. Jeong and Heyneman (1976) showed a few yellow or red coloured inclusions, spherical dark granules, mitochondrial organelles and vacuoles. These vacuoles are not necessarily around the phagocytosed material, although some of the particulate material may be present in the vacuoles as shown by Cheng and Guida (1980a).

Figs.1-16: 1, Granulocytes showing the endoplasmic granules; 2, Granulocyte showing branches of pseudopodium; 3, Merging granulocyte showing the branches of filopodium on one side; 4, Merging granulocyte showing large aggregate of cells; 5, Granulocytes showing dichotomous branching of pseudopodium; 6, Granulocyte showing the terminal ectoplasmic web of pseudopodium; 7, Granulocyte showing the bending of pseudopodium; 8, Granulocytes forming pseudopodia towards a solid particle (marked N); 9, Granulocyte showing lobopodium and oblong nucleus; 10, Granulocyte showing lobopodium and elongated nucleus; 11, Granulocyte showing kidney shaped nucleus and branched pseudopodium; 12, Granulocyte showing lobulated nucleus; 13, Multifilopodial granulocyte showing basal ectoplasmic web; 14, Hyalinocyte showing oblong nucleus and vacuoles around it; 15, Hyalinocyte merging with granulocyte; and 16, Spent granulocyte and hyalinocyte (X1000).

Table 1: **Some morphological characteristics and morphometric data (mean ± S.D.) of stained haemocytes of *Bensonies jacquimonti*.**

Mononucleated granulocytes (n = 55)

Cells		Nucleus		Cells to	Pseudopodia		
Diameter (μm)	Area $(\mu m)^2$	Diameter (μm)	Area $(\mu m)^2$	nucleus ratio	Total Number	Minimum length (μm)	Maximum length (μm)
26.15 ±14.28	910.28 ±325.44	10.45 ±1.66	101.14 ±23.21	6:1	12.12 ±4.22	7.12 ±3.25	48.24 ±12.80

Binucleated granulocytes (n = 55)

Cell		Mother nucleus		Daughter nucleus		Cell to mother nucleus ratio	Cell to daughter nucleus ratio	Pseudopodia		
Diameter (μm)	Area $(\mu m)^2$	Diameter (μm)	Area $(\mu m)^2$	Diameter (μm)	Area $(\mu m)^2$			Total Number	Minimum length (μm)	Maximum length (μm)
32.48 ±19.22	1841.34 ±629.12	9.6 ±2.03	95.21 ±28.33	7.6 ±4.21	86.21 ±22.14	7.1	65:1	10.15 ±4.13	6.24 ±2.48	38.24 ±9.21

Hyalinocytes (n = 22)

Cell		Nucleus		Cell to nucleus ratio
Diameter (μm)	Area $(\mu m)^2$	Diameter (μm)	Area $(\mu m)^2$	
29.35 ±10.21	986.21 ±548.11	10.02 ±1.34	95.24 ±18.21	9:1

Hyalinocyte

2.8% of the blood cells in the present study represented the other distinct cell type which is called the hyalinocyte (Fig.14). The hyalinocyte differ from granulocyte in the presence of a few granules in the cytoplasm, in more or less circular shape in the spread from and in possessing a comparatively hyaline cytoplasm. These also differ from granulocytes in the absence of filopodia and in a slightly high nucleus-cytoplasm ratio. Like granulocytes they can also merge with other hyalinocytes or granulocytes (Fig.15). The nucleus in hyalinocytes has also variable in shape i.e., from rounded to oval to almost oblong. Similar findings have earlier been reported by Cheng and Auld (1977, *Biomphalaria glabrata*), Cheng and Guida (1980a, 1980b, *B. glabrata*), Sminia and Barendsen (1981, *Lymnaea stagnalis*), Schoenberg and Cheng (1981, *B. glabrata*) and Tanveer (1989, 1990, 1991, *Lymnaea acuminata, Indoplanorbis eustus, Physa acuta* and *Bellamaya bengalensis*). For comparison purpose, similar findings for terrestrial snails were not available. The diameter (μm) of hyalinocytes and their nucleus measures 29.35 ± 10.21 and 10.02 ± 1.34 respectively. Their respective area $(\mu m)^2$ measures 986.21 ± 548.11 and 95.24 ± 18.21. Their cell to nucleus ratio was 9:1 (Table 1).

In the present investigation hyalinocytes were less than 3% of the total blood cells while in some earlier reports this ratio was 30% (Renwrantz *et al.*, 1979; Cheng, 1975) and 10% (Schoenberg and Cheng, 1981; Tanveer, 1989, 1990, 1991) in the molluscan blood films. The hyalinocytes in the present study generally conform to above characteristics except that the nucleus-cytoplasm ratio is lower as compared to granulycytes.

Spent granulocytes and hyalinocytes

Occasionally a haemocyte is seen (Fig.16) which is rounded and has few granules and poorly staining cytoplasm and more or less disintegrating nucleus. These probably are spent granulocytes and hyalinocytes. Similar cells have also been seen by Cheng and Guida (1980a) and they suggested them as developmental stages of blood cells.

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DISTRIBUTION OF XANTHINE OXIDASE DURING CALCIUM PARADOX CONDITIONS OF RAT HEART

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Abstract: Reintroduction of calcium into calcium depleted rat heart, so called Ca-paradox (CP), results in extensive biochemical, physiological and histological changes. Free radical producing enzyme xanthine oxidase (XO) was localized (*in situ*) in PLP fixed thick frozen sections of normal and Ca-paradox rat hearts by enzyme histochemistry using nitroblue tetrazolium (NBT) staining and also by immunocytochemistry using murine monoclonal antibodies against XO. An immunoperoxidase reaction product was accumulated in interstitial cells, endothelial cells of small capillaries and blood vessels in normal rat heart whereas it was markedly increased in Ca-paradox tissues, but XO activity was reduced by nitroblue tetrazolium staining which suggested the loss of enzyme activity. The results indicate that the XO activity during Ca-paradox conditions is increased and is restricted to interstitial and endothelial cells and its activity in sarcolemma membranes is due to rupturing and shedding of the cytoplasmic materials of interstitial and endothelial cells. The activity of XO along with CP may enhance the myocardial abnormaliies.

Key words: Calcium paradox, XO, immunocytochemistry, endothelial cells, myocardiam.

INTRODUCTION

The term "Calcium Paradox" (Ca-paradox) illustrates the reintroduction of medium containing Ca^{+2} after a brief period of perfusion with Ca^{+2} free medium. This process leads to the deterioration of structure and function, extensive biochemical, physiological and histological changes (Yate and Dhalla, 1975; Frank, 1983; Ganote *et al.*, 1983; Hulsmann, 1983) and ultimately loss of mechanical functions occur.

Calcium ions play an important role in physiological and pathological processes but the exact mechanism, which proceed the development of myocardial abnormalities are far from clearly understood. However, it is only one typical form of myocardial injury caused by excessive Ca^{+2} engulfment (Nayler *et al.*, 1983).

During pathological conditions, high energy phosphate level (ATP) is rapidly channeled to concomitant increase of ADP, AMP and purine bases, which serve as a fuel for xanthine oxidase (XO). This enzyme is mainly coupled to the degradation of xanthine and hypoxanthine to urates by producing a bulk of highly reactive cytotoxic

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superoxide radicals (Bray, 1975; Weiss, 1986) which are involved in protruding the disturbed normal physiological functions. However, the biological role of these radicals has not yet been completely known. Xanthine oxidase is normally present as dehydrogenase form (called D-type) and utilizes NAD^+ as electron acceptor (Park and Ganger, 1986) but under certain conditions, it rapidly converts into oxidase form (called O-type) and transfer electron to oxygen molecule (McCord, 1987; Schoutsen *et al.*, 1983). A number of studies (Krenitsky *et al.*, 1975) was attempted to elucidate XO activity in different organs of animals but no information is available as to which organelles of rat heart show active sites of XO during Ca-paradox conditions. It is important to examine the role of XO in myocardial abnormalities associated with Ca-paradox.

The present study was conducted to localize the XO activity in Ca-paradox rat heart using immunocytochemical methods at light and electron microscopic level and compared with enzyme histochemistry. The ultrastructural changes occurring during pathological stages have also been discussed.

MATERIALS AND METHODS

Monoclonal antibody

Murine MAb (N_2-26) against XO was characterized by enzyme linked immunosorbant assay (ELISA) and immunoblot. After checking the specificity and titer of MAb, it was used for immunolocalization studies in normal and Ca-paradox rat hearts.

Materials required for propagation of hybridoma (N2-26) secreting monoclonal antibodies (MAbs) against XO and for immunochemical tests were described in Samra *et al.* (1991). All other chemicals for routine analysis were obtained from Sigma Chemical Company.

Perfusion techniques

Normal rat heart

After being heparinized (5000 USP) and anesthetized with pentobarbitol (30 mg/kg), Sprauge-Dawley male rat (weighing 200-250 g) hearts were excised and perfused retrogradely in a non-circulating langendorff apparatus (Samra *et al.*, 1991) with Krebs-Henseleit buffer, saturated with 95% O_2 and 5% CO_2 for 15 minutes at 37°C. Perfused hearts were fixed immediately with cold periodate-lysine-paraformaldehyde (PLP) fixative in 0.4M phosphate buffer (pH 7.4) and stored at 4°C for further studies.

Calcium paradox rat heart

Again after being heparinized and anesthetized, rat hearts were removed and perfused under normal conditions as described above, followed by 10 minutes of calcium free perfusion and then 10 minutes reperfusion with buffer containing 2.5mM calcium. Treated hearts were fixed with cold PLP fixative and stored at 4°C for further studies.

Tissue embedding and homogenate preparation

Fixed and sucrose (15%) treated tissues from normal and calcium paradox rat hearts were embedded in Optimum Cutting Temperature (OCT) compound, snap frozen in dry ice ethanol bath and then stored frozen at -70°C for light and electron microscopic studies. Hearts subjected to Ca-paradox were also processed for homogenate preparation (Samra *et al.*, 1991). This homogenate was used to detect the XO activity and immunoblot analysis.

Immunoblot analysis

Purified XO and Ca-paradox rat heart homogenate were separated electrophoretically in slab sodium dodecyl sulphate-polyacrylamide gel (10%). After electrophoresis, protein samples were transferred electrophoretically on to nitrocellulose membrane (Towbin and Gordon, 1979) and proceeded for immunochemical reaction.

Immunocytochemical studies

For light and electron microscopic studies, thick cryostat sections of frozen tissues were processed for immunoperoxidase labelling (Samra *et al.*, 1991; Ashraf and Samra, 1993). For light microscopy, cryosections (6-8 μm) were cut, placed on albumin coated slides and exposed to MAbs (mouse anti-XO) to arrest its epitopes. The bound MAbs were again allowed to react with peroxidase labelled goat anti-mouse IgG. After washing, immunochemical reaction was developed by incubating the sections with 0.2% diaminobenzidine and 0.05% H_2O_2 in 0.05M Tris-HCl (pH 7.4). Rinsed tissues were dehydrated in ascending series of ethanol up to absolute ethanol and mounted in permount.

For electron microscopic studies, PLP fixed rat heart tissues were dehydrated in ascending series of ethanol. Dehydrated tissues were embedded in plastic resin (Epon) and subsequently polymerized at 60°C for 48 hour. Ultra-thin sections were cut, placed on copper grids and processed for immunoperoxidase labelling (described above) and observed with Hitachi electron microscope at accelerations voltage of 75KV. Control sections were treated with HT-medium, or secondary antibody alone.

Enzyme histochemistry

Enzyme activity (*in situ*) in normal and Ca-paradox rat hearts was also checked as described in Samra *et al.* (1991). Briefly, PLP fixed 6-8 μm sections of normal and Ca-

paradox rat heart tissues were incubated in a reaction medium (0.05M Tris-HCl, pH 7.4; 1mM Hypoxanthine; 1 mg/ml Nitroblue tetrazolium) for 20 minutes. For control sections, 1mM allopurinol (an inhibitor of XO) was also added in the reaction medium.

RESULTS

Characterization of antibodies

ELISA and immunoblot analysis showed that the murine MAbs against XO recognized the epitopes present on native XO. The specificity and monoclonality against pure XO as well as in rat heart homogenate were further examined under denaturing conditions (Fig.1). When purified XO and Ca-paradox rat heart homogenate (105,000 xg) supernatants were processed for immunoblot, a specific immune reaction with 155 KDa protein band was detected on nitrocellulose membrane. However, an additional protein bands of approximately 91 KDa in pure XO and two other protein bands of approximately 60 KDa and 27 KDa in addition to 91 KDa in rat heart homogenate were also visualized with weak staining. Immunochemical controls incubated with Hypoxanthine-Thymidine Dulbeco Modified Eagles Medium (HT-DMEM) or secondary antibody alone were devoid of reactivity.

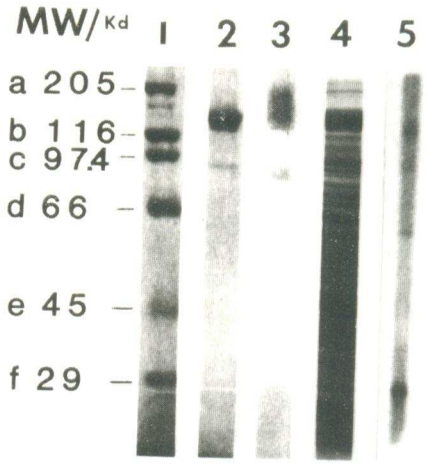

Fig. 1: **Immunoblot analysis of XO. Lane 1, standard molecular weight: (a) myosin, 205 KDa; (b) ß-galactosidase, 116 KDa; (c) phosphorylase-b, 97.4 KDa; (d) bovine serum albumin, 66 KDa; (e) ovalbumin, 45 KDa; (f) carbonic anhydrase, 29 KDa. Lane 2, pure XO. Lane 4, Ca-paradox rat heart homogenate; Lane 3 & 5, immunodetection of pure XO and also in rat heart homogenates, respectively.**

Light microscopic observation

Immunoperoxidase staining

Thick cryosections of normal and Ca-paradox rat hearts tissues subjected for immunoperoxidase labelling showed that the dark brown color reaction product was precipitated on antigenic sites. Tissue sections from normal perfused heart (Fig.3) showed weak immunostaining on interstitial cells between muscle fibres. Whereas tissue sections of rat heart underwent for Ca-paradox were signalling a copious amount of immunoreaction product distributed intensely on interstitial cells (Fig.2a), small capillaries and blood vessels (Fig.2b) along with weak immunoreaction product on heart muscles.

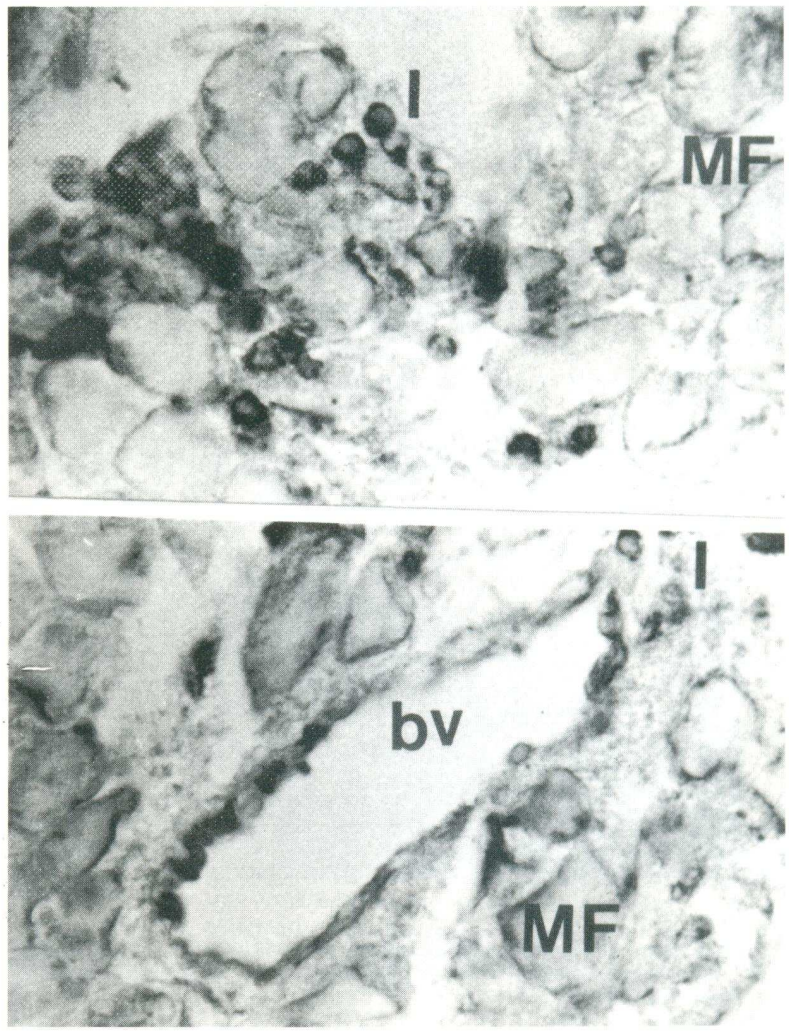

Fig. 2: An immunoperoxidase staining on frozen sections of Ca-paradox rat heart incubated with MAb against XO. The reaction product is seen on (*2a*), Interstitial cells (I), muscle fibres (MF), (*2b*), blood vessels (bv), (X 350).

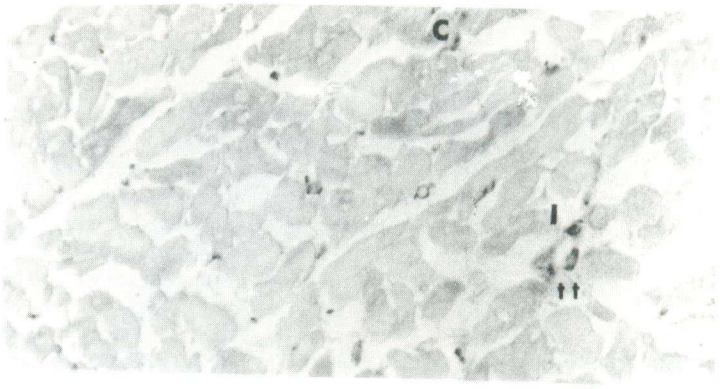

Fig. 3: An immunoperoxidase staining on frozen sections of normal rat heart incubated with MAb against XO. A reaction product is seen on capillaries (c), interstitial cells (I), (X 250).

Nitroblue tetrazolium staining

Cryosections of Ca-paradox rat heart were also analyzed for XO activity (*in situ*) by nitroblue tetrazolium (NBT) staining. It was observed that insoluble blue formazan reaction product was deposited on small capillaries and interstitial cells along with weak staining on muscle fibre (Fig.4).

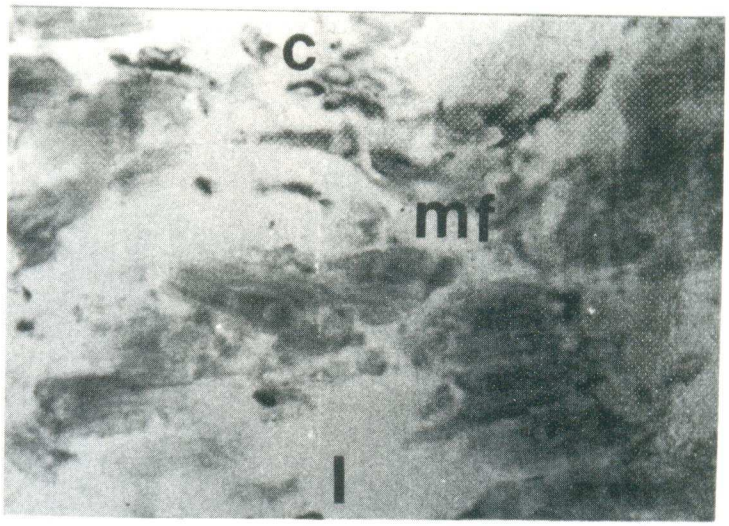

Fig. 4: Nitroblue tetrazolium staining on frozen sections of Ca-paradox rat heart. A blue formazan staining on capillaries (c), interstitial cells (I), muscle fibres (mf), (X 350).

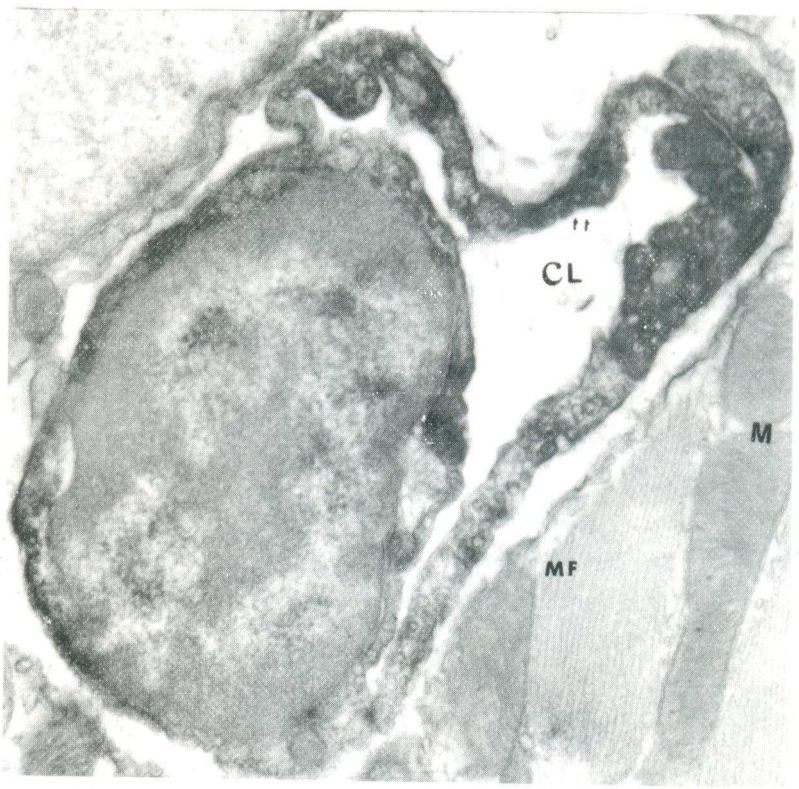

Fig. 5: **Immunoelectron microscopy of XO in Ca-paradox rat heart. The reaction product is located on (*5a*), interstitial cells (I), ruptured surrounding capillary lumen (CL), partial separated sarcolemma (S), swollen mitochondria (M) are also seen (X 8000).**

Electron microscopic observation

Observation of ultra-thin sections of epon-embedded normal and Ca-paradox rat hearts showed that the diamino benzidine (DAB) reaction product was deposited in the cytoplasm of interstitial cells, cytoplasmic rim surrouding the capillary endothelium of normal heart (Fig.6) as well as ruptured cytoplasmic rim in Ca-paradox rat heart but the staining was markedly increased in Ca-paradox conditions (Fig.5). A weak immunostaining was also observed only on the adjacent heart muscle fibres in Ca-paradox tissues. Mitochondria, nuclei of endothelial cells and interstitial cells were also not stained.

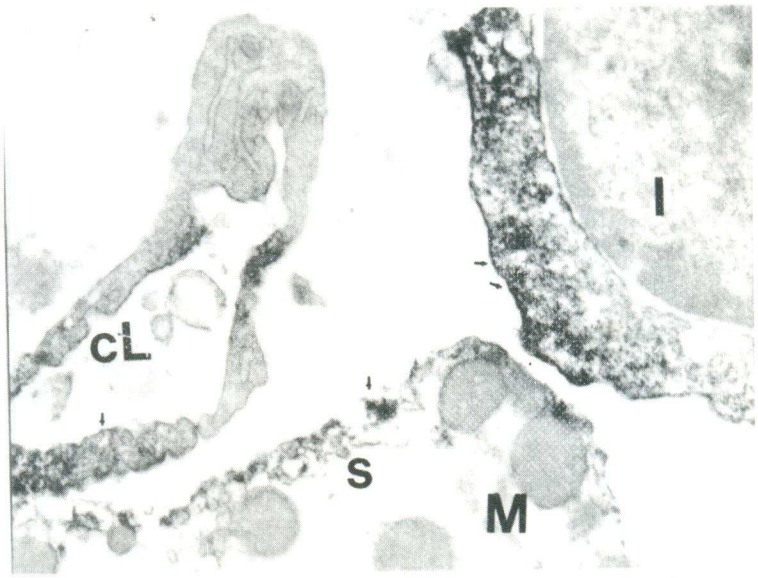

Fig. 6: **Immunoelectron microscopy of XO in normal rat heart. The reaction product (arrow head) is located on surrounding capillary lumen (CL), but muscles fibres (MF), and mitochondria (M) are not stained (X 8000).**

Morphological studies

The morphological studies of calcium reperfused rat heart tissues at light and electron microscopic level showed the severe damage of muscle fibres, partial separation of outer lamina of glycocalyx of sarcolemma, contraction of myocardial cells, swollen mitochondria and ruptured intercalated disc membrane.

Immunocytochemical control

All control sections proceeded for light and electron microscopic studies did not show staining.

DISCUSSION

In the present study, the activity of murine MAbs against XO was exploited to explicate the role of XO in normal and Ca-paradix rat hearts. The use of MAbs is superior to polyclonal antibodies due to its supreme precise reactions. The

monoclonality was corroborated with ELISA, and immunoblot analysis under native and denatured conditions respectively which encouraged for immunocytochemical studies to determine the active sites of XO in Ca-paradox rat heart.

Immunoblot analysis revealed more specific bindings of MAbs to 155 KDa protein band of pure XO as well as in Ca-paradox rat heart homogenates. Whereas the immunochemical reaction with 91 KDa in pure XO and 60 KDa, 29 KDa protein bands in addition to 91 KDa bands in rat heart homogenate suggested the breakdown polypeptide of enzyme during storage or homogenate preparation of rat heart respectively.

Immunocytochemical and enzyme histochemical techniques were used to explore the XO at light and electron microscopic level using immunoperoxidase and NBT staining, which are mainly depend upon the presence of epitopes and preservation of functional property of the enzyme respectively. An immunoperoxidase analysis at light and electron microscopic level documented the presence of XO in normal rat hearts but its activity markedly increased after reperfusion with calcium containing buffer. A weak immunostaining on adjacent muscle fibres of tissues from Ca-paradox suggested that this may be due to rupturing and shedding of the cytoplasmic materials of these cells. This finding was not seen in our previous studies (Samra *et al.*, 1991; Ashraf and Samra, 1993) which illustrated lack of immunostaining on muscle fibres during pathological conditions such as ischemia or ischemia/reperfusion.

Xanthine oxidase activity was also monitored spectrophotometrically in rat heart homogenates by detecting the conversion of hypoxanthine to uric acid at 292 nm (data not shown). In contrast to immunocytochemistry, a weak histochemical (*in situ*) staining by NBT convinced the loss of XO activity during Ca-paradox conditions. Whereas normal tissues showed the moderate enzyme activity. Allopurinol is considered as an inhibitor of XO activity and sections of normal and Ca-paradox heart tissues with this inhibitor were lack of enzyme activity. The behaviour of immunochemical activity over enzyme activity will be more reliable to make out the active sites of this enzyme.

The morphological studies by electron microscopy revealed that the Ca-paradox annulled the normal functions of heart and ultimately lost its activity. Although enzyme histochemical and immunocytochemical approaches were applied to explore the XO activity in many tissues (Park and Granger, 1986) but no data was available about the precise localization of this enzyme in Ca-paradox rat heart.

The observations in the present study suggested that the Ca-paradox induces disturbance in the xanthine dehydrogenase/ oxidase ratio and may enhance the production of oxygen derived free radicals. The presumed conversion of dehydrogenase to oxidase form in Ca-paradox presents a very important step in the series of events leading to cellular necrosis.

The results reveal the augmented activity of XO is restricted on interstitial cells and endothelial cells of small capillaries and blood vessels. The staining on adjacent muscle fibres may be due to rupturing and shedding of cytoplasmic materials of these cells. It is

concluded that oxygen free radicals produced by action of XO along with Ca-paradox condition may promote severe damage of the activity of heart muscles.

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CONTROL OF HYDATIDOSIS IN RABBITS THROUGH FEEDING LOCAL PLANTS

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Abstract: Out of twenty-six healthy rabbits acclimatized to the optimal laboratory conditions for fifteen days, 4 were considered as normal control and twenty-two were inoculated (intraperitoneally and subcutaneously) with 45000 protoscoleces of *Echinococcus granulosus* of sheep origin. They were at random divided into 4 groups. Groups 1-3 were orally given (2g/day) mashed leaves of *Aloe vera*, powdered *Punica granatum* (unripped ovary part) and fruit of *Azadirachta indica* respectively. Group 4 was considered as infected control. All the rabbits were fed on seasonal green fodder and ordinary tap water *ad libitum*. Their blood samples were pooled after every 10 days upto 70 days for biochemical analyses. Increased ($P<0.001$) body weight was noted in experimental rabbits. It was clear from the biochemical results that activities of acid phosphatase, alkaline phosphatase and glucose decreased ($P<0.001$) in rabbits treated with *A. vera*, *P. granatum* and *A. indica* as compared to normal control and the values of glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, cholesterol and total protein improved ($P<0.001$) as a result of herbal treatment. The bilirubin value improved only in rabbits treated with *A. vera* and *A. indica* ($P<0.001$). The results of our present investigation showed that alterations in different enzymes and biochemical metabolites induced by liver protoscoleces were improved in the rabbits orally given *A. vera*, *P. granatum* and *A. indica*. Although the extent of improvement varies in these plants, even then the present results are promising and it is suggested that the active ingredients of these plants should be checked for further use.

Key words: Hydatidosis, herbal control, blood biochemistry, rabbits.

INTRODUCTION

Hydatidosis is medically and economically one of the most important disease and both unilocular (*Echinococcus granulosus*) and multilocular (*E. multilocularis*) of the disease are widely and actively extending their range into areas previously considered to be free of this infection (Schwabe, 1986). Sometimes it look like an out break in different localities like England and Wales (Stallbaumer *et al.*, 1986), in Morocco (Pandey *et al.*, 1988) and in China (Chi *et al.*, 1990). It is also an important zoonosis and the chances of its high infection in man are more in those areas where sheep and cattle grazing is carried out with the help of dogs (FAO report, 1993) as in Pakistan. Its wide prevalence in the live stock of Pakistan has been given consideration by Khan and Haseeb (1984), Iqbal *et al.* (1986), Pal and Jamil (1986) and Iqbal *et al.* (1989). Its seriousness in human beings is also far greater than what the published record shows and its incidence is much higher then what is generally believed to be (Junejo *et al.*, 1995). Its pathology resulted due to various haematological and biochemical alterations have been given consideration (Pandey, 1971; Schantz,

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1984) with the ultimate finding that such changes were attributed to the hydatid cyst fluid that seeps out of the hydatid cyst wall thereby causing necrosis of the surrounding cells with the ultimate result in the increase of enzymatic level in the blood.

Cyst can be removed only by surgery (Schantz, 1984) that is expensive, debilitating and entail risk. Therefore, programs have been implemented to control the parasite in regions where it is endemic. Most control programmes are aimed at eliminating the source of infection to man and other intermediate hosts. Besides this two benzimidazole derivatives, namely mebendazole and albendazole are now routinely used in cystic echinococcus (Davis *et al.*, 1986; Horton, 1989; Matossian *et al.*, 1992). Many plant drugs have also been in practice since ancient times for the treatment of parasitic infection in man and animals (Nadkarni, 1954; Chopra *et al.*, 1956; Said, 1969).

Since Pakistan has a great variety of herbs and medicinal plants grown widely in the northern hilly areas. Unfortunately a large number of the indigenous medicinal plants and herbs have not been investigated by the modern phytochemical and pharmacological methods except Said (1969), Ikram and Hussain (1978), Ohigashi *et al.* (1982), Sanyal *et al.* (1985), Akhtar (1986).

Akhtar (1986) studied the anthelmintic efficacies of indigenous plant and herbs alledgedly used to treat various parasitic infections by the practitioners of old and traditional medicines so as to find a scientific use of these herbs for veterinary therapeutic purposes. He also studied the anthelmintic activities of several powdered plant drugs and their extracts in goats, buffaloes and calves. Akhtar (1987) found out a number of plant drugs and their extracts that possess interesting potent and safe anthelmintic principle for veterinary usage. Anwar *et al.* (1997a) tested two indigenous plants to control hydatidosis in experimentally infected rabbits along with the common fodder and their effects were studied on different haematological and biochemical parameters. Beside this plants extract was used to control the larval stages of *E. granulosus* (Anwar *et al.*, 1997).

In the present work 3 plants *(Aloe vera, Punica granatum, Azadirachta indica)* were used to control this disease. These plants were orally given to experimentally infected rabbits with common fodder, and their effects were studied in terms of their biochemical parameters which may give a clear picture of the changes they have produced in curing the disease.

MATERIALS AND METHODS

Maintenance of rabbits

Twenty-six healthy rabbits maintained under optimal conditions were fed twice a day on seasonal green fodder. Few crystals of $KMnO_4$ were added to their drinking water to minimize the chances of infection. $KMnO_4$ solution was used to disinfect the utensils and cages. Rabbits were individually weighed in the beginning of experiment and then before each inoculation.

Hydatid cyst fluid

Hydatid cyst fluid (HCF) was aspirated from the cyst present in liver, lungs and spleen of infected sheeps at local slaughter house and only viable protoscoleces were selected for inoculation. Hydatid cyst fluid was centrifuged at 500 rpm for 5 minutes and the pallet thus formed was washed with saline solution, containing the Penicillin and Streptomycin sulfate as described by Ohnishi (1985). The protoscoleces were counted in the chamber of white blood cells of haemocytometer. Two inoculums each having 22500 protoscoleces were injected sub-cutaneously and intra-peritoneally in the left flank of rabbit after taking all antiseptic precautions.

Grouping of rabbits

Rabbits were divided into 5 groups. For each plant treatment 3 treated (six rabbits each) and one experimental control (having 4 rabbits) along with one normal control (n=4) were maintained. Following plants and their parts were used:

The leaves of *Aloe vera* (Kwargandal), ovary part of *Punica granatum* (Anar) and fruit of *Azadirachta indica* (Neem) were used for feeding animals.

Preparation of plant/herbal extracts

The dried plants and their parts (Anar, Neem) were ground to fine powder. 2 g of each powdered plant was mixed with 25 ml of tap water and then given to rabbits. The fresh leaves of kwargandal were mashed and 2 g mixed with 25 ml of water was given to rabbits. Three treated groups were orally given 2 g/day of *A. vera, P. granatum* and *A. indica* up to 70 days. The experimental control (without herbal treatment) and normal control were also maintained.

Blood sampling and biochemical analyses

After every 10 days about 4 ml blood was pooled from the marginal vein of each rabbit and refrigerated at 4°C for 15 minutes and then centrifuged at 3000 rpm for 20 minutes. Unhaemolysed and clear serum was used for estimating acid phosphatase, alkaline phosphatase, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), bilirubin, cholesterol, glucose and total protein according to standard kits (Randox, U.K.), following Dacie and Lewis (1991).

RESULTS

Group I (treated with leaves of Aloe vera)

The initial body weight (g) before any treatment in normal control, experimental control and treated rabbits was 1056 ± 65.23, 1075 ± 68.23 and 1054.16 ± 164.63 respectively. It decreased (-0.35%) after 10 days in normal control but increased (0.76% and 0.39%) in experimental control and rabbits orally given leaves of *A. vera* (2 g/day)

($P<0.001$, Table 1, Fig.1). Afterwards, body weight of normal control rabbits fluctuated a lot with an overall increase of 29.37%, till the end. In case of experimental control and treated rabbits body weight increased upto 26.5% and 13.6% after 50 days. After 60 days their body weight decreased upto -5.65% and -5.15%. After 70 days there was again an increase (29.34% and 23.58%) in the body weight of both experimental control and treated rabbits ($P<0.001$) (Fig.1).

After 10 days the specific growth rate (%) increased by 0.02% and 0.07% in both normal control and experimental controls. It was noted that specific growth rate (%) of treated rabbits faced a slight decrease (0.04%) after 10 days, (0.11%) 20 days, followed by an increase of 0.78%, 0.40%, 0.6% after 30, 40 and 50 days, respectively. The values again decreased (-4.9%) after 60 days with an increasing trend (1.73%) after 70 days.

Blood biochemistry

The biochemical effect of *A. vera* on the blood of rabbits have been studied in terms of changes it produced in the activity of some enzymes such as acid phosphatase (AcP), alkaline phosphatase (Ap), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT). The changes produced in the concentration of certain biochemical metabolites like bilirubin, cholesterol, glucose and total protein were also studied.

The initial value of AcP (IU/dl) for normal control, experimental control and treated rabbits was 80.25 $\pm$ 1.29, 81.14 $\pm$ 1.83 and 96.96 $\pm$ 2.11, respectively. Later on, there were great fluctuations in these values and after 70 days the AcP value increased by 9.65% for normal control along with a prominent decrease in experimental control and treated rabbits (-245.80% and -90.45%) ($P<0.001$, Fig.2). The value of Ap (IU/dl) gradually decreased from 13.29 $\pm$ 7.69 to 10.02 $\pm$ 5.06 for normal control rabbits. However, the overall values for Ap in the experimental and treated rabbits showed sharp decline (-689.7% and -200.7%) ($P<0.001$, Fig.2). The GOT value (IU/dl) of normal control rabbits showed an overall increase (16.55%) while the similar values for the experimental control declined upto -58.5%. In case of treated rabbits GOT value improved by 5.29% in the end of treatment ($P<0.001$, Fig.2). There was overall increase (13.15% and 10.48%) in the GPT value (IU/dl) for both normal control and treated rabbits while experimental control faced decline by -8.80%. Changes in GPT values were found statistically significant ($P<0.001$) (Fig.2).

In the beginning of experiment the average bilirubin value (mg/dl) for normal control rabbits was 0.86 $\pm$ 0.67. This group, however, faced decline (-26.31%) till the termination of experiment. In case of experimental control rabbits great fluctuations were noted in the bilirubin value with an overall decline of -15.20%. Moreover, the bilirubin values for the treated rabbits showed an overall increase (34.7%) ($P<0.001$, Fig.2). It was noted that cholesterol level (mg/dl) increased continuously from 70.34 $\pm$ 10.23 to 80.49 $\pm$ 13.30 for normal control rabbits. Although experimental control rabbits showed great fluctuations but their cholesterol contents increased by 80.3% till the end of experiment. The treated rabbits also faced increased (59.45%) cholesterol values ($P<0.001$, Fig.2). Glucose level (mg/dl) showed variations with an overall

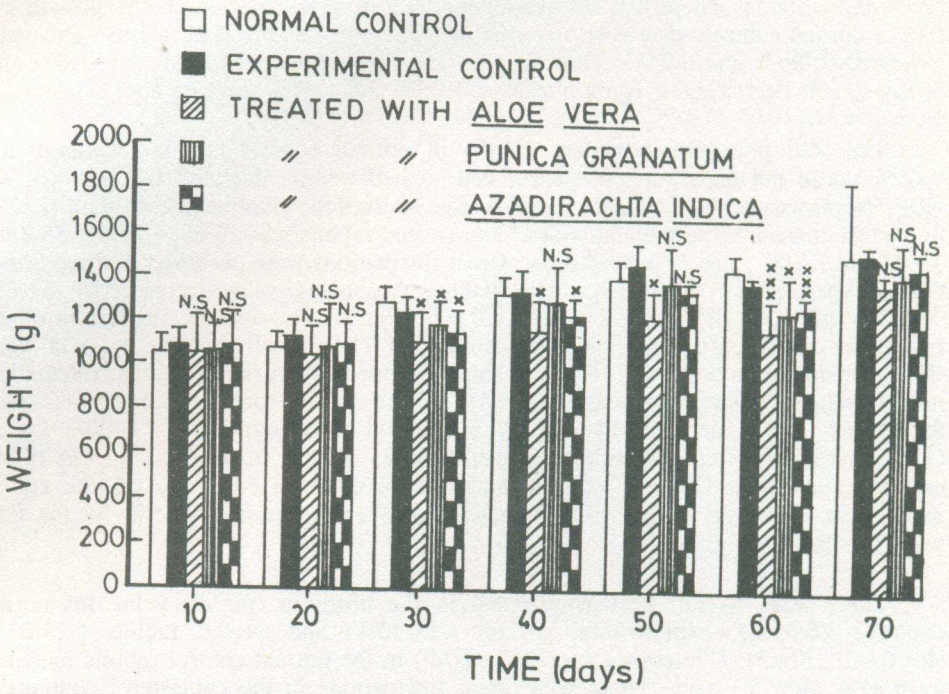

Fig. 1: **Changes in the body weight of rabbits, normal control, experimental control and treated with *Aloe vera* leaves, *Punica granatum* fruit and ovary part and *Azadirachta indica* fruit (2 g/day). Values given are mean ± S.D. of 4 control, 3 experimental control and six treated rabbits for 70 days. The statistical significance has been determined by Student's 't' test and probability represented by stars: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.**

decrease in all the rabbits. Glucose level for the normal control and experimental control groups decreased by -103.42% and -195.7% while for the treated rabbits the decreased noted was -34.73% till the end of experiment ($P<0.001$, Fig.2). The total protein value (g/dl) at the start of experiment was 6.025 ± 0.434 and it changed a little till the end for normal control. In case of experimental control variations were noted in the values with an overall increase of 17.20%. The amount of total protein increased by 11.23% for treated rabbits ($P<0.001$, Fig.2).

Group II (treated with ovary part and flowers of Punica granatum)

The average weight (g) of normal control, experimental control and treated rabbits increased from 1056 ± 65.23 to 1512.5 ± 335.8, 1075.0 ± 68.23 to 1533.33 ± 14.43 and 1056.5 ± 184.83 to 1433.33 ± 137.53 respectively. The body weight of treated rabbits increased by 22.9% after 50 days and decreased by -11.66% after 60 days.

Again there were increasing trend (24.99%) in the body weight of treated rabbits ($P<0.001$, Fig.1). There was an overall increase (0.41%) in the specific growth rate (%) of normal control. However, in experimental control and treated rabbits. This value increased (0.96% and 0.81%) after 50 days and then decreased (-0.51 and -1.09) after 60 days. This decrease was again improved 1.05% and 1.37% increase after 70 days.

The acid phosphatase values (IU/dl) in normal control rabbits increased upto 9.65% while the experimental control and treated rabbits declined by -245.8% and -192.5% respectively ($P<0.001$, Fig.3). After 10 days the alkaline phosphatase (I.U/dl) in normal control, experimental control and treated rabbits was 13.29 ± 7.69, 55.33 ± 43.92 and 72.00 ± 18.50 respectively. Great fluctuations were observed in three groups during experiment. After 70 days their alkaline phosphatase value decreased by -32.6%, -689.7% and -263.26% respectively ($P<0.001$, Fig.3). The decline was less in treated rabbits as compared to the experimental control. The overall increase in GOT value (IU/l) for normal control was 16.55%. The experimental control have great fluctuations in their values showing a decrease of -58.5% till the end of experiment. The GOT values for treated rabbits increased by 38.63% at the end of experiment ($P<0.001$, Fig.3). GPT value (IU/l) of normal rabbits changed from 25.25 ± 2.62 to 35.62 ± 3.68 till the end of experiment. GPT of experimental control declined (-8.80%) till the end of experiment while the treated rabbits showed an overall increase (9.45%) in the GPT value ($P<0.001$, Fig.3).

There were overall decreasing trend in the bilirubin (mg/dl) value for normal control (-26.31%), experimental control (-15.20%) and treated rabbits (-23.07%) ($P<0.001$, Fig.3). Cholesterol contents (mg/dl) in the normal control rabbits increased by 5.87% after 70 days. There were great fluctuations in the cholesterol contents of experimental control and treated rabbits and their values increased by 80.3% and 52.00% respectively ($P<0.001$) (Fig.3). The glucose level (mg/dl) at the start of experiment for normal control, experimental control and treated rabbits was 179.52 ± 12.86, 153.83 ± 13.04 and 109.52 ± 9.53 respectively. There were great fluctuations in their glucose values during the experiment with a decline of -103.42%, -195.7% and -38.79% at the end of experiment ($P<0.001$, Fig.3). Initial and final total protein (g/dl) values were almost the same (6.025 ± 0.434 to 6.28 ± 1.24, 8.182 ± 2.94 to 9.88 ± 1.38) for both normal control and experimental control. In case of treated rabbits the total protein value increased by 2.29% at the end of experiment. The results were found statistically significant ($P<0.001$) (Fig.3).

Group III (treated with fruit of Azadirachta indica)

The body weight (g) of both experimental control and treated rabbits increased till 50 days and it was followed by decrease (-5.65% and -5.88%) after 60 days. After 70 days their body weight again increased (29.34% and 26.27%) ($P<0.001$) (Fig.1). There were continuous increase (0.58%, 0.96% and 0.87%) in the specific growth rate (%) of normal control, experimental control and treated rabbits respectively upto 50 days. After 60 days it decreased (-0.51% and -0.49%) followed by an increase of 1.05% and 1.56% at the end of experiment for both experimental control and treated rabbits.

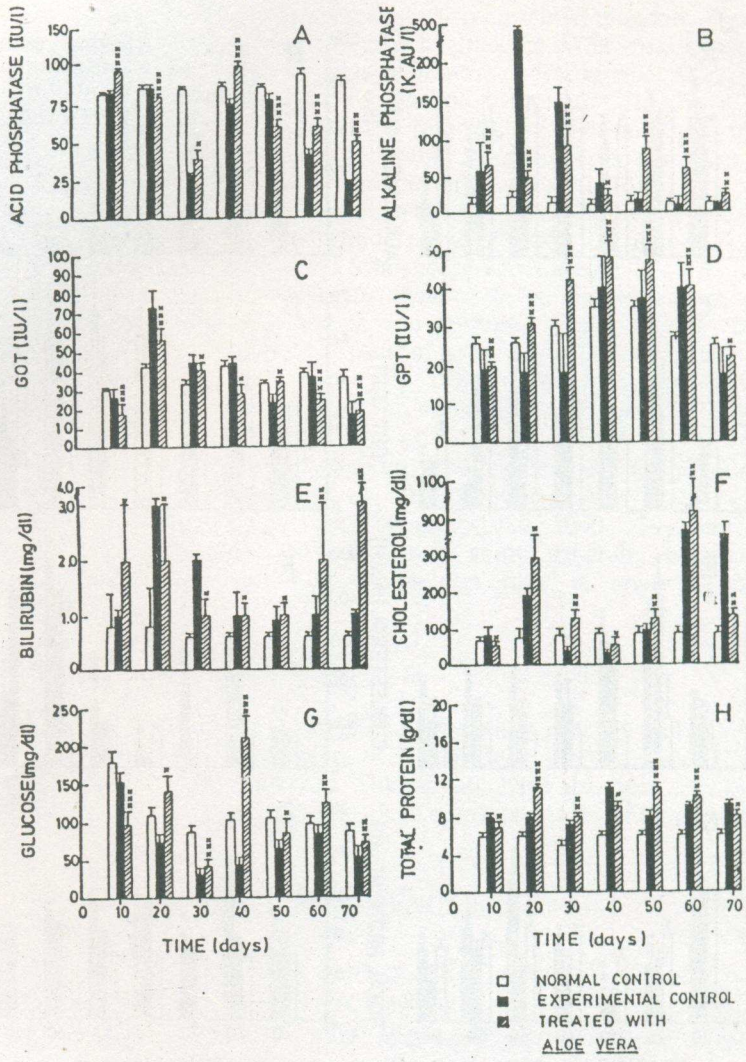

Fig. 2: **Biochemical changes in rabbits (intraperitoneally and sub-cutaneously injected with 45000 protoscoleces) after oral administration of *Aloe vera* leaves (2 g/day). Values given are mean ± S.D. of 4 control, 3 experimental control and six treated rabbits for 70 days. The statistical significance has been determined by Student's 't' test and probability represented by stars: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.**

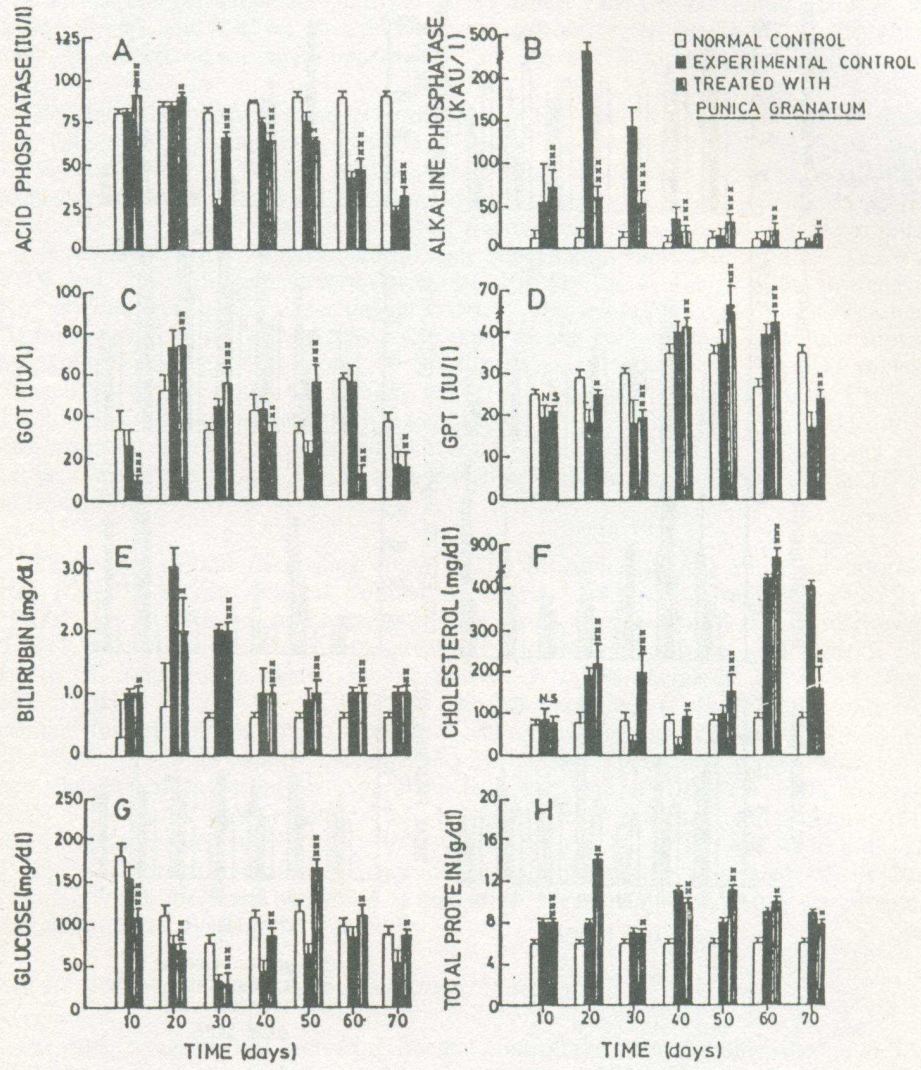

Fig. 3: Biochemical changes in rabbits (intraperitoneally and sub-cutaneously injected with 45000 protoscoleces) after oral administration of *Punica granatum* flower and ovary part (2 g/day). Values given are mean ± S.D. of 4 normal control, 3 experimental control and 6 treated rabbits for 70 days. The statistical significance has been determined by Student's 't' test and probability represented by stars: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

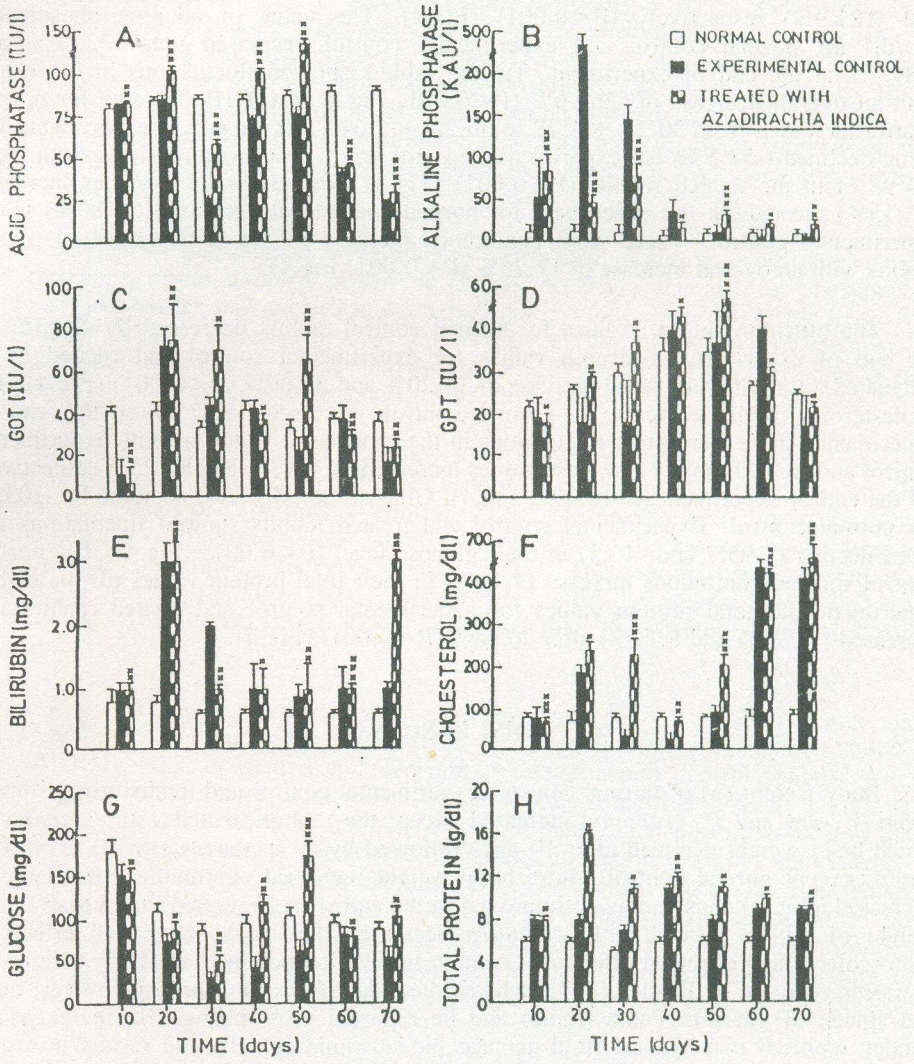

Fig. 4: Biochemical changes in rabbits (intraperitoneally and sub-cutaneously injected with 45000 protoscoleces) after oral administration of *Azadirachta indica* fruit (2 g/day). Values given are mean ± S.D. of 4 normal control, 3 experimental control and 6 treated rabbits for 70 days. The statistical significance has been determined by Student's 't' test and probability represented by stars: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

The acid phosphatase (AcP) (IU/dl) increase (9.65%) throughout the experiment for normal control but decreased in experimental control and treated rabbits (-245.8% and -183.9%) respectively ($P<0.001$) (Fig.4). The value of alkaline phosphatase (IU/dl) for normal control and experimental control decreased upto -32.60% and -689.7% at the end of experiment. Treated rabbits showed fluctuations in the values with an overall decrease of -261.6% ($P<0.001$, Fig.4). GOT (IU/l) value for normal control increased from 30.25 $\pm$ 1.27 to 36.25 $\pm$ 3.64% till the end of experiment. The value declined (-58.5%) for experimental control group along with an increasing trend (14.98%) in the treated rabbits ($P<0.001$) (Fig.4). The GPT (IU/l) values increased (13.15%) throughout the experiment for normal control but decreased (-8.80%) in the experimental control. There were continuous increase in the GPT values of treated rabbits with an overall increase of 17.26% ($P<0.001$, Fig.4).

The bilirubin (mg/dl) values for normal control rabbits decreased by -26.3% till the end of experiment. Bilirubin values for experimental control and treated group fluctuated a lot with an overall increase of 15.20% and 57.69% ($P<0.001$) (Fig.4). The cholesterol (mg/dl) contents in normal control increased (5.87%) at the end of experiment. There were great fluctuations in the cholesterol values of both experimental control and treated rabbits and these values increased by 80.3% and 88.25% respectively till the end of experiment ($P<0.001$, Fig.4). Glucose (mg/dl) level decrease by -103.42 for normal control. Experimental control and treated rabbits showed fluctuations and faced decline (-195.7 and -46.3) in their glucose level ($P<0.001$) (Fig.4). The normal control showed continuous increase (4.14%) in their total protein values till the end of experiment and total protein values for experimental control and treated rabbits also increased (17.20% and 8.11%) after 70 days ($P<0.001$) (Fig.4).

DISCUSSION

Body weight (g) of normal control, experimental control and treated groups orally given *A. vera* and *P. granatum* increased except the rabbits treated with *A. indica* in which body weight declined after 10 days followed by an increase up to 50 days in all groups except normal control whose body weight increased continuously but later on decreased after 60 days and again improved in the end. It is suggested that initially body weight of rabbits treated with *A. indica* decreased due to the combined effects of protoscoleces and chemical effect of *A. indica* leaves. Reduction in the body weight has also been reported by Pandey (1971) who studied the effect of hydatidosis in liver, lungs and spleen of goats in Patna (India) and he reported gross changes in these tissues. Pandey reported that hydatid fluid damage the surrounding cells and results in organs condemnation which lead to weight reduction. Economic losses due to hydatidosis through low quality and reduced yield of milk, meat and retarded growth have also been reported by Anonymous (1985) and Schwabe (1986). Protein deficiency and reduction of weight took place in animals treated with hydatid fluid have also been studied by Iqbal *et al.* (1989). Similar findings have earlier been reported by Anwar *et al.* (1997a) in rabbits experimentally infected with *E. granulosus* and treated with *Prosopis glandulosa* (leaves) and *Embelia ribes* (fruits).

In the present investigation, the body weight of treated rabbits improved either due to the development of resistance, or due to certain inhibitory effect of plants against the protoscoleces. The increased body weight can also be attributed to some of pregnant female rabbits. Then decrease noted in the body weight of experimental control and treated groups may be due to some bacterial infections and finally there was again increasing trend in the body weight of rabbits which showed that plants *A. vera*, *P. granatum* and *A. indica* have developed some resistance and they have antagonized the effects produced by the incoming protoscoleces. Increase in the body weight of treated rabbits was a curing mechanism.

Two types of phosphatase are commonly estimated in serum. Acid phosphatase (AcP) that helps in the autolysis of cells after their death whereas alkaline phosphatase (AP) is a brush border enzyme rich in epithelial lining of bile canaliculi of the liver (Ali and Shakoori, 1993). Increase in ACP activity may be due to pre-necrotic changes in different tissues including liver and increase in AP activity may be due to lesions in the biliary epithelium, or lesions in the intestinal brush border.

The results of our present finding showed decreased acid and alkaline phosphatase activity in experimental control and treated rabbits as compared to normal ones. In the beginning the values of experimental control and treated groups were almost same but with the passage of time the treated rabbits recovered from disease and values became almost normal as compared to experimental control. This normal trend showed that plants *A. vera*, *P. granatum* and *A. indica* were effective against the toxicity of protoscoleces. The decrease in values may be due to affected lysosomal acid phosphatase.

GOT is found in high concentration in liver, heart, kidney, skeletal muscle and pancreas while GPT is much abundant in liver. Results of present investigation showed increased GOT, GPT in treated and decrease in experimental control rabbits. After herbal treatment these values improved in treated rabbits somewhat close to the normal values that may be due to certain inhibitory effects of plants against protoscoleces or due to pathological response of the hepatocytes instead of induction (Sanchez and Sanchez, 1971), or hemolysis of blood or gentamycin's effect or skeletal muscle damage (Khan, 1990). However, Frayha and Haddad (1980) reported that enzymes present in the protoscoleces could alter the permeability of cell membrane of hepatocyte due to which level of transaminase became high in blood serum or it may be due to leaching of enzyme in blood serum after destruction of pathological cells formed by toxicity and enzyme activity of protoscoleces.

Bilirubin level is an index applied for detecting (*i*) liver disease (hepatic jaundice), (*ii*) hemolytic anaemia, and (*iii*) degree of jaundice. The extent of increase in bilirubin level determines the type of jaundice (Khan, 1990). In the present findings the bilirubin level increased in rabbits treated with *A. vera* and *A. indica* as compared to experimental control and values of rabbits treated with *P. granatum* showed decline but were more nearer to normal control as compared to experimental control. Increased bilirubin level can be attributed to some damage in kidney and liver cells which ultimately resulted in enormous RBCs breakdown and disintegration of haemoglobin molecules and obstruction in biliary tract (Khan, 1990). Decreased bilirubin level showed that although

production of haemoglobin in this group increased but the chemicals of *Punica granatum* have inhibited the breakdown of haemoglobin, thereby keeping the bilirubin at normal level. Increased bilirubin by toxic effect of protoscoleces in rabbits have also been reported by Anwar *et al.* (1997a).

Cholesterol is a component of cell membrane and precursor of the steroid hormones. It mainly occurs in the liver and synthesized by virtually all tissues in humans (Guyton and Hall, 1996). In the present study although cholesterol content increased in all the groups, but the values of treated rabbits were more nearer to the normal control as compared to the experimental control which showed that treatment with plants was a curing mechanism against hydatidosis. In present work increased cholesterol level indicated that it was not used in biosynthesis of steroid hormone. The increased cholesterol content can also be attributed either to worm load retained in host system or metabolic disturbance and immunobiochemical reactions of the host, or hormonal metabolism that have disturbed in *de novo* synthesis of cholesterol in liver and intestine (Vardhani and Rao, 1995).

Glucose forms different compounds in the body and it is instant source of energy. In the present investigation glucose level decreased most of the time thereby resulting in hypoglycemia in all normal control, experimental control and treated groups, but the values of glucose in treated groups orally given *A. vera*, *P. granatum* and *A. indica* became close to normal control as compared to experimental control. It may be suggested that glucose level decreased because it might be forcefully catabolized by the aerobic and anaerobic enzymes present in the protoscoleces (Agosin *et al.*, 1957).

Anonymous (1985) and Schwabe (1986) observed the economic losses due to hydatidosis in domestic livestock through reduced quality and yield of milk, meat and retarded growth. Iqbal *et al.* (1989) observed that due to hydatidosis protein deficiency results in infected animals. Decreased protein values may be due to direct proteolytic effect of hydatid cyst fluid as it contained many lytic enzymes (Frayha and Haddad, 1980) or due to some interference in the process of synthesis in liver or due to some malfunctioning of kidney (Khan, 1990). However, increased protein level may be attributed to the formation of antibodies against the antigen present in the protoscoleces inoculated in the rabbits. In the present investigation total proteins increased in normal control, experimental control and treated rabbits orally given *A. vera*, *P. granatum* and *A. indica* respectively which means that treated rabbits develop resistance against protoscoleces. This means that plants were effective in improving the total protein in treated groups as compared to experimental control. This increase in total protein may be due to i, increased seepage of soluble protein including enzymes through the abnormal liver parenchymal cells, which affects the cell membrane permeability; ii, due to increased protein synthesis in the liver; iii, utilization of free amino acid contents for energy needs in the absence of glucose oxidation (Abdel-Salam et al., 1982); or iv, as a result of some catabolic reactions protein synthesis yet arrested and amino acids which are absorbed are not incorporated and hence level of total protein increases. Another strong reason for the increased protein contents is the heavy inflow of proteins through the regularly inoculated protoscoleces.

In the present work, results of all biochemical parameters indicated that plants *Aloe vera*, *Punica granatum* and *Azadirachta indica* are equally effective against hydatidosis and showed the curing mechanism in rabbits infected with protoscoleces. This can provide basis for the treatment of hydatidosis which can secure man and his livestock. Among the plants tested in the present investigation, *A. indica* have also been reported as effective protoscolecidal by Anwar *et al.* (1997). Keeping in view their findings role of *A. indica* was further checked in curing hydatidosis. In the end it can be suggested that these plants were equally effective in curing hydatidosis and further investigations are needed to find out their active ingredient.

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A STUDY OF CUTANEOUS LEISHMANIASIS IN BALOCHISTAN, PAKISTAN: A FORGOTTEN DISEASE OF THE TROPICS

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Abstract: Fifty cases, Army soldiers, with cutaneous leishmaniasis were observed during a survey carried out (1995-96) by a joint team of Armed Forces Medical College and Defence Science and Technology Organization (DESTO) in different areas in the Province of Balochistan. Causes of prevalence as well as suggestive measures for prevention are also discussed.

Key words: Leishmania, protozoan parasite, gross pathology.

INTRODUCTION

Cutaneous leishmaniasis is endemic in more than 80 countries, notably those of South-West Asia (Desjeux, 1996). It attacks 400,000 new people every year and currently afflicts 12 million people in the world. It is the disease ignored as much by science as by the general public. Its victims are largely the rural poor population of the tropics and sub-tropics.

It is an important public-health problem for the Army personnels stationed in Baluchistan, Pakistan, in whom the disease often reaches the level of an epidemic. Its incidence is high in the winter and early spring, because this is the period when troops camp outside in the field, as a result the man vector-contact is augmented in zoonotic foci of the disease. Successful infection of a mammalian host by the human pathogen *Leishmania* involves the promastigote form inoculated by the sandfly vector of the subgenus *Phlebotomus* (Le Blancq *et al.*, 1986), invading a macrophage, transforming to the amastigote form and surviving against the microbicidal activity of the host cell.

Balochistan, the southwestern Province of Pakistan, is spread over an area of about 347,000 km^2 which comes to about 43.6% of the total land area of Pakistan. The whole province with the exception of a few areas, comprises arid zones with marked diurnal variation in temperature, dry barren rocks or sandy deserts. This province is famous for its arid character and lies outside the monsoon range. The province also faces extreme summer and winter temperatures. In summer it frequently touches 50°C in Kachhi, Turbat and Nokundi whereas in winter it falls down to -15°C in certain area of Kalat. The humidity level is generally low throughout the province. Rodents, canids, caprids, gazelles and various partridges are important animal groups inhabiting this province. Such animals do not spread the disease to humans, but do provide a reservoir of the

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parasite from which the sandfly draws the parasite. Leishmaniasis is one of those interesting conditions in which the lower animals play an important role as reservoir hosts. Cutaneous leishmaniasis locally called as "Quetta" or "Kandahar Kaldana" prevails over several areas in this province. Jan (1984) reported cases of leishmaniasis from local people and Afghan refugees residing in this province, belonging to very low socio-economic group of population, comprising males, females and children.

Much of the research concerning the epidemiology of leishmaniasis has been done in the former Soviet Central Asia (Kozevnikov, 1941; Latyshev and Krivkova, 1942). Nothing has been done uptil now on the epidemiology of this disease in Pakistan.

MATERIALS AND METHODS

This survey was conducted from September 1995 to February 1996 at Combined Military Hospital (CMH) Quetta and M.I. Rooms of Sibi, Maiwand, Kohlu, Lorali and Dera Bughti by a joint team of Armed Forces Medical College (AFMC) and Defence Science & Technology Organization (DESTO). Fifty cases of cutaneous leishmaniasis were examined. Data collected from patients were according to age, ethnic group type, number, location and duration of the lesions and the localities where infection was acquired. After taking a brief history, representative lesions were cleared with 70% alcohol. The diagnosis was parasitologically confirmed by amastigote - positive Giemsa/ Leishman stained smears from the lesions. Biopsy specimens collected by needle were inoculated into modified Tobie's medium (Evans *et al.*, 1984) or "NNN" medium in the laboratory. For culture studies, specimens incubated at 22°C for upto one month with periodic examination for promastigotes. 85% of the isolates aspirated from the patients' lesions became promastigotes-positive.

The leishmanin test was also done on the spot. Leishmanin is a suspension of 10^6 washed promastigotes of leishmania per ml of 0.5% phenol in saline. An intradermal injection of 0.1 ml on the volar surface of the fore arm is examined after 48-72 hours. Induration is measured by the "ball-point-pen" technique and recorded. A positive test indicates delayed hypersensitivity to leishmanial antigens.

OBSERVATIONS

Fifty cases of cutaneous leishmaniasis were examined in the winter months of the years 1995-96 (Table I). All were males belonging to different age-groups serving in Pakistan army admitted in the Combined Military Hospital, Quetta and M.I. Rooms of visited areas. Many of the patients had multiple lesions and the most common sites were forearms, hands and fingers of feet. By caste the patients were Punjabis and Pathans. Both age and caste factors were observed. The results are shown in Table II.

Caste-wise overall incidence was observed as 72% in Punjabi and 28% in Pathan groups (Table II).

Age was also considered as epidemiological factor, the results observed are given in Table II-A, showing that young people are more susceptible than older ones. The infection was found as 58% in younger group, 34% in middle group and 10% in older group of the patients.

Within each age-group (Table II-B) caste-wise infection was about 32% in Punjabis and 24% in Pathans in younger group, 30% in Punjabis and 4% in Pathans in middle group and 10% only in the elder group of Punjabis. None of them was local resident of this province. This shows that non-immune expatriates appear to be particularly at risk (Al-Gindan *et al.*, 1984).

Table I: Summary of 50 cases of cutaneous leishmaniasis

Sr. No.	Age (years)	Caste	Site of infection	Duration	Gross pathology
1.	45	Punjabi	Left wrist	1 year	Ulcer
2.	34	Pathan	Right forearm	1 year	Nodule
3.	32	Pathan	Both forearms	1 year	Ulcer
4.	36	Punjabi	Left forearm	8 months	Ulcer
5.	30	Pathan	Left forearm	9 months	Ulcer
6.	38	Punjabi	Forehead	9 months	Ulcerated nodules
7.	30	Punjabi	Right hand	9 months	Ulcerated nodules
8.	44	Punjabi	Toes of feet	1½ years	Ulcer
9.	21	Punjabi	Left iliac region	4 months	Plague
10.	40	Punjabi	Dorsum wrist	6 months	Ulcer
11.	42	Punjabi	Left small finger	2 years	Ulcer
12.	24	Punjabi	Cheek	6 months	Papule with crusting
13.	25	Punjabi	Right arm	4 months	Ulcer
14.	28	Punjabi	Left thigh	6 months	Ulcer
15.	25	Punjabi	Right ear lobe	6 months	Ulcer
16.	40	Punjabi	Left foot	1 year	Ulcer
17.	40	Punjabi	Right foot	1 year	Ulcer
18.	32	Punjabi	Right lower leg	1½ years	Ulcer
19.	28	Punjabi	Left upper arm	10 months	Ulcer
20.	13	Punjabi	Right hand	9 months	Ulcer
21.	30	Punjabi	Calf of left leg	9 months	Ulcer
22.	24	Pathan	Neck	8 months	Ulcer
23.	26	Punjabi	Left wrist	6 months	Ulcer
24.	33	Punjabi	Right arm	5 months	Ulcer
25.	26	Pathan	Left forearm	5 months	Ulcer
26.	27	Punjabi	Elbow	5 months	Ulcer

Continued on the next page

27.	27	Punjabi	Toes of left foot	4 months	Ulcer
28.	22	Pathan	Right hand	3 months	Ulcer
29.	26	Pathan	Toes of right foot	3 months	Ulcer
30.	25	Pathan	Left thumb	2 months	Ulcer
31.	26	Punjabi	Cheek	4 months	Ulcer
32.	24	Pathan	Left scapular region	3 months	Ulcer
33.	30	Pathan	Neck	1 year	Ulcer
34.	28	Pathan	Left wrist	1 year	Ulcer
35.	40	Punjabi	Right ear lobe	1½ years	Ulcer
36.	31	Punjabi	Elbow	2 years	Ulcer
37.	34	Punjabi	Right wrist	1 year	Ulcer
38.	40	Punjabi	Toes of right foot	1 year	Multiple ulcer
39.	42	Punjabi	Elbow	1 year	Single sore
40.	23	Pathan	Left upper arm	9 months	Ulcer
41.	32	Punjabi	Left thumb	7 months	Ulcer
42.	35	Punjabi	Neck	6 months	Ulcer
43.	26	Pathan	Forearm	5 months	Multiple ulcer
44.	26	Punjabi	Left hand	1 months	Ulcer
45.	25	Punjabi	Elbow	6 months	Ulcer
46.	22	Punjabi	Right arm	4 months	Ulcer
47.	24	Punjabi	Toes of right foot	4 months	Multiple ulcer
48.	22	Pathan	Both forearms	2 months	Multiple ulcer
49.	27	Pathan	Toes of both feet	6 months	Multiple ulcer
50.	31	Punjabi	Both forearm	2 months	Multiple ulcer

As far as survey of local people are concerned very little information exists. Jan (1984) examined about 100 patients including males, females and children both from local population and Afghan refugees. Out of 100 patients, 75 were children. Most of them had more than one lesion, most commonly on cheeks, nose, legs and hands. All of the patients belonged to a low socio-economic group.

Now the question is why it is more common in this Province of Pakistan? It needs investigation to elucidate parasitological, entomological and overall epidemiological studies in this Province. Bray (1974) reported that *L. major* is widely distributed across the old world in arid tropical and sub-tropical regions, accounting for much zoonotic cutaneous leishmaniasis (ZCL), although it is not only aetiological agent of old world ZCL. The factors which are responsible for the prevalence of this disease specially in this province are described below:

Agricultural activities are very little as most of the land is barren, arid or sandy providing suitable habitats for insects especially sandflies and other wild fauna acting as carriers, vectors or reservoirs for this as well as many other diseases.

Table II: Showing age-wise and caste-wise incidence of cutaneous leishmaniasis observed in the Province of Balochistan

A. Age-wise overall incidence:

	21-30 yrs	31-40 yrs	41 and above
Total number of patients	28	17	5
Percentage	56%	34%	10%

B. Caste and age-wise incidence:

	Punjabi (21-30 yrs)	Pathans (21-30 yrs)	Punjabi (31-40 yrs)	Pathans (31-40 yrs)	Punjabi (41 and above)	Pathans (41 and above)
Total No. of patients	16	12	15	2	5	-
Percentage	32%	24%	30%	4%	10%	-

C. Caste-wise overall incidence:

	Punjabi	Pathan
Total number of patients	36	14
Percentage	72%	28%

As this disease is very common in Afghanistan (Nadim *et al.*, 1979) and Iran, from both these countries, the free movement of refugees throughout Pakistan with their cattle, dogs and cats is responsible for spreading and exposure to leishmaniasis.

Most of the wildlife have also migrated from Afghanistan towards Pakistani areas near the boundary line which may be called not only the reservoir of leishmania but many other zoonotic diseases.

Rodent burrows provide ideal resting and breeding sites for sandflies (Naumov and Lobachev, 1975), since the internal microclimate is humid and stable in an otherwise arid environment. Thus, the sandfly vectors live in intimate association with these rodents that are potential host.

The situation of the Arabian Sea in the South of the Province has also facilitated the transmission of this disease from the Mediterranean and Middle East countries.

Health units as well as trained parasitologists are not available in this Province who can initiate research on this problem.

The medical doctors who hesitate to go and serve in the far flung areas of this Province are also responsible for this health problem.

Suggestive measures

a. The life cycle of leishmania involves an insect *i.e.*, sandflies of the genus *Phlebotomus*, serving as a vector, a rodents or canids serving as reservoirs or carriers and human beings as a final definitive host and hence representing a dreadful zoonotic problem which needs a detailed study on biological and epidemiological aspects of this disease. The basis of epidemiological studies must be adequate identification of the parasites.

b. Effective insecticide and rodenticide spray programmes should be started, keeping in view the side effect of these chemicals on wild fauna and flora. The spraying of such insecticides as DDT during anti-malaria campaigns also cuts down sandfly numbers. But the use of such chemicals against malaria has fallen off as mosquitoes grew resistant.

c. Mobile squads consisting of veterinary scientists, wildlife experts should patrol the natural habitats and the endemic areas for providing the health cover needed.

d. Dogs and cats positive for this parasite should be killed and buried in the ground. Chinese officials claim a programme of rounding up stray dogs and dissuading the population from keeping pets has eradicated the internal form which once menaced China.

e. A detailed programme should be initiated on zoonotic problems of this country at various R&D organizations. Soviet Union and Israel have elaborated a vaccine against particular species of leishmania; in our country planning would be done to develop an effective, standardized and safe vaccine producing long-lasting immunity against all types of leishmaniasis.

f. Public awareness should be created about all parasitic diseases by different mass media in the country and translated in all local languages.

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PREVALENCE OF GASTRO-INTESTINAL PARASITES IN SHEEP AND GOATS SLAUGHTERED AT LAHORE ABATTOIR

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Abstract: The prevalence of Gastro-intestinal parasites of both sheep and goats brought for slaughtering at Lahore abattoir was studied. The overall prevalence was recorded to be 92.35% including 28.13% infected with one species while 64.22% infected with more than one species of parasites. During this study *Haemonchus contortus* (39.41%), *Trichostrongylus* spp. (22.94%) and *Fasciola hepatica* (14.11%) were found major parasites in these animals. The effect on various blood parameters showed a decrease in total leukocytic count, haemoglobin level and increase in erythrocyte sedimentation rate values in infected sheep and goats.

Key words: GIT infection, parasites, haematology, caprine.

INTRODUCTION

Pakistan is an agricultural country and most of the population is dependent on agriculture and animals along with their allied industries, to which sheep and goats have a significantly important portion. Sheep and goats are not only main source of protein but their products such as bones, skin, hides, hair and goods made from them fetch more than 4 billion rupees annually in the form of foreign exchange having sizable contribution in GNP. In Pakistan, the sheep population was 28.3 million and goats population was 34.2 million (Durrani and Khan, 1993). About 90% of livestock population in our country has various parasitic diseases (Rauf, 1984). Very little attention has been paid towards their health and production status. The monetary losses due to sheep and goat mortality caused by parasitic diseases were estimated about Rs.80,000 in Punjab only (Choudhry and Khan, 1984). These parasites caused watery diarrhoea, weakness, weight loss, damage to wool production and leads to other secondary infections. The most important factor affecting the sheep health in the country is the parasitic infection (Sarwar, 1963). In Australia, the losses caused by parasites to sheep industry were over 7,000 Australian dollars per farm and half of these losses were due to internal parasites, causing damage to wool production (Beck *et al.*, 1985). These parasites are responsible for decrease in total leukocytic count, haemoglobin and packed cell volume in infected animals (Singh *et al.*, 1984). No study seen to have been undertaken to assess the magnitude of this problem in Lahore area. This article showed the prevalence of gastro-intestinal parasites and haemotological studies of both infected and non-infected sheep and goats slaughtered at Lahore abattoir.

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MATERIALS AND METHODS

This study was conducted on 170 animals of both sheep and goats irrespective of age and sex brought for slaughtering at Lahore abattoir. Faecal samples from 170 sheep and goats were collected directly from the rectum. All the samples were subjected to salt floatation method (Soulsby, 1982) and examined carefully under the low power of the microscope for the presence/ absence of parasites eggs. Identification of parasites ova was done following Thiepont *et al.* (1979). For haemotological study 5 ml of blood was collected directly from the jugular vein before slaughtering and put into a test-tube containing a few drops of 1% EDTA. Total leukocyte count (TLC) was determined according to the method suggested by Benjamin (1978). Haemoglobin (Hb) estimation was conducted by using Kit method (Cyanmethemoglobin method). Erythrocyte sedimentation rate (ESR) was determined according to Wintrobe's method.

RESULTS AND DISCUSSION

A total of 170 faecal samples were studied for the prevalence of gastro-intestinal tract (GIT) parasites and 157 were found positive with parasitic infestation. The overall prevalence of GIT parasites were recorded 92.35%. During this study, 7 species of GIT parasites were found. Name of the different species with their relative prevalence was presented in Table I. Amongst the nematodes the prevalence of *Haemonchus contortus* was recorded to be the highest (39.41%) followed by *Trichostrongylus* spp. (22.94%). Amongst the cestodes, prevalence of *Moniezia expansa* (6.47%) was the highest. The results are not in agreement with Durrani *et al.* (1981) who recorded an overall incidence of 73.33% and 60.29% in sheep and goats, respectively in NWFP province. Khan (1993) found ten species of GIT parasites and recorded 88.47% overall incidence in both species in Rawalpindi area. The difference in prevalence might be due to variation in the geo-climatic conditions of these areas of study. The results of haemotological studies are presented in Table II in both infected and non-infected animals. The result revealed that these parasites caused decrease in TLC, Hb and increase in ESR values in infected sheep and goats. The results are in agreement with

Table I: Prevalence of different species of GIT parasites in sheep and goats slaughtered at Lahore abattoir

Species of parasites	Infected samples (n=157)	Infection incidence (%)
Haemonchus contortus	67	39.41
Trichostryngylus spp.	39	22.94
Fasciola hepatica	24	14.11
Chabertial ovina	13	7.64
Moniezia expansa	11	6.47
Dictyocaulus filaria	3	1.76

Rowlands and Clampitt (1979) and Hawkins (1984) who found drop in concentration of Hb in infected sheep and goats. Furmage *et al.* (1975) studied white cell system in the course of experimental fascioliasis in rabbit and sheep and recorded increase in number of cosinophilic and neutrophilic leukocytes while drop in lymphocytes. Ajaz (1993) studied decrease in TLC, Hb and PCV values in parasitic infection.

Table II: Haematological values of sheep and goats suffering from GIT parasites

Parameters	Infected (n=157)	Non-infected (n=13)
Total leukocytic count (10^3/mm)	6.25±0.51	9.81±0.72
Lymphocytes (%)	48.62±3.01	52.20±2.11
Neutrophils (%)	41.09±1.29	45.96±1.08
Monocytes (%)	2.13±0.12	3.29±0.11
Eosinophils (%)	9.40±1.30	4.17±0.24
Basophils (%)	1.16±0.02	2.13±0.03
Hb (g/dl)	8.54±1.42	14.26±1.04
ESR (mm/hr)	3.20±0.43	1.07±0.08

Abbreviations used: Hb, haemoglobin; ESR, erythrocyte sedimentation rate.

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DEVELOPMENTAL DEFECTS INDUCED BY METHAMIDOPHOS IN CHICK EMBRYOS

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Abstract: The embryotoxic and teratogenic potential of methamidophos, an organophosphorus insecticide, was tested in chick embryos. Different concentrations of methamidophos ranging from 3 to 15 μg per egg were injected into the yolk of chick eggs before incubation. A higher embryonic mortality was observed in dose groups of 9, 12 and 15 μg per egg. Besides reduced body size the gross malformations including microcephaly, exencephaly, anophthalmia, micromelia, short neck and beak, twisted spinal cord and ectopia cardis were found in treated embryos. These malformations were more severe in higher dose levels.

Key words: Organophosphorus insecticide, methamidophos, embryotoxic, teratogenic, *Gallus domesticus*, chick, birds, aves.

INTRODUCTION

With the diminished utilization of chlorinated hydrocarbons, organophosphorus insecticides have started being used quite heavily (Davis and Richardson, 1980). These insecticides are considered relatively safe, especially in the sense of these being biodegradable and thus non-cumulative. Unfortunately, however, these compounds have also been seen to be quite harmful to the non-target organisms (Durham and Williams, 1972; Jennings *et al.*, 1975; Harbison, 1975). A survey by WHO tested about hundred organophosphorus insecticides and found "acute toxicity for (non-target) experimental animals" (WHO report, 1988). A study in Pakistan also showed that in one medical unit there were 755 cases of organophosphorus insecticide poisoning reported, which were almost 40% of the total poisoning cases (Jamil, 1989). The harmful effects of these compounds have been primarily attributed to their acetylcholinesterase (AchE) inhibition properties (Harbison, 1975; Richardson, 1983). Ishikawa *et al.* (1975) reported that acetylcholine induced cardiac anomolies in nine of 23 chick embryos at a total dose of 20 mg given for a period of 3 hours and 20 minutes. The anomolies induced ventricular septal defect, atrial defect and double aortic arch. Some other studies have also shown that many of the

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commonly used organophosphorus insecticides are embryotoxic in birds. For example, it was discovered that Phosphamidon not only caused brain defects, dwarfism and stunted growth in chick embryos (Mufti and Dad, 1977), but many internal organs such as heart and kidneys were also adversely affected (Mufti and Nasim, 1987).

All these studies indicated that organophosphorus insecticides are potentially dangerous to the avian embryos due to their potential teratogenic properties. It is quite apparent that much more research will have to be carried out before we know more specifically, about the effects of these chemicals on the unborn and thus plan and design ways and means for the use of these insecticides in the most appropriate way. The present research was designed as a step in that direction.

MATERIALS AND METHODS

One hundred and forty fertilized eggs of the white leghorn breed of *Gallus domesticus* were obtained from the Veterinary Research Institute, Lahore. These eggs were divided into 7 groups, each of 20 eggs. Five groups were administered with 0.1 ml/egg of different aquous concentrations of methamidophos (60 SL, Welgreen Chemical Ltd.) containing 3, 6, 9, 12 and 15 μg insecticide. The other two groups which were vehicle control (VC) and control (C) were treated with 0.1 ml/egg of distilled water and no treatment, respectively. The eggs were randomly selected and cleaned with a piece of cotton soaked in 70% alcohol. A small window, for the insertion of needle, was made in shell of each egg (except the C group eggs), without rupturing the shell membrane. With the help of a micro-applicator, 0.1 ml of each concentration was injected into the yolk of each egg under sterile conditions. After injection, the hole in the egg shell was immediately sealed with liquid paraffin wax. After administration of the insecticide the eggs along with VC and C groups eggs were placed in an incubator adjusted at 38 ± 0.5°C. Humidity was provided by placing a water filled beaker in each shelf of the incubator. The eggs were rotated twice a day.

The embryos were taken out of the eggs on 7th day of incubation, observed for the live/dead embryos, fixed in freshly prepared Bouin's fixative, cleared in 70% alcohol and preserved in 80% alcohol for morphological and morphometric studies. The embryos were studied macroscopically and microscopically for teratological observations. The selected embryos were macrophotographed with the help of a zoom-lens fitted camera.

RESULTS

There was 100 percent embryonic mortality at doses of 9, 12 and 15 μg per egg, while at comparatively lower doses of 3 and 6 μg per egg, embryonic mortality was 45 and 78%, respectively (Table I). All the embryos recovered from treated groups were abnormal. The rate and severeness of malformation increased with an increase in dose. The crown-rump length was decreased significantly ($P > 0.001$) in all treated embryos (Table I). The teratogenic effects of this insecticide were observed all over the body of the embryos in all dose groups. Microcephaly was observed in all treated embryos.

Table I: Developmental anomalies induced by methamidophos in embryos of *Gallus domesticus*.

Parameters	Methamidophos treatment (μg/egg)						
	C	VC	3	6	9	12	15
No. of eggs treated	20	20	20	20	20	20	20
No. of embryos recorded	18	18	20	18	18	20	20
Dead/resorbed embryos (%)	0.00	0.00	45.00	78.00	100.00	100.00	100.00
Abnormal embryos (%)	11.11	11.11	100.00	100.00	100.00	100.00	100.00
CR length (mm±S.D)	17.28 ±0.59	17.41 ±0.38	9.63*** ±1.81	10.58*** ±1.98	9.09*** ±2.35	8.94*** ±2.83	8.77*** ±2.34
Abnormal brain parts (%)	0.00	0.00	45.00	83.83	83.83	100.00 (25)*	95.00 (5)
Defective eye (%)	0.00	0.00	80.00	88.88	88.88	95.00 (5)**	100.00
Defective beak (%)	0.00	0.00	80.00	94.44	94.44	100.00	100.00
Short neck (%)	0.00	0.00	75.00	100.00	100.00	100.00	100.00
Twisted spinal cord (%)	0.00	0.00	75.00	94.44	100.00	100.00	95.00
Ectopia cardis (%)	0.00	0.00	75.00	88.88	94.44	85.00	100.00

*, Exencephaly; **, Anophthalmia; ***, Significantly decreased ($P>0.001$); C, Control; VC, Vehicle control; CR, Crown-rump.

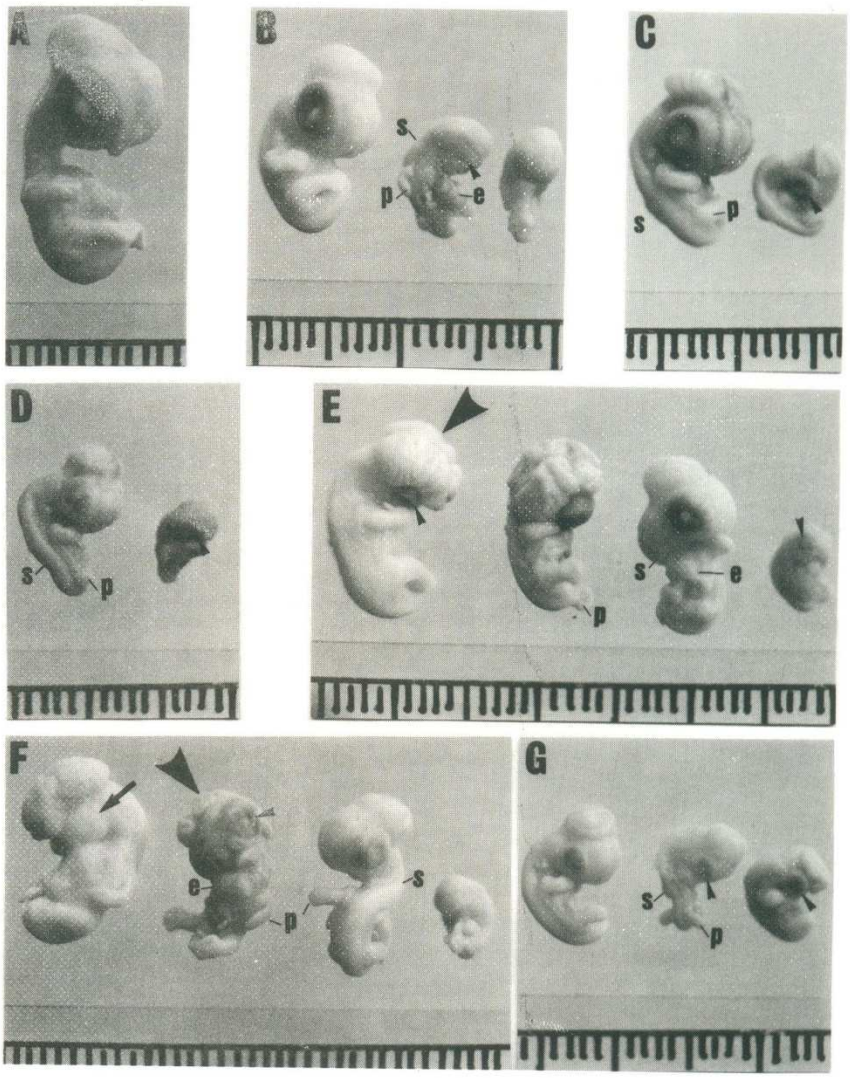

Fig. 1: Chick embryos extracted from eggs of experimental groups incubated for 7 days. (A) An embryo from control group with normally developed body organs; (B) embryos from dose group of 3 μg/egg; (C) 6 μg/egg; (D) 9 μg/egg; (E,F) 12 μg/egg; (G) 15 μg/egg. Note: abnormal development of body organs induced by methamidophos: ectopia cardis (e), peddle shaped limbs (p), twisted spinal cord (s), exencephally (large arrow head), degenerated eye (small arrow head) and anophthalmia (arrow).

Twenty five percent embryos with exencephaly were found at dose of 12 μg per egg (Table I; Fig.1E,F). Eyes were developed abnormally with a case of anophthalmia in 12 μg per egg dose group (Fig.IF). A higher percentage of gross abnormalities including short neck, micromelia, everted viscera, ectopia cardis, reduced beak size and twisted spinal cord was found in all treated embryos (Table I; Fig.1).

DISCUSSION

In all dose groups a higher embryonic mortality and severe embryonic resorptions were noted. The gross morphological and anatomical abnormalities including microcephaly, exencephaly, anophthalmia, abnormal neck, micromelia, everted viscera, ectopia cardis, short beak and twisted spinal cord were found almost in all dose levels tested during this study (Table I; Fig.1). In spite of being comparatively safe, organophosphorus insecticides are harmful and dangerous to embryonic development which has been found during the present study. This also has been supported by many studies already carried out to investigate harmful effects of this group of insecticides on embryonic development. Many studies have further shown that these insecticides may also be teratogenic. The harmful effects of these compounds especially to avian embryos have been shown quite convincingly (Khera, 1966; Khera and Bedok, 1967; Meiniel and Autossier-Navarro, 1980; Fishbein, 1975; Meiniel, 1976; Sternberg, 1978; Wyttenbach and Thompson, 1985). In most of these studies it has been shown that even very small quantities of organophosphates induced gross embryonic malformations which included microcephaly, eye catracts, ascites, hepatic degeneration, micromelia, ectrosyndectyly and many other musculo-skeletal abnormalities. Khera and Bedok (1967) studied teratogenicity of diazinon in ducks. They injected diazinon into yolk sac before incubation or four days after incubation at the rate of 1 mg/egg. The vertebral column was found to be twisted, shortened and composed of abnormal vertebral bodies. The teratogenic effects of diazinon with regard to skeletal development, particularly extremities and vertebrae were also 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.

Henderson and Kitos (1982) and Kushaba-Rugaaju and Kitos (1985) studied the effects of diazinon on nucleotide and amino acid contents in chick embryos. Teratogenic dose of diazinon was administered by the intravitelline route of chick egg at day 3 of incubation. Analysis by day 10 of development showed that the levels of free tryptophan and histidine were decreased while the levels of threonine and aspartic acid were increased. All other amino acids, however, remained unchanged in response to Diazinon. NAD+ contents of the hindlimbs of embryos also showed decrease by day 10, which further decreased by day 15 and severe type I and type II teratogenic responses were observed (Henderson and Kitos, 1982). The presence of tryptophan served to maintain the NAD+ levels of diazinon in treated embryos close to normal. A possible involvement of tryptophan in inducing micromelia, parrot beak and abnormal feathering in chick embryo is considered (Kushaba-Rugaaju and Kitos, 1985).

Many recent studies have shown that diazinon is both embryotoxic and teratogenic in mice, if it is orally administered during pregnancy. A dose level of 1 mg/g body wt.

of diazinon proved 100% lethal to pregnant mothers. A dose as low as 50 μg/g body wt. resulted into a tendency in embryos towards dwarfism and decrease in body weight. Defects in spinal cord, ventricular septum and myocardial atrophy were common observations made by Mufti and Asmatullah (1991). Malathion, Malaoxon, Parathion and Paraoxon caused dose-dependent defects, such as abnormal pigmentation, abnormal gut development, notochordal defects and reduced growth in African clawed frog (Snawder and Chambers, 1990). Greenberg and LaHam (1969) found that Malathion caused shortening of hindlimbs, shortening of plumage and beak defects in chick embryos.

The results of this study suggest that methamidophos is potentially dangerous to chick development under present experimental conditions.

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REGENERATION OF EXTENSOR DIGITORUM LONGUS MUSCLE GRAFTS IN TESTOSTERONE PROPIONATE SUPPLEMENTED RATS*

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Abstract: Exogenous administration of testosterone propionate to normal male rats accelerated the process of skeletal muscle fibre's regeneration following orthotopic transplantations of extensor digitorum longus muscles. At one-week postgrafting period the hormone supplemented muscle grafts were found to attain significantly higher average cross-sectional area of the regenerated muscle fibres than those in the control transplants. The steroid exerted its pronounced anabolic effects during the early phase of muscle regeneration and the experimental grafts looked also better in terms of less deposition of connective tissue and earlier completion of degeneration-regeneration course than the control regenerates. In the later stages of regeneration cross-sectional areas of the regenerated muscle fibres were comparable in both categories of the grafts, however, number of the regenerated muscle fibres appeared higher in the hormone supplied transplants accompanied with little connective tissue deposition.

Key words: Androgens, muscle transplantation, testosterone propionate.

INTRODUCTION

Damaged skeletal muscles regenerate and regain structural and functional characteristics of varying degrees depending on amount and nature of the injury incurred (Carlson, 1985; Brooks and Faulkner, 1990; Islamov *et al.*, 1991; Zacharias and Anderson, 1991; Robertson *et al.*, 1993; Sesodia *et al.*, 1994). Likewise, skeletal muscles also regenerate following various types of graftings (Carlson and Gutmann, 1973; Mufti and Ali, 1977; Carlson *et al.*, 1983; Martin *et al.*, 1990; Qazi and Mufti, 1990; Zamir and Oron, 1993; Chuang *et al.*, 1994). Among the different grafting procedures the model of free skeletal muscle transplantation has been used widely in various experimental studies to understand the mechanism of the regeneration (Grim *et al.*, 1986; Phillips *et al.*, ,1987; Schultz *et al.*, 1988; Gulati and Swamy, 1991; Qazi and Mufti, 1997). Concerning clinical applications, free skeletal muscle transplantations have successfully been attempted in a number of cases to cure paralyses of different muscles (Thompson and Wynn-Parry, 1981), anal and urinary incontinences (Gierup and Hakelius, 1978; Hakelius *et al.*, 1978) and for upper limbs

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

reconstructions (Grotting *et al.*, 1990; Chuang *et al.*, 1994) in addition to many cosmetic surgeries.

At time of transplantation a freely grafted skeletal muscle is an avascular, denervated and tenotomized mass, kept within a bed of surrounding tissues. These conditions subject the graft to necrosis followed by appearance of regenerating muscle fibres within the transplant accompanied by revascularization and reinnervation processes (Carlson and Faulkner, 1983; Womble, 1983; Grim *et al.*, 1986; Carlson, 1989; Schultz, 1989). Ultimately, the regenerated skeletal muscle becomes integrated with its surroundings and starts functioning, however, the structural and functional recoveries of a mature regenerate are only about 50% of the original grafted tissue (Carlson and Faulkner, 1983; Faulkner and Côte, 1986; Baker and Poindextor, 1991).

For its wider clinical applications many efforts have been made to improve the quality of skeletal muscle regenerates. Such studies include ablation of muscles synergistics to the graft (Coan and Tomanek, 1981; Mufti and McNemar, 1986), addition of extra satellite cells or soluble factors from crushed muscle (Bischoff and Heintz, 1994) and application of laser irradiation (Bulyakova, 1992; Weiss and Oron, 1992). One of the ways considered to obtain better skeletal muscle transplants is to study the effects of anabolic hormones, especially androgens on the process of regeneration (Carlson and Faulkner, 1989; Qazi and Mufti, 1989, 1997; Ullman and Oldfors, 1991). Testosterone is a highly myotropic hormone (Allen *et al.*, 1983; Kuhn and Max, 1985) and its deficiency causes muscle atrophy (Miyamoto *et al.*, 1989). Role of this hormone in the process of skeletal muscle regeneration has been studied, in detail, for a testosterone dependent muscle, the levator ani (Max *et al.*, 1981; Mufti, 1985; Mufti and Chaban, 1987; Mufti and Raouf, 1990). Regarding the effects of androgens on regenerating skeletal muscles other than the levator ani, practically little information is available. However, Qazi and Mufti (1989, 1997) have reported that the extensor digitorum longus (EDL) muscle grafts regenerated poorly in orchidectomized rats and the transplants responded positively to exogenous supply of the hormone. The present study was undertaken to observe the effects of testosterone propionate on regenerating EDL muscle grafts in normal (uncastrated) rats. The information reported here is useful for improving the quality of skeletal muscle regeneration and adds further to the understanding of the mechanisms underlying this process.

MATERIALS AND METHODS

Experimental animals

Twenty-seven adult, *Rattus norvegicus*, males were used in this research. The rats weigh at start of the experiment was in the range of 121 to 210 g. They were kept in an animal house with roughly 12 hours dark/light cycle and fed on Lab. prepared food comprising of poultry feed, fish meal, wheat flour, molasses and water (see Ali and Shakoori, 1990). The animals were provided a constant supply of water.

Surgical procedure and hormone administration

The rats were anaesthetized with ether and othotopic transplantations of both EDL muscles were performed under disinfected conditions. The operated animals were supplied with 0.06% terramycin in drinking water for 3-4 days postoperative. Details of the surgical protocol have been mentioned elsewhere (Qazi and Mufti, 1997). Following the graftings of the muscles the rats were categorized into two groups. One set of the animals (TP) was injected with a solution of testosterone propionate in corn oil in an amount of 1.0 mg/100 g body weight (bw). Second group of the rats received 0.2 ml of corn oil/100 g bw and served as vehicle injected controls (cv). The drug and the vehicle were administered intraperitoneally, each day starting from the day of muscle transplantation.

Processing of grafts for various observations

The operated animals were anaesthetized as mentioned above and the EDL muscle grafts were recovered at each week within a period of one month postgrafting. The regenerates were isolated from the surrounding tissues removed out by cutting the tendinous connections and immediately immersed in 0.9% saline solution to protect them from drying. They were soon weighed and calibrated as mg/100 g bw. Tendons of both sides of the regenerates were then cut away, while mid portions were processed for histological and morphometric analyses. The tissues were fixed in Bouin's fluid for at least 4-6 hours and processed routinely for paraffin embedding and sectioning. Cross and longitudinal sections were cut at 8 μm on a Cambridge rotary microtome. The sections were subsequently stained with haematoxylin and eosin. Photomicrographs of representative sections were taken on a camera fitted microscope.

Morphometric analysis of the regenerated muscle fibres was performed by measuring average cross-sectional area of the fibres within graft. This procedure involved projection of six areas from a cross-section of a given regenerate onto paper with the help of camera lucida. While observing at 400X magnification through the microscope, outlines of individual regenerating/ed muscle fibres falling within 17x14 cm sample area on the paper were traced for each of the six areas. Magnification of the microscopic image on the paper was determined by projecting and tracing the divisions of a stage micrometer. Cross-sectional areas of outlines of the muscle fibres were then measured by a K-E polar Planimeter. Readings of the planimeter $(cm)^2$ were calibrated as $(\mu m)^2$ by the following formula:

$$\text{Cross-sectional area } (\mu m)^2 = \frac{\text{Planimeter reading} \times 10^8}{(\text{Magnification})^2}$$

Average cross-sectional area of regenerated muscle fibres was calculated for each cross-section representing a given sample. Statistical difference of the experimental EDL muscle grafts from the controls was then assessed by applying Student's 't' test.

RESULTS

Weight of animals and EDL muscle grafts

A gradual increase in weights of the control rats was observed throughout the study period and upto 2-week postgrafting there was no difference in the % body weight gain in the CV and TP rats. However, at 3- and 4-week stages the increase in body weights of the hormone supplemented animals was found significantly less than the respective controls (Table 1).

Table I: **Percent gain in the body weights (A) and percent recovery in weights of the EDL muscle regenerates (B) during the postgrafting periods in the control (CV) and testosterone propionate supplemented (TP) rats.**

Experimental group	Time postgrafting (weeks)							
	1		2		3		4	
	A	B	A	B	A	B	A	B
CV	10.47[a] ±0.96 (4)	65.77[b] ±4.26 (3)	18.41 ±3.39 (4)	39.58 ±0.62 (4)	33.67 ±4.83 (4)	40.91 ±6.39 (3)	49.99 ±5.90 (4)	32.53 ±2.62 (4)
TP	11.71 ±3.81 (4)	53.05 ±4.87 (4)	18.76 ±1.22 (4)	38.34 ±3.85 (3)	5.75** ±5.57 (4)	40.45 ±5.06 (4)	5.93* ±10.90 (4)	55.04* ±5.51 (4)

a: body weights taken in grams; b: weights of EDL muscles regenerates calibrated as mg/100 g b.w. Values are means ± SEM; asterisks show significant difference. * = $P<0.05$; ** = $P<0.01$. Number in parenthesis indicates sample size (Student's 't' test).

Weights of the muscle regenerates in the TP rats were significantly higher at 2- and 4-week stages, than the respective control transplants. Average weights of 2-week EDL muscle regenerates in CV and TP rats turned out as 22.45 ± 0.82 and 29.71 ± 2.81 (mg/100 g bw), respectively, while the corresponding figures for one-month old regenerates were 19.96 ± 1.34 and 32.23 ± 3.64. When grafts were calibrated as % of original mass, the 4-week TP transplants showed a significant increase as compared to the controls (Table I).

Histological observations

One-week EDL muscle grafts in the TP rats, looked quite better than the respective CV transplants (Fig.1A-F). One-week control graft contained a considerable number of centrally located necrotic and peripheral original surviving muscle fibres. Fascicles of regenerating muscle fibres in such grafts were seen well separated from each other with elaborate amount of connective tissue in between them (Fig.1A,C). The hormone supplemented transplants looked entirely different as there were no central necrotic muscle fibres, thus the degeneration phase had already completed its course and the

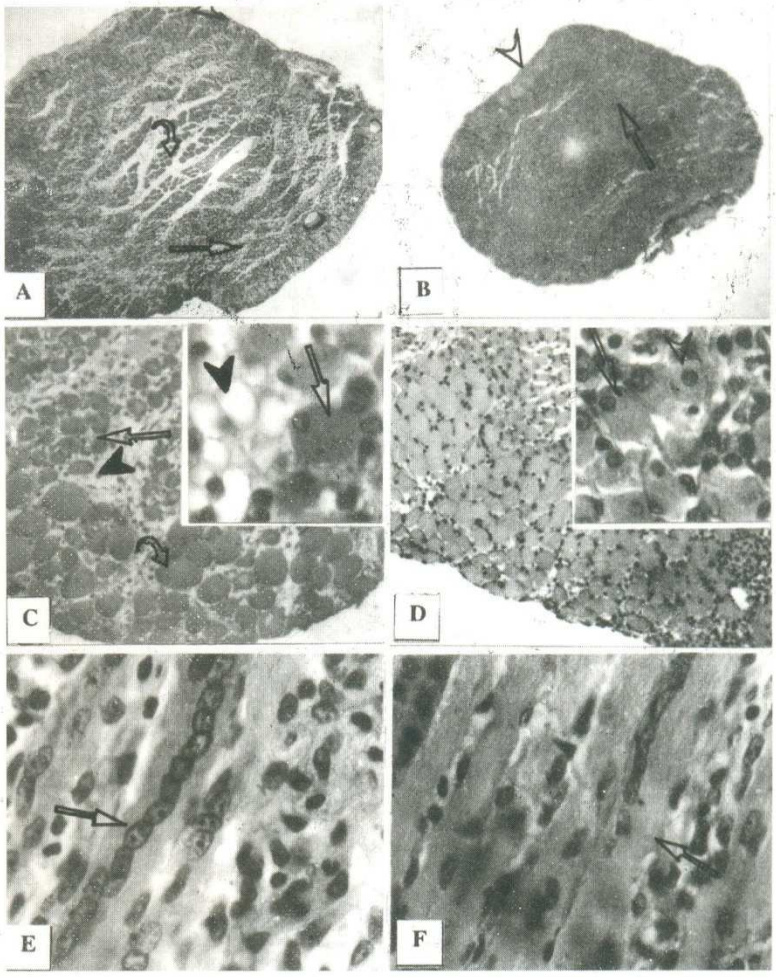

Fig. 1: One-week control (CV) and testosterone propionate supplemented (TP) EDL muscle grafts. A. CV graft showing regenerating (arrow), necrotic (curved arrow) and original surviving muscle fibres (o.s.m.f.) (arrow head). C.S. 40X. B. TP graft indicating regenerated muscle fibres (r.m.f.) (arrow) and tendon (arrow head), C.S. 40X. C. CV graft, showing fascicles of regenerating myotubes (arrow), (o.s.m.f.) (curved arrow) and connective tissue (arrow head), 100X. Inset: A well developed r.m.f. (arrow) and fat deposition (arrow head), C.S. 400X. D. TP graft showing fascicles of r.m.f. with very little connective tissue, 100X. Inset: Compactly arranged regenerated (arrow) and regenerating (arrow head) muscle fibres, C.S. 400X. E. CV graft, showing r.m.f. (arrow) and connective tissue, L.S. 400X. F. TP graft illustrating closely packed mature r.m.f. (arrow) with very little connective tissue, L.S. 400X. *Note*: All the sections were stained with H&E.

regeneration processes were operative well in advance as compared to the situation found in the control regenerates (Fig.1B,D). The muscle grafts in the TP rats were filled with compactly arranged regenerated muscle fibres, with scanty amount of connective tissue in between the fascicles, while regenerated muscle fibres were very thinly distributed and varied greatly in size in the control grafts which also contained extensive amount of connective tissue. Notable amount of adipose tissue formation was also observed in the CV transplants, but the situation was not encountered in the TP regenerates (Fig.1C,D). Longitudinal sections of the grafts also presented the same picture. The CV regenerates contained elaborate amount of connective tissue and the regenerating muscle fibres were observed at an early stage of regeneration; with chain of centrally located nuclei (Fig.1E). On the other hand, in TP grafts there was very little connective tissue and the transplants contained well developed and mature muscle fibres, with peripherally located nuclei (Fig.1F).

At 2-week stage the TP grafts contained relatively lesser amount of connective tissue and appeared to have many more regenerated muscle fibres (hyperplasia) than in the respective controls. These fibres, however, appeared to have cross-sectional areas comparable to the fibres in the control grafts (Fig.2A,B). At 3-week postgrafting the regenerated muscle fibres within the control grafts had undergone further growth and development and connective tissue had correspondingly decreased (Fig.2C). The situation was more or less similar in the TP grafts and the overall quality of muscle regeneration in such grafts did not differ much from the controls (Fig.2D). By the end of 4th week, the control as well as the hormone supplemented grafts were observed to contain developed, mature regenerated muscle fibres, compactly arranged into muscle fascicles. The fibres in both categories of the muscle regenerates at this stage appeared of about same dimensions. However, number of the muscle fibres in the TP grafts looked more than in the controls and they were found compactly arranged with polygonal morphologies in cross-sections. The two types of the grafts also differed in having the amount of connective tissue which was more in the CV transplants (Fig.2E,F).

Morphometric observations

The morphometric data were found in fair agreement with the histological observations. The average cross-sectional area (ACSA) of the regenerated muscle fibres in 1-week TP grafts was about 55% larger than in the control grafts. Except for the significant difference at this stage there were almost no differences between the ACSAs of the regenerated muscle fibres of the CV and TP grafts, for the remaining study periods (Table 2). It appeared that in the latter stages of regeneration the EDL muscle grafts responded to the hormone supply via hyperplasia (Fig.2C,D), while hypertrophy of the regenerated muscle fibres was observed in the earlier phase.

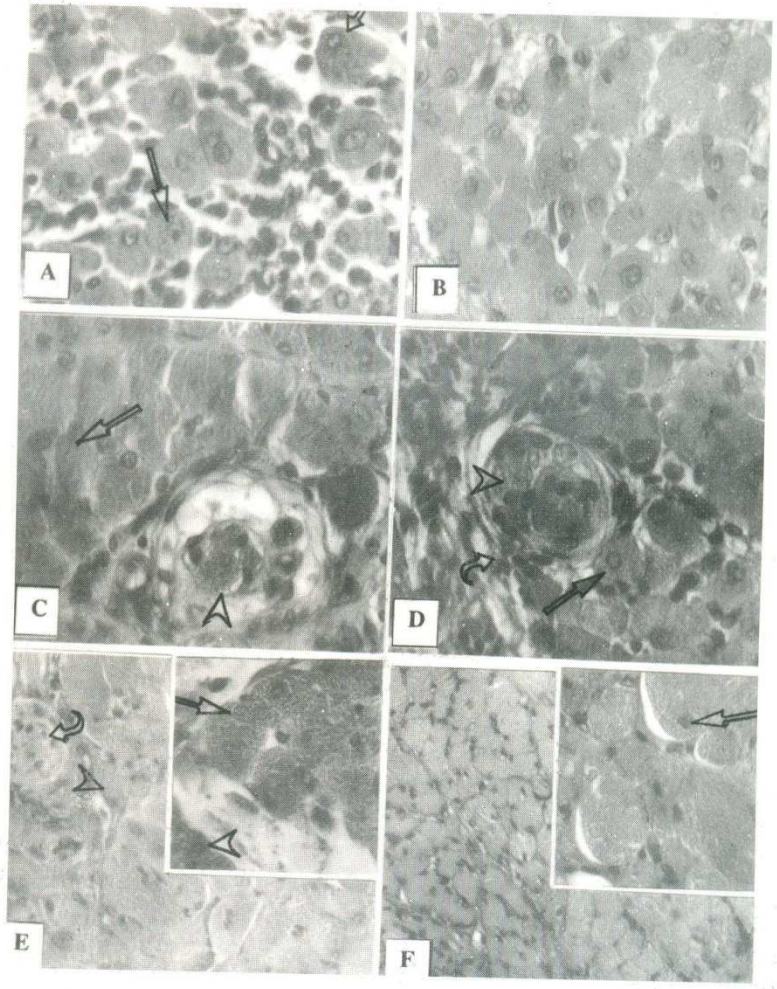

Fig. 2: A. Two-week CV graft, showing newly r.m.f. (arrow), some developed ones (curved arrow) and connective tissue, 400X. B. Two-week TP graft illustrating r.m.f. with very little connective tissue, 400X. C. Three-week CV graft showing r.m.f. (arrow) and a muscle spindle (arrow head), 400X. D. Three-week TP graft illustrating r.m.f. (arrow), connective tissue (curved arrow) and a muscle spindle (arrow head), 400X. E. Four-week CV graft showing mature r.m.f. fibres of smaller sizes (arrow head) and a nerve (curved arrow), 100X. Inset: A fasicle of developed r.m.f. (arrow) and some with centrally located nuclei (arrow head), 400X. F. A four-week TP graft, showing hyperplasia and polygonal shapes of r.m.f., 100X. Inset: Peripheral position of nuclei in mature r.m.f. (arrow) with very little connective tissue in between them, 400X. *Note*: All the photographs represent cross-sections stained with H&E.

Table II: **Average cross-sectional areas (μm)^2 of regenerated muscle fibres of the EDL muscle grafts in the CV and TP rats.**

Type of graft	Stages of regeneration			
	1 week	2 week	3 week	4 week
CV	121.73[a] ±17.23 [b]N=567 (3)	213.54 ±9.00 N=464 (3)	198.82 ±29.69 N=558 (3)	239.68 ±9.71 N=605 (4)
TP	189.09* ±16.34 N=379 (3)	196.87 ±22.01 N=658 (4)	182.59 ±40.06 N=467 (3)	215.37 ±35.56 N=770 (4)

a: Mean ± SEM; b: total number of the regenerated muscle fibres of all samples taken into account for measurement of cross-sectional areas. Asterisk shows significant difference ($P<0.05$). Number in parenthesis indicates the sample size (Student's 't' test).

DISCUSSION

Administration of testosterone propionate to normal rats did accelerate the earlier phase of the process of regeneration following orthotopic transplantation of EDL muscles. As at 1-week stage the muscle regenerates in the hormone supplemented animals attained mature regenerated muscle fibres with significantly higher cross-sectional areas than those found in the control grafts. Connective tissue element and fat deposition were much less in the TP grafts and these regenerates did not contain central mass of necrotic muscle fibres. However, the significant hypertrophy of the regenerated muscle fibres in the TP rats at the first sampling period was not observed for the remaining stages. Rather the hormone treated muscle transplants in the later stages indicated hyperplasia and the one-month old regenerates showed significantly higher % recovery in terms of the wet weight than the control grafts.

The results indicate synthesis of androgen receptors in the earlier phase of the muscle fibres' regeneration. It is well known that response of target tissue to steroid hormones is mediated and dependent on variations of cellular content of receptor proteins (Saartok *et al.*, 1984; Baulieu and Master, 1989). Formation of androgen receptors has been reported to occur in regenerating levator ani muscles in rats within few days following crush injuries (Max *et al.*, 1981; Mufti, 1985). Another mode of action of androgens on regeneration of skeletal muscle is mediated through the process of reinnervation: Re-establishment of neural connection with a muscle graft is pre-requisite for development, growth and final functional differentiation of the regenerated muscle fibres (Carlson, 1988, 1989; Melone *et al.*, 1990; Sesodia *et al.*, 1994). In this connection it is important to note that the regeneration of axotomized motorneurons has

been shown to be influenced positively by androgens in various experimental animals such as in rats (Kujawa *et al.*, 1993) and in hamsters (Jones and Oblinger, 1994; Kinderman and Jones, 1994).

Apart from the growth promoting effects of testosterone propionate on regenerating muscle fibres, acting via better reinnervation of the muscle grafts the hormone also appeared to enhance the process of revascularization. Breakdown and removal of necrotic muscle fibres within a graft is accomplished by cell-mediated destruction. On the invasion of ingrowing capillaries to the ischaemic zone of a muscle graft, large number of macrophages phagocytize necrotic sarcoplasm of the dead original muscle fibres (Hansen-Smith and Carlson, 1979; Carlson, 1982). And speed of regeneration of muscle fibres in ischaemic EDL muscles in rats has been shown to be directly proportional to the rate of revascularization (Jennische, 1985, 1986). Results of this investigation indicated that the degeneration/ regeneration processes in the hormone supplemented grafts commenced quite earlier and by the end of first week such transplants comprised of developed regenerated muscle fibres. On the other hand, one-week control grafts contained a considerable number of central necrotic muscle fibres still awaiting for the vascular approach for their destruction and subsequent disappearance. To conclude it is stated that anabolic effects of the steroid on the processes of regeneration can be visualized in terms of rapid revascularization and reinnervation, well grown regenerated muscle fibres, and scarce development of adipose and connective tissues within the muscle transplants.

Significant hypertrophy of regenerated muscle fibres in the experimental grafts observed at the first but not for the remaining sample periods could be related to alterations in the levels of growth hormone. There is ample evidence that anabolic effects of androgens are closely associated with the presence of adequate levels of growth hormone (Kawai *et al.*, 1982; Griffin and Wilson, 1987). And it is also well known that circulating growth hormone concentrations are increased after various kinds of injuries including surgical stress (Charters *et al.*, 1969; Carey *et al.*, 1971; Wright and Johnston, 1975). In the light of these findings it is tempting to speculate that increase in the level of growth hormone might had occurred in the rats following the muscle grafting surgery and elevated level of this hormone enhanced the growth promoting effects of the steroid during the initial phase of the muscle fibres' regeneration. It is, therefore, hypothesized that anabolic effects of androgen on regenerating skeletal muscle fibres may be accentuated by enhancing availability of growth hormone.

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STUDIES ON THE TOXICITY OF LINDANE IN ALBINO RAT: HISTOPATHOLOGICAL EFFECTS IN LIVER

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Abstract: An organochlorine insecticide, lindane (gamma-HCH) was administered to three groups of Sprague Dawley rats, @ 30 mg/kg body wt. once, 18 mg/kg body wt./day and 9 mg/kg body wt./day, for 48 hours, 15 days and 18 months, respectively. Following stipulated durations, animals were dissected, their livers were removed, fixed quickly and processed further by routine histological techniques. Liver sections, 6-8 μm thick were cut, stained with haematoxylin and eosin technique and examined under microscope for morphometric and pathological changes. Lindane treatment produced significant increase (10-59%) in hepatic cell size in all experiments. The nuclear and nucleolar size also showed similar increase with 26% and 73% maximum change. The number of cells/microscopic field decreased by 18%, 22% and 26% in 48 hour, 15 days and 18 month experiments. No significant change was found in number of nuclei/cell and number of nucleoli/nucleus. The liver sections revealed moderate to high cellular pathology in different treatments. Cellular and nuclear hypertrophy was evident in almost all treatments. The clear areas in the cytoplasm showed proportionate increase with increase in duration of insecticide treatment. Increased sinusoidal spaces, condensed/hyperchromatic and distorted nuclei, fatty changes, increase in kupffer cells and clear areas around the nuclei were the other prominent changes.

Key words: Chlorinated hydrocarbons, gamma-benzenehexachloride (gamma-BHC), gamma-hexachlorocyclohexane (gamma-HCH), insecticides, liver histology, liver pathology, morphometric changes, organochlorines, pesticides, mammals, vertebrates.

INTRODUCTION

Pesticides play a very significant role in boosting agriculture production. In present times, practically we can not feed the world by excluding pesticides from the agriculture sector.

Lindane (gamma-HCH) is one of such pesticides, belonging to organochlorine group, which has been extensively used in the near past for controling crop pests in agriculture and vector-borne diseases in public health and veterinary sectors. This chlorinated insecticide has shown great cummulative potential due to hydrophobic nature of its benzene ring, the characteristic which enable this insecticide to bind with lipoprotein membrane system in the cell. The principle binding force, of course, is hydrophobic interaction between the insecticide molecules and cell membranes. Due to this property, the residues of these chlorinated insecticides are being reported from soil, water, animals and plant tissues throughout the world (Lopez Carrillo *et al.*, 1996; Dua *et al.*, 1996). In living systems, the residues and their metabolites frequently induce

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serious consequences such as reduction in weight, liver injury, (Ali and Shakoori, 1990, 1996; Mari *et al.*, 1994; Ito *et al.*, 1996) convulsions and tremors followed by death (Nagata and Narahashi, 1995; Nagata *et al.*, 1996; Blaszczak and Turski, 1998).

Toxicity of sublethal doses of lindane and other related organochlorine insecticides in animals has already been reported from different laboratories (Blaszczak and Turski, 1998). According to one study, lindane significntly reduced the T3 and T4 concentrations while TSH level was increased (Akhtar *et al.*, 1996). Previous studies have also shown that chlorinated insecticides are liver tumor promoters in rat and other animals (Davis *et al.*, 1993; Leiss and Savitz, 1995; Lopez Carrillo *et al.*, 1996; Ito *et al.*, 1996; Rougnt *et al.*, 1998).

In the present study histopathological effects of lindane are being reported in the liver of rat because liver is invovlved in the further processing and transformation of insecticides and considered as the main target of foreign chemical invasion.

MATERIALS AND METHODS

Experimental animals and their maintenance

Three groups of albino rats (Sprague Dawley strain) were fed on three different dose levels of lindane, orally, alongwith the feed for the total duration of 48 hours, 15 days (strong dose experiments) and 18 months (week dose experiment). The animals were kept in iron cages under optimum conditions in small groups. The feed and water was provided to rats ad libitum. The rat feed was formulated as mentioned in Ali and Shakoori (1988).

Toxicant used and its administration

A polychlorinated organic compound, lindane [gamma- hexachloro-cyclohexane (HCH), gamma-benzene hexachloride (BHC)] was obtained as 26% powder from Jafar Brothers (Pvt.) Ltd. Shadman, Lahore. The insecticide was administered to rats @ 30 mg (=0.33 LD_{50}), 18 mg (=0.20 LD_{50}) and 9 mg (0.10 LD_{50})/kg body wt./day for three different durations *i.e.*, 48 hours, 15 days and 18 months, respectively.

The insecticide-mixed diet for first strong dose (30 mg) experiment was prepared by adding 3.84 g of lindane (26%) per kg of rat feed. In second strong dose (18 mg) experiment 2.31 g of lindane/kg was mixed with rat feed while weak dose feed was prepared by the addition of 1.15 g of lindane/kg of rat feed thoroughly with the help of suitable amount of water to prepare semi-solid cakes.

Experimental procedure

Three to five animals each from treated and control groups were dissected to collect the liver samples. Small pieces of liver were quickly fixed in Bouin's fixative and processed further for routine paraffin embedding technique. Sections of suitable thickness (6-8 μm) were prepared and stained with haematoxylin and eosin counter staining technique. The histological sections were examined under microscope for morphometric changes and for induction of any other pathology by lindane.

Ocular micrometer, pre-calibrated with stage micrometer, was used for all measurements. For counting cells their nuclei and nucleoli 500X magnification while for measuring size 1250X magnification was used.

RESULTS

Tables I-III and Figures 1-3 show the effect of feeding gamma-HCH mixed diet, @ 30 mg, 18 mg and 9 mg/kg body wt./day for the total period of 48 hours, 15 days and 18 months, respectively on various morphometric parameters of rat liver. As it is obvious from the data that hepatic cells, their nuclei and nucleoli were hypertrophied after insecticide treatment. The hepatic cell size increased 14% and 39% after 24 and 48 hours of toxicant feeding. The nuclear size increased by 26% and 18%, while nucleolar size increased 42% and 35% during the same fedding duration, respectively. The number of nuclei/cell and number of nucleoli/nucleus did not show any alteration after insecticide treatment @ 30mg/kg body wt./day for 48 hours (Table I, Fig.1).

During 15 day lindane treatment the hepatic cell size increased gradually and showed 30% increase after 15 days of insecticide feeding. The nucleolar size increased 20% during the same period and showed 52% increase during first week of insecticide administration. The size of the nucleoli no longer showed any significant deviation from control during later part of the experiment. The number of nuclei/cell and the number of nucleoli/nucleus did not show any change, although the number of cells/microscopic field decreased by 22% (n=90) after 15 days of gamma-HCH feeding (Table II, Fig.2).

Table I: Effect of feeding gamma-HCH mixed diet (30 mg/kg body weight/day) for 48 hours on the various histological parameters of rat liver.

Parameters	Control	gamma-HCH feeding	
		24 hours	48 hours
No. of cells/ field (n=9)	276.54[a] ±9.62	247.92 ±11.39	226.13* ±14.45
No. of nuclei/ cell (n=90)	1.12 ±0.33	1.19 ±0.05	1.26 ±0.15
No. of nucleoli/ nucleus (n=90)	1.52 ±0.17	1.77 ±0.09	1.63 ±0.13
Size of cell (μm^2; n=90)	269.27 ±8.49	306.91** ±4.92	373.95** ±9.09
Size of nucleus (μm^2; n=90)	37.71 ±0.92	47.39*** ±1.18	44.45** ±1.33
Size of nucleolus (μm^2; n=90)	2.81 ±0.18	3.98* ±0.30	3.80** ±0.21

[a]Mean±SEM. Student's 't' test; *P<0.05; **P<0.01; ***P<0.001

Following 18 months of uninterrupted gamma-HCH feeding @ 9 mg/kg body weight/day, the hepatic cells, their nuclei and nucleoli registered a significant increase. The average hepatic cell size increased 34, 28 and 59%, respectively, after 6, 12 and 18 months toxicant feeding, while the nucleus showed 20, 13 and 14% and nucleolus 73, 42 and 37% increase during the same period. The number of nuclei/cell and the number of nucleoli/nucleus did not show any significant deviation. The number of cells/microscopic field were decreased due to the hypertrophy of the hepatic cells (Table III, Fig.3).

Figures 8-29 show the histological structure of rat liver following lindane feeding at various dose levels for 48 hours, 15 days and 18 months. During 48 hours insecticide feeding, the hepatolobular structure was maintained, though very prominently hypertrophied hepatic cells with vesiculated nuclei, prominent nucleoli and granulated cytoplasm was observed (Figs. 10 and 12) when compared with the normal section (Fig.7). Slight cellular disorganization was evident from irregular arrangement of cells in addition to distorted and condensed nuclei (Figs. 8 and 11).

Table II: Effect of feeding gamma-HCH mixed diet (18 mg/kg body weight/day) for 15 days on the various histological parameters of rat liver.

Parameters	Control	gamma-HCH feeding				
		3 days	6 days	9 days	12 days	15 days
No. of cells/ field (n=9)	274.64[a] ±12.47	253.32 ±8.40	231.17* ±9.63	223.54* ±10.19	220.40* ±11.21	213.15** ±10.44
No. of nuclei/ cell (n=90)	1.08 ±0.04	1.17 ±0.04	1.34 ±0.20	1.16 ±0.04	1.10 ±0.03	1.14 ±0.04
No. of nucleoli/ nucleus (n=90)	1.49 ±0.11	1.54 ±0.09	1.71 ±0.26	1.51 ±0.08	1.62 ±0.10	1.65 ±0.10
Size of cell (μm^2; n=90)	289.23 ±8.81	317.01* ±5.61	330.32** ±5.03	345.42** ±6.45	335.95** ±7.84	375.11*** ±10.94
Size of nucleus (μm^2; n=90)	39.45 ±1.18	45.79* ±1.49	47.93** ±1.44	47.90** ±1.42	46.44** ±1.20	47.52** ±1.28
Size of nucleolus (μm^2; n=90)	2.57 ±0.30	3.68* ±0.22	3.90* ±0.25	3.44* ±0.20	3.19 ±0.17	3.33 ±0.18

[a]Mean±SEM, Student's 't' test; *P<0.05; **P<0.01; ***P<0.001

After continuous gamma-HCH feeding for 15 days (Figs. 13 to 22), the hepatic lobular structure remained unchanged during initial period of the experiment. The nuclei were compact with distinct vacuolar spaces which appeared quite prominent during the first 3 day treatment (Fig.14). The hepatic sinusoid showed slight dilation in 6 and 9 day treatments (Figs. 15 and 17) when compared with control (Fig.5). In low power objective, lightly and darkly stained areas can be differentiated (Fig.13). Any alteration in staining characteristics of the tissue is itself a pathological change. The cytoplasm was not granulated until day 15, when vacuoles appeared in the cytoplasm and slight granulation was visible (Figs. 22 and 28). Although the hepatic cells and nuclei appeared to be larger in size, but the typical indication of insecticide toxicity *i.e.*,

vacuolation is not distinctly visible in other treated groups. Irregular shaped condensed nuclei were frquently observed in the hepatic cells, in addition to normal nuclei. The number and size of kupffer cells also increased (Figs. 16-18). Almost similar changes were observed in 12 and 15 day gamma-HCH treated rat livers. The cytoplasmic margins were distinctly away from the nuclei, leaving clear spaces around them in 12 and 15 day treatment (Figs. 20 and 22). The clear areas around the nucleus may either be due to fatty degeneration or indicate hepatic glycogenesis.

Table III: Effect of feeding gamma-HCH mixed diet (9 mg/kg body weight/day) administered for 18 months on the various histological parameters.

Parameters	6 months gamma-HCH feeding experiment		12 months gamma-HCH feeding experiment		18 months gamma-HCH feeding experiment	
	Control	Treated	Control	Treated	Control	Treated
No. of cells/ field (n=9)	263.31[a] ±13.42	197.24* ±15.63	258.87 ±11.21	180.55** ±12.82	252.77 ±14.51	161.80** ±9.72
No. of nuclei/ cell (n=90)	1.14 ±0.09	1.32 ±0.26	1.19 ±0.13	1.40 ±0.30	1.12 ±0.11	1.27 ±0.07
No. of nucleoli/ nucleus (n=90)	1.25 ±0.05	1.38 ±0.07	1.28 ±0.07	1.50 ±0.17	1.21 ±0.47	1.30 ±0.10
Size of cell (μm^2; n=90)	272.41 ±9.13	365.34*** ±10.75	302.92 ±11.81	388.12** ±11.27	310.63 ±16.89	496.88** ±19.21
Size of nucleus (μm^2; n=90)	42.78 ±1.29	51.35** ±1.57	38.57 ±1.14	43.48 ±2.50	40.84 ±1.37	46.80* ±1.95
Size of nucleolus (μm^2; n=90)	2.88 ±0.31	4.97** ±0.25	2.64 ±0.41	3.74 ±0.35	2.71 ±0.19	3.72 ±0.33

[a]Mean±SEM. Student's 't' test; *P<0.05; **P<0.01; ***P<0.001

Figures 23 to 24 show the effects of lindane feeding for 6 months on the hepatic histological structure, while Figures 25-27 and Figures 28-31 indicate the effect of insecticide feeding in rat liver for 12 and 18 months, respectively. Although well defined liver cell hypertrophy alongwith condensed and hypertrophied nuclei can be observed (compare Figures 24 and 27 with Figure 7), the most prominent and significant change was noticed in 18 month group, where extensive cytoplasmolysis was seen with vacuolated and marginated cytoplasm. The nuclei of the hepatic cell were considerably condensed (Figs. 26, 27 and 29). Clear areas around nuclei were also visible in 12 and 18 month treatments (Figs. 25-27 and 29) which was an indication of slight cytoplasmic degeneration. The hepatic cord structure, generally, looks disturbed in long term study (Figs. 23, 25 and 28). The nuclear distortion (pycnosis) increased gradually in case of uninterrupted gamma-HCH feeding for 6-18 month period (Figs. 24, 27 and 29). The

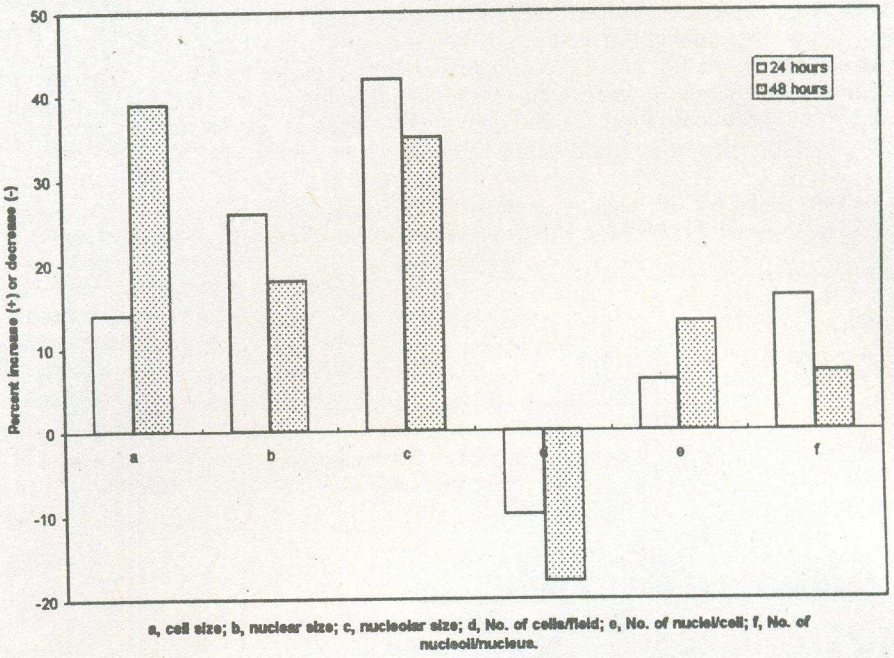


Fig.1: **Percent increase or decrease in various morphometric and histological parameters of rat liver fed on lindane-mixed diet (30 mg/kg body wt./day) for total period of 48 hours.**

liver of insecticide fed rats was marked by distended central vein, prominent blood vessels and bile canaliculi on the outer margins of hepatic cord cells during 18 month treatment (Figs. 28-29)

DISCUSSION

The size of hepatic cell, its nucleus and nucleolus increased considerably after lindane treatment, the extent of which was highest in long term experiments. Increase in the size of above parameters has also been reported from different laboratories in various experimental animals by treating with other chemicals and organochlorine (OC) insecticides, like heptachlor, DDT, aldrin, dieldrin and endrin (Shakoori and Ali, 1986; Shakoori and Haq, 1987; Ali and Shakoori, 1990, 1996). Similar pattern of changes were also recorded in another study from this Lab in rats with chlorinated insecticide, endrin. According to this study the hypertrophy of the nuclei and nucleoli was due to the change in the fluid conntent of these organelles caused by changes in permeability of the nuclear membrane (Ali and Shakoori, 1993). Changes in permeability and other properties of the cell membrane may be responsible for induction of cellular hypertrophy.

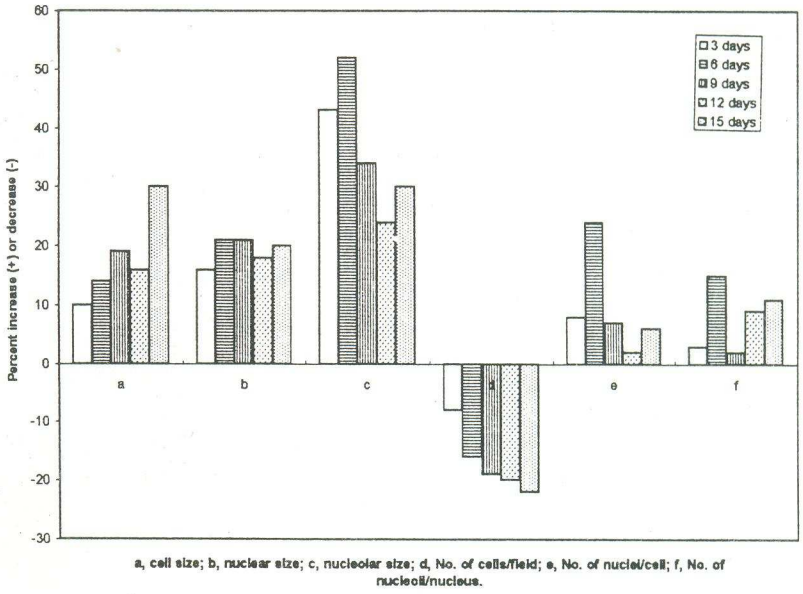


Fig.2: Percent increase or decrease in various morphometric and histological parameters of rat liver fed on lindane-mixed diet (18 mg/kg body wt./day) for total period of 15 days.

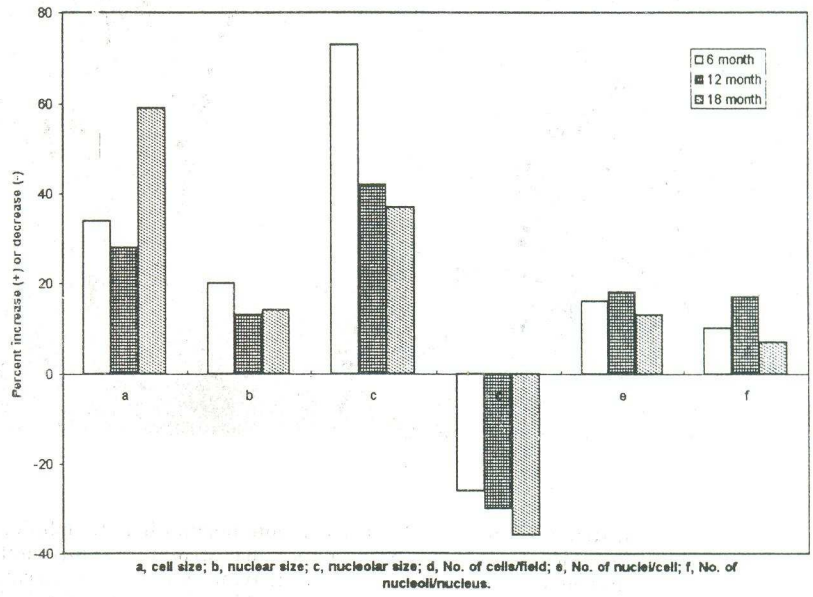
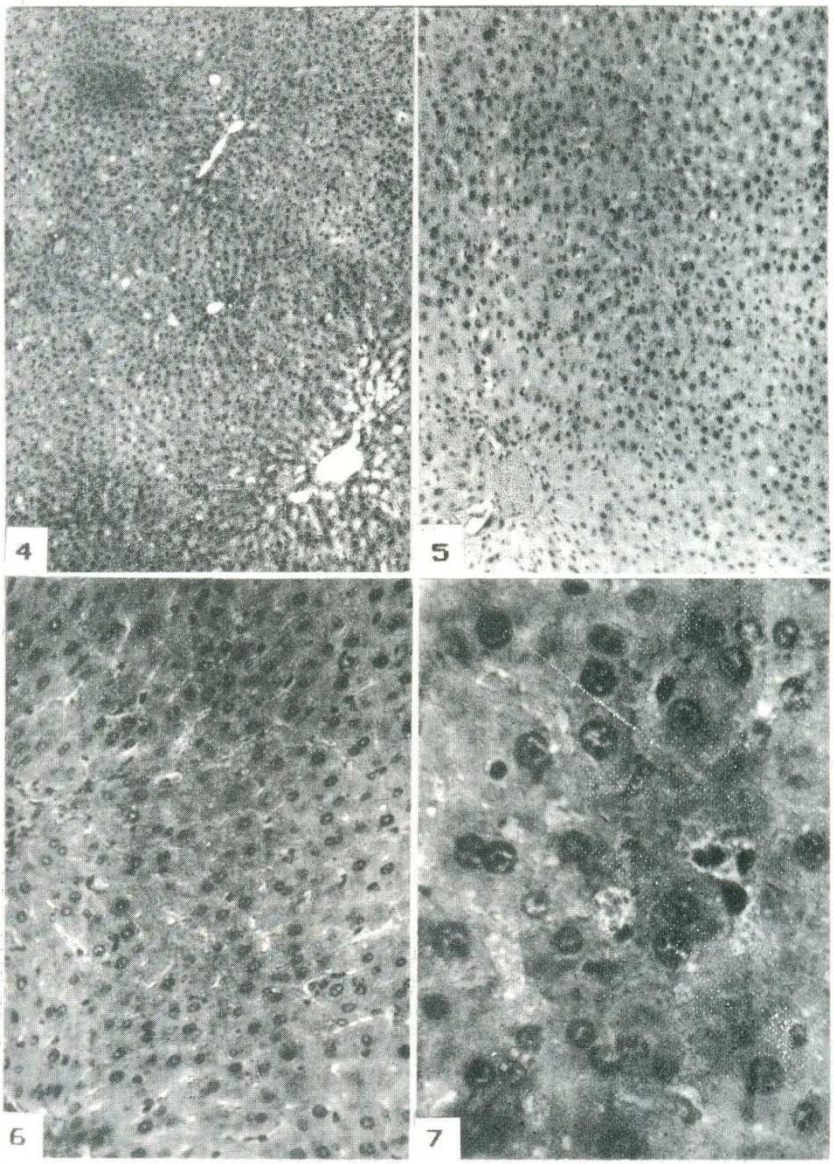
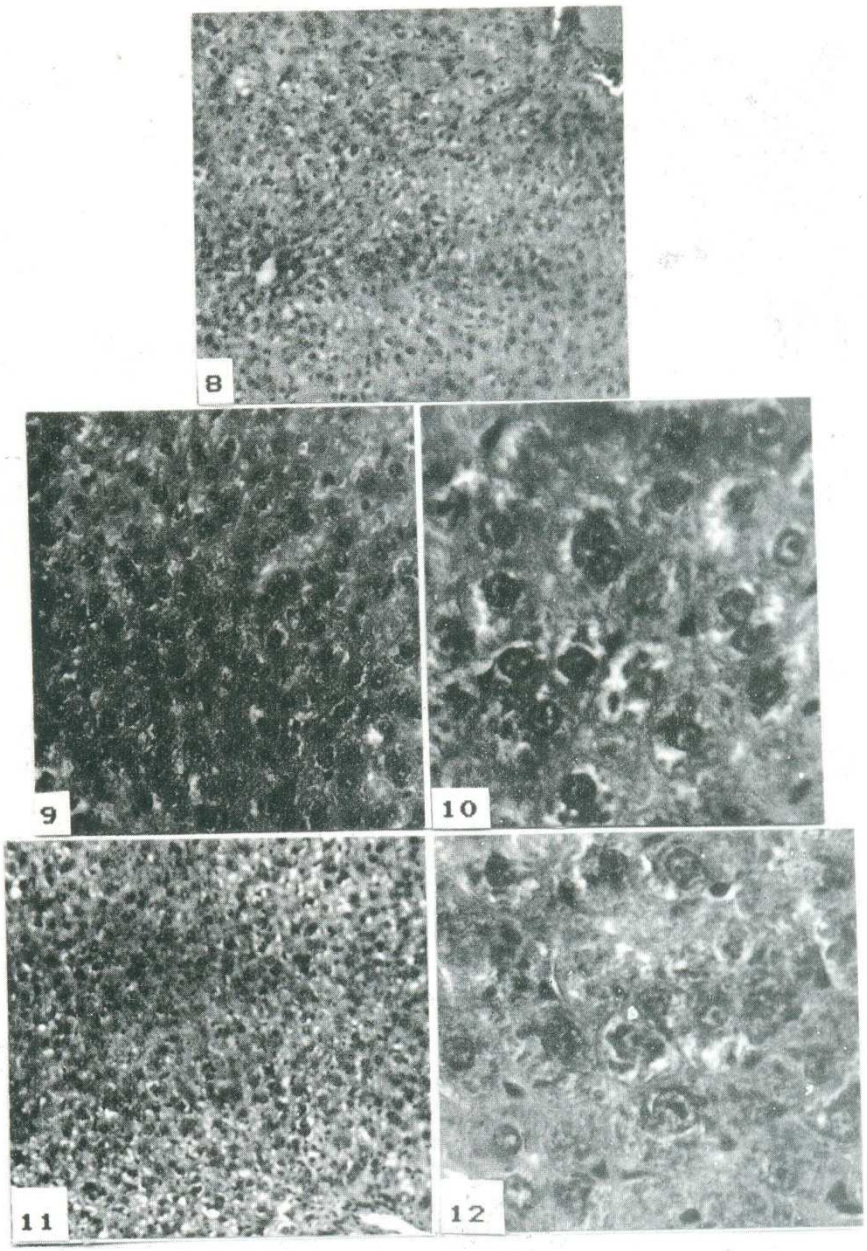


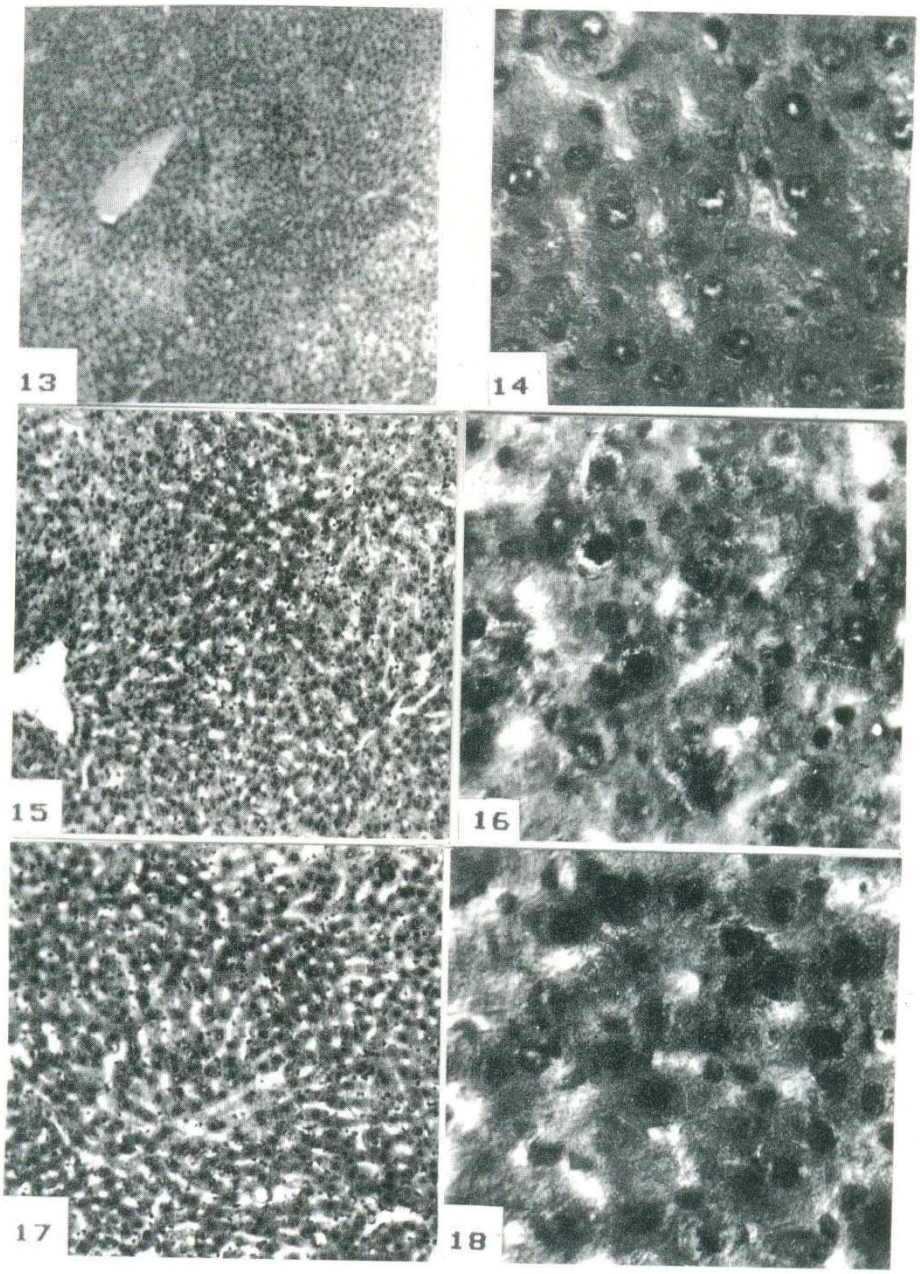
Fig.3: Percent increase or decrease in various morphometric and histological parameters of rat liver fed on lindane-mixed diet (9 mg/kg body wt./day) for total period of 15 days.



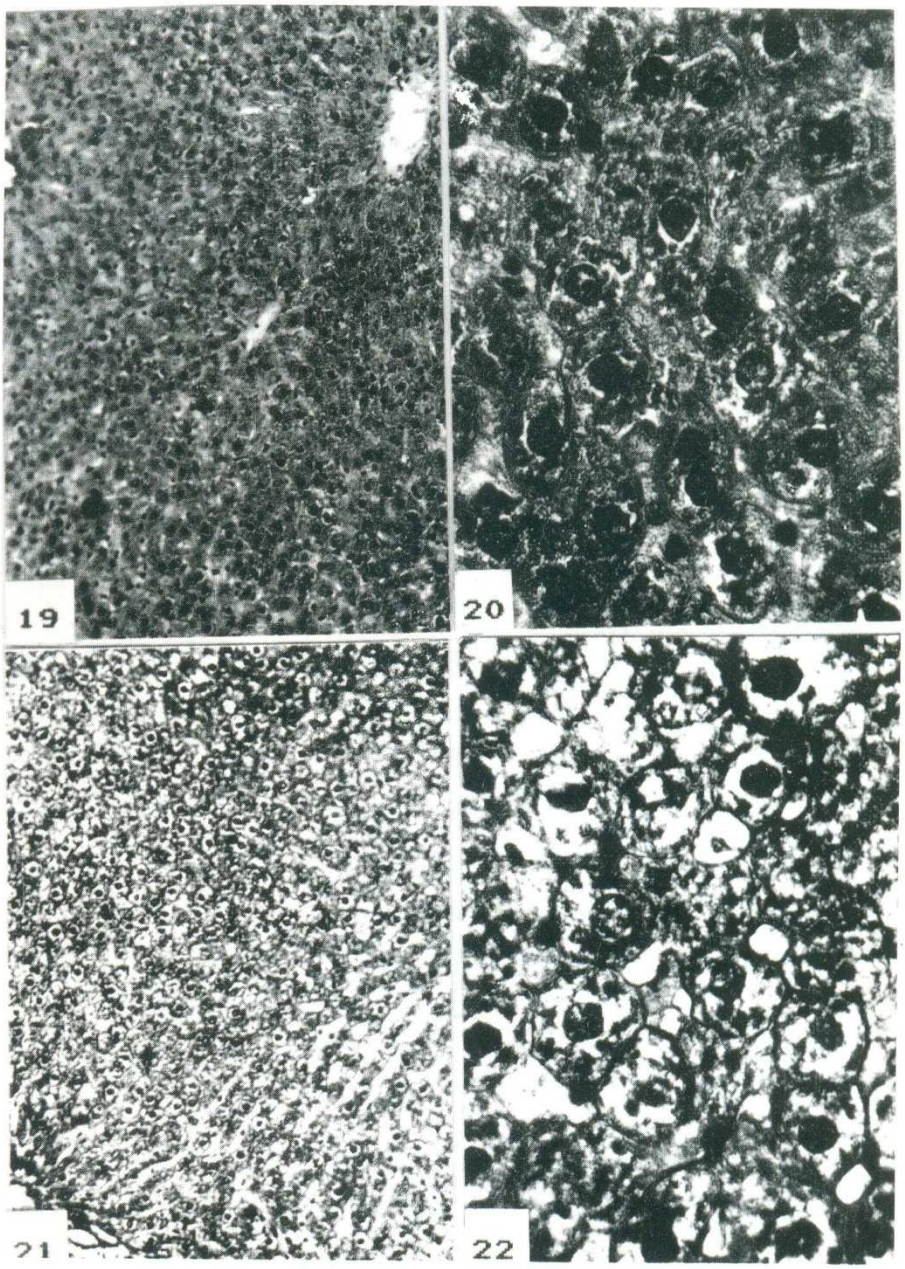
Figs. 4-7: Histological structure of normal rat liver. Note normal hepatic lobule, central veins with portal areas (Fig. 4-5), hepatic cord-structure, sinusoidal spaces with rod shaped kupffer cells, liver parenchyma and nuclear arrangement (Figs.6-7). Stain: Haematoxylin and Eosin. Magnifications: Fig.4, 25X; 5, 50X; 6, 100X; 7, 250X.



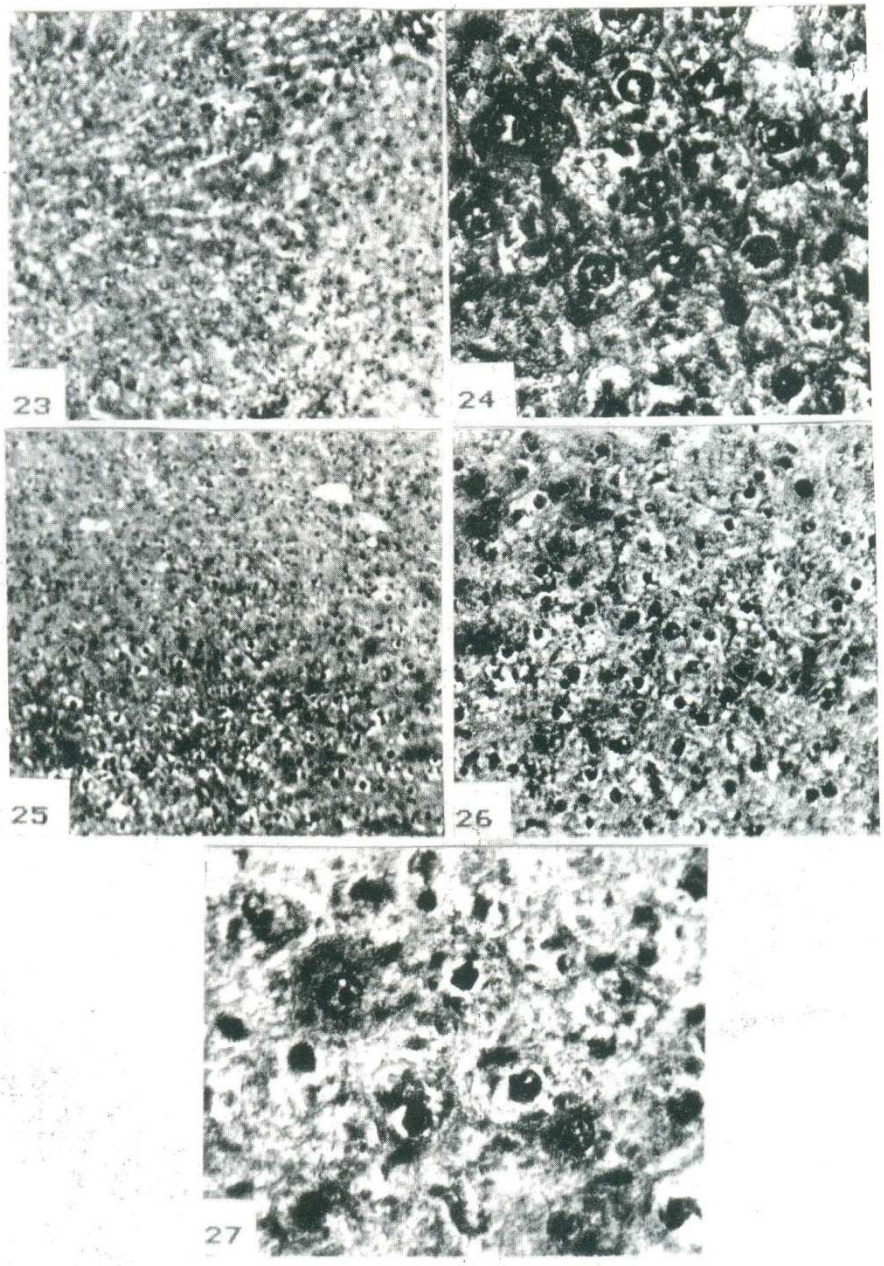
Figs. 8-12: Histological structure of rat liver fed on lindane-mixed diet for 24 hours (Figs.8-10) and 48 hours (Figs.11-12). Note disorganised lobular structure, and clear areas around the nuclei (Figs. 8 & 11) hypertrophied cells (Figs. 10 & 12)irregular shaped, picnotic nuclei (Figs. 9 & 11) and increase in clear areas (Figs. 10-11). Stain: Haematoxylin and Eosin. Magnifications: Figs. 8 & 11, 50X; 9, 100X; 10 & 12, 250X.



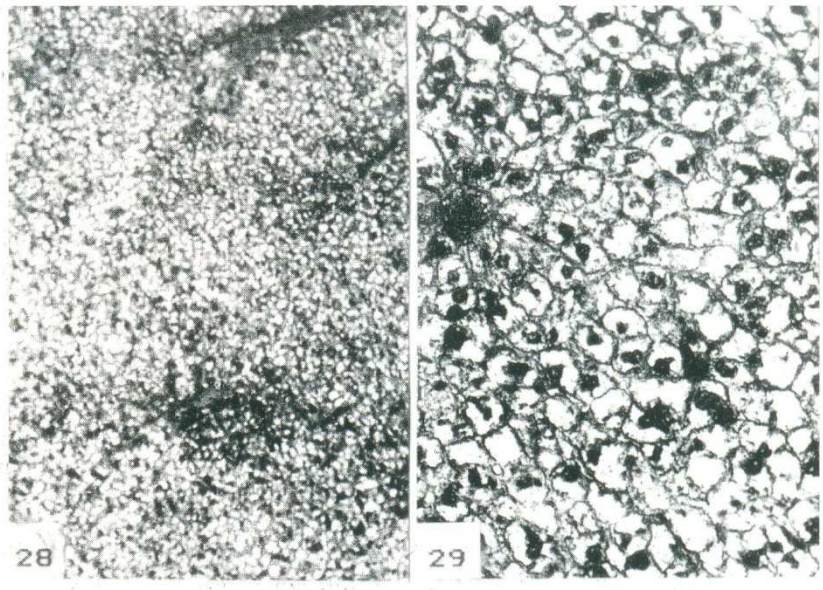
Figs.13-18: Histological structure of rat liver fed on lindane-mixed diet for 3 days (Figs. 13-14) 6 days (Figs. 15-16) and 9 days (Figs. 17-118). Note lobular structure with slightly disturbed cords (Figs. 13,15 & 17), lightly stained zones (Fig. 13), increased sinusoidal spaces and kupffer cells (Figs. 15-18), numerous clear areas around the nuclei (margination) and in the cytoplasm (Figs. 15 & 17), condensed, distorted, hyperchromatic nuclei (Figs. 16 & 18). Stain: Haematoxylin and Eosin. Magnifications: Figs. 13 & 15, 25X; 17, 50X; 14, 16 & 18 250X.



Figs.19-22: Histological structure of rat liver fed on lindane-mixed diet for 12 days (Figs. 19-20) and 15 days (Figs. 21-22). Note disturbed lobular architecture with disorganised regions (Figs. 19 & 21), hyperchromatic and picnotic nuclei with clear areas around them (Figs. 20-22)and fatty degeneration (Figs. 21-22). Stain: Haematoxylin and Eosin. Magnifications: Figs. 19 & 21, 50X; 20 & 22 250X.



Figs.23-27: Histological structure of rat liver fed on lindane-mixed diet for 6 months (Figs. 23-24) and 12 months (Figs. 25-27). Note totally disorganised regions of hepatic lobule with disturbed cord structure (Figs. 23, 25 & 26), dilated sinusoidal spaces (Fig. 23), hypertrophied cells and nuclei alongwith irregular shaped picnotic nuclei (Figs. 24-27). Also note fatty changes and clear areas (vacuolation) around the nuclei and in the cytoplasm (Figs. 23,26 & 27). Haematoxylin and Eosin. Magnifications: Figs. 23 & 25, 50X; 26, 100X; 24 & 27, 250X.



Figs.28-29; Histological structure of rat liver fed on lindane-mixed diet for 6 months (Figs. 23-24) and 12 months (Figs. 25-27). Note totally disorganised lobular structure with heavy vacuolation and almost invisible sinusoidal spaces (Fig. 29). Regions with variable staining (Fig. 28) thick membraned hepatic parenchyma alongwith picnotic nuclei (Fig. 29) were also evident. Haematoxylin and Eosin. Magnifications: Figs. 28, 25X, 29; 100X.

Increase in hepatic cell size during the present study, may be related to hypertrophy of the liver tissue which is a typical response of liver to foreign toxic compounds and most probably, is due to the rise in the relative weights of the liver (RLW), observed following exposure of animals to lindane and other OC insecticides (Shakoori *et al.*, 1984; Narayan *et.al.*, 1990; Katayama, 1993; Ali and Shakoori, 1996). Prominent increase in the average size of the hepatic cell (15%) and its nuclei (10%) has been shown by oral feeding of a pyrethroid insecticide (cypermethrin) to rats @ 420mg/kg body wt./day (Shakoori *et al.*, 1988). The findings in the present series of experiments have also been supported by studies on aldrin feeding in rats for 18 month period (Ali and Shakoori, 1990). Chatervedi (1993) noticed a dose-dependent increase in liver/body weight ratio by 24-79 % in ICL male mice fed on mixture of 10 pesticides, including lindane (gamma-HCH), aldrin, dieldrin, DDT, endosulfan and alachlor. Liver cytochrome p-450 also increasec after gamma-BHC treatment @ 20 ppm for 15 and 30 days in the rats which in fact, is a consequence of raised smooth endoplasmic reticulum in the hepatic cell (Barros *et al.*, 1991). Drug mtabolism is one of the major functions associated with smooth endoplasmic reticulum and is carried out through induction of mixed-function oxygenase system utilizing cytochrome P-450 mediated pathway. Mikol *et al.* (1980) reported hepatic enzyme induction in rats by feeding gamma-HCH while raised synthesis of cytochrome P-450 content of the hepatic cell was shown by Kurihara *et al.* (1984).

Liver structure under light microscope revealed loss of cytoplasm and vacuolation at certain treatment durations. These findings also coincides with the study of Nigam *et al.* (1982) and Swaroop and Upadhyay (1985). Liver necrosis was also reported in rats by Barros and Saliba (1978). In another study, carbaryl has been shown to induce patchy lesions in the liver and developed endothelial damage to centro-lobular veins (Mari *et al.*, 1994). Various histological, histochemical and ultrastructural changes by pesticide exposure in animals have been reported from different labs (Zufarov *et al.*, 1975; Dikshith *et al.*, 1978a,b; Jeanne, 1979; Shivanandappa and Krishnakumari, 1981; Gupta and Singh, 1982, Nigam *et al.*, 1982, 1984a,b; Qin *et al.*, 1982; Preza *et al.*, 1983; Akhtar *et al.*, 1996; Ito *et al.*, 1996). Extensive accumulation of glycogen in liver (glycogenesis) of mice was reported after gamma-BHC feeding for 10 months (Nigam *et al.*, 1984a).

Rivett *et al.* (1978) however could not find any detectable histopathological changes after treatment of dogs with 100 and 200 ppm of lindane for 104 weeks. The degenerative changes in liver after 12 weeks of lindane feeding were reported by Su and Zhou (1986) and according to this report lindane induced granular hyaline degeneration of hepatic tissue. Lindane-induced peripheral necrosis with haemorrhagic foci in the liver of rat were shown by Junqueira *et al.* (1991). Gamma-HCH also induced injury and lesions in other tissues. Baronia and Sahai (1990) observed lindane-induced adrenal pathology in rats, with necrotic changes, ruptured cells, pycnotic nuclei and increase in spaces and vacuolation at 200mg/kg body weight dose level.

Fatty changes, degeneration, necrosis, cytoplasmic vacuolation and lipid peroxidation are the other pathologies observed in the hepatic and renal tissues of rats, mice guinea pigs and hamsters with oral administration of OC insecticide, endrin, alongwith the corn oil (Hassan *et al.*, 1991).

Lindane also stimulates hyperplasia (cell proliferation) in the liver (Brade *et al.*, 1974). Hyperplasia normally occurs due to increased mitotic activity which, in turn, is a consequence of increased DNA synthesis in the tissue (Yusof and Edwards, 1990;

Waller *et al.*, 1996; Rought *et al.*, 1998). This observation was further confirmed by the findings of another part of this study (Ali, 1988) in which it has been noted that gamma-HCH produced 123% and 55% increase in DNA of rat liver after 15 days and 18 month experiments, respectively. Development of liver and other tissue cancer has been reported in different animals (Ito *et al.*, 1975; Herbst, 1975; Reuber, 1979; Kashyap *et al.*, 1979; Bhatt *et al.*, 1981d; IARC, 1982; Nigam *et al.*, 1982, 1984; Oesch *et at.*, 1982; Ahlborg *et al.*, 1995; Ito *et al.*, 1996; Rosa *et al.*, 1996) but in the present study we could not find any malignant change in rat liver.

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EFFECT OF A BIOINSECTICIDE AGAINST *LUCILI A CUPRINA* (WIED) (CALLIPHORIDAE : DIPTERA)

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Abstract: The toxic effect of *Bacillus thuringiensis* on the haemolymph of adult *Lucilia cuprina* was observed. LD_{50} was calculated. The treatment resulted in the destruction and vacuolization of plasma and haemocytes. Differential haemocyte counting of the treated flies showed quantitative changes in the haemocytes as compared to the control ones.

Key words: *B. thuringiensis, Lucilia cuprina*, haemolymph.

INTRODUCTION

The unplanned and indiscriminate use of various insecticides to control them is a serious threat to human health as well as to other biotic factors of the ecosystem and environment. The death of the target as well as the non-target organisms is also a major drawback of these insecticides (Edward *et al.*, 1987; Khillare and Waghs, 1988; Reddy and Bashamohideens, 1989). The other major drawback is the development of resistance which has been reported for a variety of insects *e.g.*, insecticides such as organophosphates, carbamates and pyrethroid have failed to control German cockroach (Schal, 1988; Cochran, 1989; Zhai and Robinson, 1994), Diamond back moth (Tabashnik, 1994), sheep blow fly (Kotze, 1995; Levot, 1995), house fly (Lalah *et al.*, 1995) and mosquito larvae (Mazarri and Georghiou, 1995; Karunaratue *et al.*, 1995) due to the development of resistance.

Keeping in view the hazards of the chemical insecticides, the alternate and relatively safe means *i.e.*, biological pesticides are now kept in focus. One of the strategies involves the use of microorganisms to control those insects whose activities pose serious problems for the mankind (Mittal *et al.*, 1991; Pietrantonio and Gill, 1992; Orduz *et al.*, 1992).

The stress has been imposed on the spore forming bacteria, some of which have proved to be the best pathogens against them. *Bacillus thuringiensis* Kurstaki, HD-1 is one of the widely used bioinsecticides (Teiltelson *et al.*, 1992). The crystal proteins of *B. thuringiensis* have been extensively studied because of their pesticidal properties (Crickmore *et al.*, 1998). It is a rod shaped aerobic, gram-positive spore forming bacterium, which during sporulation produces crystalline structure. These crystals possess insecticidal crystal proteins ICP or endotoxin (Hofte and Whitlley, 1989; Lereclus *et al.*, 1989; Adang, 1991). It is non-pathogenic for mammals including man.

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These proteins can be mixed with food attractants, which increase the normal rate of feeding. Manasherob *et al.* (1994) found the toxicity of *B. thuringiensis* israelensis increased three times when used after encapsulation in *Tetrahymena pyriformis*. This decreased the natural life span and gave efficient control of *Aedes aegypti* larvae.

The object of the present work was to find the susceptibility of *Lucilia cuprina* to *B. thuringiensis* Kurstaki. This blow fly was selected because of its veterinary and medical importance. It is major contributor to fly strike in Australia (Graham, 1979) and is found in more than 70% of all strikes in New Zealand (Heath and Bishop, 1995) and other sheep rearing countries.

MATERIALS AND METHODS

Rearing of the insects

The colonies of flies were maintained at 30°C, 12 hours photoperiod and relative humidity ranging from 65% to 70%. The larvae hatched out from the eggs after about 24 hours. Two more moultings occurred at two days interval. The 3rd instar larvae thus obtained moved away from the food approximately at the end of the second day but remained mobile for 4-6 hours after which they settled down. It indicated the onset of the pupal life. The adults emerged nearly after five days.

Bacterial inoculation

The strain of *Bacillus thuringiensis* Kurstaki, HD-1 was obtained from Centre of Excellence in Molecular Biology (CEMB) (Fig.1). These bacteria were reared on nutrient agar. For the inoculation of bacteria into the insects 10,000 times dilution of the nutrient broth was prepared and number of colonies obtained after 24 hours on the nutrient agar plate were calculated.

One ml of sterilized milk-sugar solution along with 8 ml of bacterial culture was poured in each jar. This was considered to be the treated dose. For the control only 8 ml of nutrient broth was given along with 1 ml of milk sugar solution.

Estimation of LD_{50}

Three concentrations (2 ml, 4 ml, 6 ml) were selected for calculating LD_{50}. Three replicates were set up. Mortality was noted after 48 hours.

Blood film formation

For the blood film formation the insects were exposed to hot glacial acetic acid vapours for 5 to 10 min. Then a drop of blood was obtained on the slide. After spreading it with the help of a coverslip, the film was stained with Giemsa's or Wright's stain. It was then cleared in xylene and mounted in Canada balsam.

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 200 cells/ experimental stage were

counted and classified. Those cells which appeared to be intermediate between any two types were divided equally between two types following the method adopted by Nappi (1970).

RESULTS

The blood or haemolymph of *Lucilia cuprina* is contained in the general body cavity, as is the case in all the insects and has two components, the plasma, which is the liquid part and the haemocytes or the blood cells.

Eight types of haemocytes were distinguished on the basis of light microscopy, which are, prohaemocytes, plasmatocytes, podocytes, granular cells, cystocytes, oenocytoids, vermiform cells and spherule cells.

Prohaemocytes (Fig.2a)

These are small to medium sized cells varying from 6.0 to 11.0 μm. They 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 have smooth and regular boundary. The nucleus is usually spherical or ovoidal ranging from 3.5 to 6.5 μm in diameter. These cells are usually deeply basophilic but the nucleus always stains more intensely. In the large cells however, it is slightly eosinophilic and chromatin is then clearly granular and evenly distributed. Many of these cells can be seen undergoing both equal and unequal division. These are germ or stem cells (Rowley and Ratcliffe, 1981).

Plasmatocytes (Fig.2b)

They are highly polymorphic haemocytes and larger in size as compared to prohaemocytes. Although typically they tend to be ovoidal in shape but round, fusiform, spindle-shaped and irregular forms are also common. They have generally centrally located large nucleus which in some cells almost fills the entire cell body. The round plasmatocytes are less than 17 μm in diameter. These cells have moderately basophilic cytoplasm but the nuclei are eosinophilic with granular chromatin. The vacuoles are probably the result of the release of the granules from the cell body. In this preparation many intermediate forms between prohaemocytes and plasmatocytes can be seen. Plasmatocytes have more cytoplasm surrounding their nuclei as compared to that of prohaemocytes, very few haemocytes of this class were seen dividing.

Podocytes (Fig.2h)

These cells have long and tapering cytoplasmic extensions of variable length. The length of these extensions, arms or filopodia varies from 5 to 25 μm beyond the cell body which is in these cases oval. These arms were fixed in position and are not pseudopodial in nature. Some of these cells have fusiform bodies with the two tapering sides extending into long arms. The cytoplasm of these cells is basophilic and finely granular, while nucleus is eosinophilic. Fusiform cells vary from 16x8 μm to 25x10 μm in dimensions.

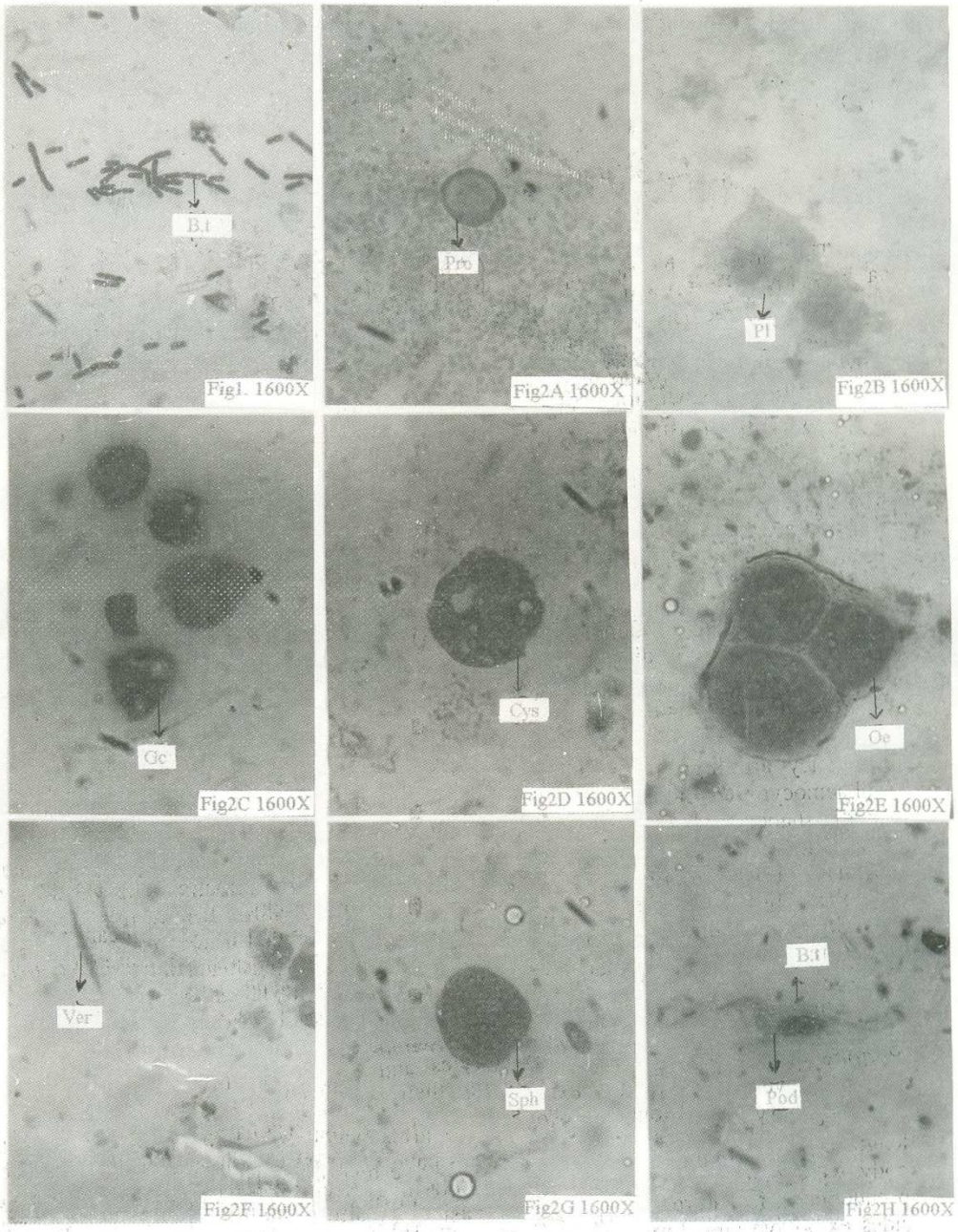

Fig. 1: Clusters of *B. thuringiensis* chains

Fig. 2: Control (a-h) a, Prohaemocyte; b, Plasmatocyte; c, Granulocyte; d, Cystocyte; e, Oenocytoid; f, Vermiform cells; g, Spherule; h, Clumping of cells.

Granular cells (Fig.2c)

These 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. The size of these granules can vary between 2 and 3 μm in diameter. These cells when round vary from 10 to 25 μm in diameter but when oval they are from 10 to 25 μm wide and 15 to 35 μm long. Their nuclei are eosinophilic and smaller in size as compared to plasmatocytes nuclei and vary from 5 to 10 μm in diameter. Apart from the granules, the cytoplasm also contains vacuoles and vesicles of different sizes.

Many intermediate forms between plasmatocytes and granular cells were observed. Some of the transitional forms were difficult to place as the resemblance was so close to the plasmatocytes.

Cystocytes (Fig.2d)

These cells are round, ellipsoidal or slightly irregular in shape with an occasional broken cell wall. They have a small nucleus as compared to that of the plasmatocytes or granular cells. The cytoplasm is either moderately or slightly basophilic with a round acentric eosinophilic nucleus. These cells vary from 2 to 4 μm in width and 8 to 12 μm in length.

Oenocytoid cells (Fig.2e)

These are usually slightly larger in size than granular cells and are round, ovoidal and sometimes irregular in shape. Their size varies from 12 to 30 μm in width and 15 to 40 μm in length and when round from 12 to 35 μm in diameter. They have a large quantity of homogenous cytoplasm which is almost neutrophilic. The nucleus range from 5 to 8 μm in width and 6 to 10 μm in length.

Vermiform cells (Fig.2f)

These cells are extremely elongated and thin with finely granular basophilic cytoplasm that extends and tapers into long arms. They vary from 16 to 50 μm in length but their width is very small varying from 3 to 6 μm. The comparatively thick central part sometimes also houses an elongated eosinophilic nucleus ranging from 6x3 μm to 9x5 μm in dimensions.

Some of these cells are without any apparent nucleus and have a finely granular cytoplasm in their bodies. Granules are not closely packed. Tuzet and Manier (1959) have called them the "giant fusiform cells".

Spherule cells (Fig.2g)

These cells are very conspicious of their large size and spherular inclusions. They are round, ovoidal and sometimes irregular in shape. They vary from 20 to 100 μm in diameter when round and are from 20 to 80 μm wide and from 25 to 120 μm in diameter. Spherule cells have very little amount of cytoplasm as most of the cell body is filled up with spherules, the large number of which often obscures the nucleus and also

distend the cell periphery. All the cell contents are basophilic. The nucleus is more deeply stained than the cytoplasm.

When *Lucilia cuprina* was fed on different concentrations of *B. thuringiensis*, LD_{50} was noted to be 7 ml of its liquid culture.

Blood smear analysis

The blood of the flies fed with the liquid culture of *B. thuringiensis* was studied at twelve hourly intervals upto 48 hours when death occurred. A large number of abnormalities were noted which have been explained here in some detail.

After the first 12 hours of treatment, the direct microscopic observations of the blood showed many of the bacteria approaching the haemocytes and ultimately attaching themselves to the cell membranes (Fig.3a). After 24 hours, many bacteria were seen entering into the haemocytes themselves (Fig.3b,c). After 36 hours, clumping of

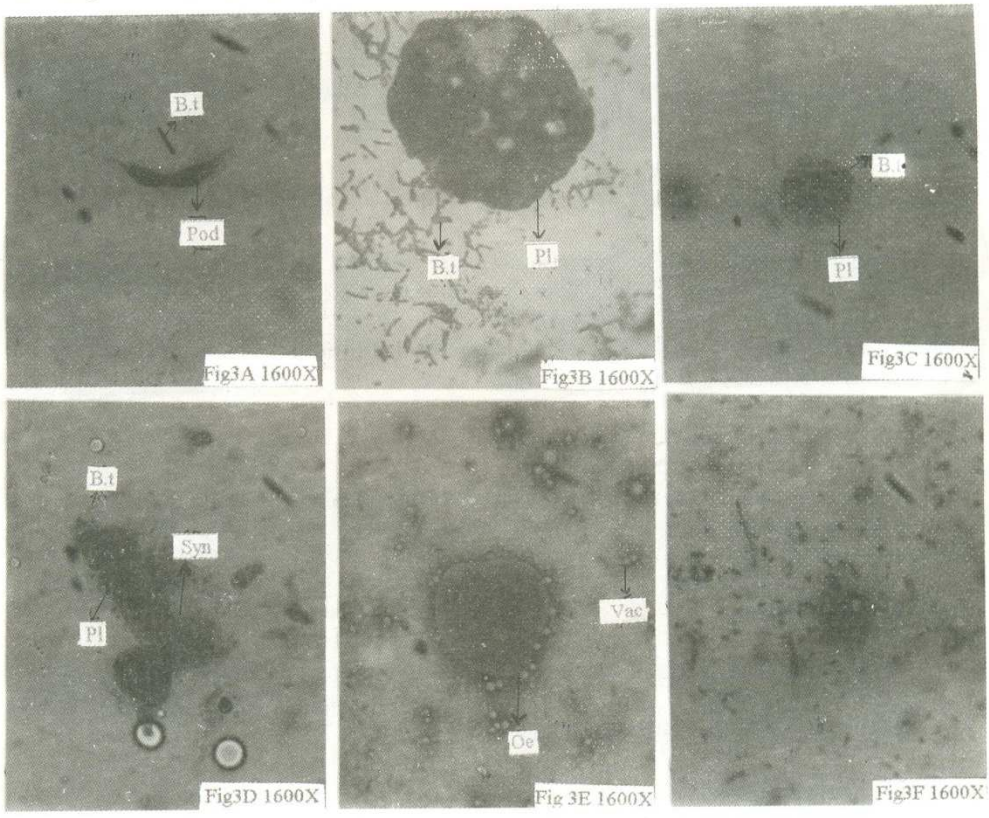

Fig. 3: *B. thuringiensis* treatments (a-f) a, *B.t* in the vicinity of Podocytes after 12 hours; b, Attachment of *B.t* to cell membrane of Plasmatocyte and vacuolization after 24 hours; c, *B.t* ingested by Plasmatocyte after 24 hours; d, Scattered cytoplasmic contents and synctium formation after 36 hours; e, Vacuolization in plasma after 36 hours; f, Destruction of plasmatocyte after 48 hours.

Table 1: **Effect of *B. thuringiensis* treatment on differential haemocytes count (DHC) at 40 x**

	Cell types																
	Pro		PC		GC		OE		POD		Sph		Cys		Verm		No.of cells Counted
	Total	%	Total	%	Total	%	Total	%	Total	%	Total	%	Total	%	Total	%	
Control																	
	20	10	112	56	6	3	8	4	16	8	6	3	8	4	24	12	200
Experimental (B.t. fed)																	
12h	12	6	114	57	10	5	2	1	14	7	10	5	10	5	28	14	200
24h	18	9	124	62	8	4	4	2	16	8	8	4	8	4	14	7	200
36h	20	10	126	63	6	3	-	-	14	7	8	4	4	2	22	11	200
48h	32	16	128	64	4	2	-	-	12	6	4	2	-	-	20	10	200

Pro: Prohaemocytes; GC: Granular cells; Pod: Podocyte; Cys: Cystocyte; PC: Plasmatocytes; OE: Oenocytoids; Sph: Spherule cells; Ver: Vermiform cells.

Table 2: **Results of effects of haemocyte number at different durations (Mean of three replicates was taken).**

Types of haemocytes	Treatments									
	Control				B.t treatment				L.S.D. values of different durations of treatments	L.S.D. values of different durations of strains
	12 hrs	24 hrs	36 hrs	48 hrs	12 hrs	24 hrs	36 hrs	48 hrs		
Prohaemocytes	20	20	20	20	12	18	20	32	13.317	18.833
Plasmatocytes	112	112	112	112	114	124	126*	128*	9.89	13.98
Granulocytes	6	6	6	6	10	8	6	4	4.11	5.80
Oenocytoids	8	8	8	8	2	4	0*	0*	3.046	4.30
Podocytes	16	16	16	16	14	16	14	12*	2.59	3.67
Spherale cells	6	6	6	6	10	8	8	4	6.75	9.55
Cystocytes	8	8	8	8	10	8	4	0	7.05	9.98
Vermiform cells	24	24	24	24	28	14	22	20	9.19	12.98

Note: L.S.D. value at p=0.05

haemocytes took place. The membranes of these cells broke up and synctia were formed (Fig.3d). Plasma became thickened and vacuolization occurred (Fig.3e). After 48 hours, most of the haemocytes were found to be distorted which made it difficult to distinguish the different types of the blood cells (Fig.3f).

The bacteria were found to be mostly entering the plasmatocytes and granular cells, although former were affected more.

Differential haemocytes counting (DHC) (Table 1)

DHC showed that in the control male flies, prohaemocytes were found to be 10% plasmatocytes were 56%, granular cells were 8%, spherule cells were 3%, cystocytes were 4% and vermiform cells were 12%.

Prohaemocytes increased in percentage from 10% to 15% while plasmatocytes also increased from 56% to 64% as compared to the control. Granular cells decreased from 3 to 2%. Oenocytoids also decreased to 2% as compared to the 4% of the control specimen and disappeared altogether after 24 hours of the treatment.

Podocytes also decreased to 6% as compared to its 8% ratio in control flies. Similarly, spherule cells, cystocytes and vermiform cells also decreased to 2%, 2% and 10% respectively as compared to the 3%, 4% and 12% ratio in the corresponding controlled flies.

Statistical analysis

When compared statistically, it was found that there existed significant difference in the number of plasmatocytes, oenocytoids and podocytes in the treated as compared to the controls.

DISCUSSION

Microbiological control of insects is considered as an important aspect of biological control and can be defined as the use of entomopathogenic microorganisms for insect control (Ertola, 1988). One of the important reasons for current interest in the entomopathogens lies in the facts that they are sufficiently specific and do not affect beneficial insects. Nearly all entompathogen bacteria are from class "Schizomycetes".

Bacteria belonging to genus *Bacillus* produce endotoxins which are toxic to insects. The different species of *Bacillus* have already been used as bioinsecticides by various scientists. Chak and Young (1990) found *B. thuringiensis* toxic against *Bombyx mori*, *Aedes aegypti* and *Heliothis* sp.

Bioinsecticidal activity of *B. sphaericus* have also been tested against various insects (Krammer, 1990; Rady *et al.*, 1990). Several strains of *B. thuringiensis* have been found to be toxic to *Aedes aegypti*, *Anopheles gembiae* and *Culex quinquefasiatus* (Federici, 1995; Smith *et al.*, 1996).

The microbial insecticides are also facing resistance problem but rate of the development of resistance has been found to be very slow (Rao *et al.*, 1995). The

diamond back moth *Plutella xylostella* was the first insect to evolve resistance against *B. thuringiensis* in open field population (Tabashnik *et al.*, 1997).

Recently insecticidal toxins from *B. thuringiensis* have also found to be toxic to some non-target species (Tapp and Stotzky, 1997). But on the other hand, this is highly effective against some important crop pests (Broza and Brauch, 1994; Moar *et al.*, 1995; Perez *et al.*, 1995).

The toxic effects of *B. thuringiensis* have been found in insects other than crop pests, *e.g.*, in 1997, Akhurust *et al.* found that larvae of *L. cuprina* were susceptible to some strains of *B. thuringiensis*.

In the present study, treatment with *B. thuringiensis* resulted in certain abnormalities, disruption of haemocytes and the ultimate death of these flies after 48 hours of the treatment.

Haemolymph

The blood film studies revealed that the haemolymph was affected both in its cellular contents and the plasma.

Plasma

The plasma became coagulated due to the scattering of the cytoplasmic contents and fragmentation of the cells and the bacteria became entangled in this thickened, coagulated plasma. This seemed to be the first defence against the invasions by these foreign particles.

Haemocytes

Phagocytosis is by far the most spectacular of the haemocyte function. The different steps involved in this process could be seen clearly such as attachment of *B. thuringiensis* to the cell membranes of the various haemocytes and their ingestion by these cells. Insect haemocytes have been found to be implicated in the immune responses against invading microorganisms and the detoxification of poisons by other workers and also by Gupta (1985). This is done by haemocytes phagocytosis and encapsulation of entomopathogenic microorganisms and also storing the antibacterial enzyme, lysozyme (Zachary and Hoffman, 1984).

The most affected cells were found to be the plasmatocytes. These cells have been shown to be involved in phagocytosis also by different scientists for various insects (Salt, 1970; Rowley and Ratcliff, 1981). Granular cells were also involved in this process, but to a lesser extent. The cystocytes also disintegrated. These changes involved the rapid degranulation and the loss of cytoplasmic contents. In most of the insects studied in the past, cystocytes were found to be performing the major role in coagulation (Gregoire, 1974). The oenocytoids did not seen to take part in the phagocytosis in this fly as also found by other workers like Gupta (1979).

Differential haemocyte count (DHC)

DHC of the control and the infected flies was done in order to correlate the resultant qualitative changes. During the present study the DHC revealed that nearly

20% of the haemocytes burst and their contents became scattered around them in the infected haemolymph. Upto 90% cell lysis has been reported by other workers in some other insects depending upon the pathogenecity 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. Plasmatocytes and the prohaemocytes increased in numbers in response to the treatment. Plasmatocytes played a major role against the bacteria. They became disrupted but their constant transformation from the prohaemocytes could easily explain the increase in their number while the prohaemocytes probably increased in number by cell division.

Statistical analysis

Statistical analysis revealed that a significant difference existed between the plasmatocyte oenocytoid and podocyte cell number of the control and *B. thuringiensis* treated flies.

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CYPERMETHRIN TOXICOSIS IN THE CHICKS OF DOMESTIC FOWL, *GALLUS DOMESTICUS*: HAEMATOLOGICAL STUDIES

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Abstract: Cypermethrin was administered orally to two groups of chicks @ 250 mg/kg body weight (strong dose, only once) and 150 mg/kg body weight/day (weak dose) for a total duration of 40 hours and 12 days, respectively. A group of five chicks were dissected at the intervals of 5, 10, 20 and 40 hours of toxicant feeding in strong dose (short term) experiment and at 3, 6 and 12 days durations in weak dose (long term) experiment. Blood samples were collected and processed for various haematological parameters which did not show any severe abnormality in both strong and weak dose experiments, except some changes in Hb and MCHC contents. The Hb content increased significantly by 18% within 5 hours, followed by decreasing trend. At 10 hours cypermethrin treatment increase was 16% which was reduced to 6% at 20 hours while at 40 hours significant decrease of 11% was observed. MCHC content raised by 13% and 14% at 5 and 10 hours of insecticide feeding. The only parameter, that showed significant change in both experiments was WBC count, which was increased by 75%, 48%, 86% and 62% at 5, 10, 20 and 40 hours in short term experiment, while in long term treatment 28% rise was observed at 6 days insecticide treatment. All other compounds such as RBC, PCV, MCV and MCH remained unaffected in both treatments.

Key words: Insecticides, pyrethroids, cypermethrin, haematology, blood cells, erythrocytes, leukocytes, RBC, WBC, haemoglobin, haematological indices, poultry, birds.

INTRODUCTION

The pyrethroids are third generation insecticides, playing very significant role in controlling insect pests of agriculture, vetrinary medical and house hold importance (Class and Kintrup, 1991; Lee and Clark, 1998; Martinez-Torres *et al.*, 1998). Most of the early developed pyrethroids are unstable in air and light (Elliott *et al.*, 1973, 1978). This property restricts their use particularly against pests of field crops regardless of their other favorable properties. The modern and more stable pyrethroid insecticides are not only more effective in controlling insect pest population but are also more harmful towards mammals and other non target systems.

Cypermethrin is one of these pyrethroids which has been extensively used to control wide range of insect pests. Most of the initial work performed with these insecticides was on toxicity, metabolism, metabolic fate and excretion in target and non target animal systems (Kaneko *et al.*, 1987; Herzberg, 1988; Akhtar *et al.*, 1989;

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Gupta, 1990; Hodgson and Levi, 1992, 1996; Hodgson *et al.*, 1995). These pesticides and their residues, due to their stability produced variety of harmful effects in non target living systems (Mugambi *et al.*, 1989). Substantial amount of work has been published on the effects of pyrethroids on the central nervous system which is the principal site of action of these toxic compounds in the animal system (Staatz *et al.*, 1982; Akhtar *et al.*, 1985, 1987; Hutson and Stoydin, 1987; Saleh *et al.*, 1987; Reddy *et al.*, 1991).

Childhood cancers in North America, according to 31 studies review, was also due to paternal occupational exposure with pesticides (National Research Council, 1993; Daniels *et al.*, 1997). It has also been reported that in addition to nervous system (Saunder and Harper, 1994), these pesticides induce their toxic effects on other tissues and systems of the body (Kagan *et al.*, 1986; Guguen-Guillouzo *et al.*, 1988; Ansari and Kumar, 1988; Shakoori *et al.*, 1991, 1992b, 1994). Cypermethrin and deltamethrin induced chromosomal abberations in human lymphocytes have been reported (Dolara *et al.*, 1992; Osman *et al.*, 1995). Several workers have studied and reported their effects on liver, kidney and other tissues systems of the animals. Similar type of work with pyrethroids and other insecticides has already been reported from this laboratory in chick, rat and rabbit (Shakoori *et al.*, 1988, 1990, 1992a; Ali *et al.*, 1994, 1997). The toxicity of these compounds may vary depending upon the route of administration and if the route is oral, as is the case in the present study, generally, the blood and its various cellular components will the first target of insecticide in the body.

The main objective of the present study is to investigate the harmful effects of cypermethrin on the blood and its cellular components in the chick model.

MATERIALS AND METHODS

Experimental animals and their maintenance

Fifty two, one day old broiler chicks were obtained from Coccation Breeders, Shadman market, Lahore. They were kept in the cages (125 cubic feet size)in the animal house of Zoology Department under cotrolled temperature conditions $20 \pm 1°C$. During this period the chicks were fed on commercial poultry feed, purchased from the local market. The feed and water was provided to the animals *ad libitum*. The chicks were allowed to acclimatize for about two weeks before dose administration.

Toxicant used

Cypermethrin as Ripcord, 100 EC, [α-cyano-3-phenoxybenzyl 2,2-dimethyl-3-(2,2-dichlorovinyl) cyclopropane carboxylate], a highly active synthetic pyrethroid and a product of Shell, was used for the experiment.

Administration of toxicant

Two sublethal doses (weak and strong) of cypermethrin were administered keeping in view the LD_{50} values of the insecticide against chicks.

A strong dose of cypermethrin was administered orally only once to chicks at a dose of 250 mg/kg body weight for a total duration of 40 hours. In another experiment,

a weak dose of insecticide was administered @ 150 mg/kg body weight/day for a total period of 12 days.

Experimental procedure

In short term experiment, a group of twenty chicks with almost same body weight and age, after weighing, were administered with strong oral dose (250 mg/kg body weight) of cypermethrin. After the stipulated periods of 5, 10, 20 and 40 hours a group of five birds were anaesthetized and dissected for sampling. A group of four chicks processed similarly, except insecticide treatment was used as control.

In Long term experiment, thirty chicks after weighing, were divided into three groups of ten animals (six treated and four control). Weak dose (150 mg/kg body weight/day) of cypermethrin was administered orally to six animals in each group. The blood samples were collected at 3, 6 and 12 days intervals from control and treated groups with the help of sterlized syring and quickly transferred to EDTA coated tubes with gentle rotation for further studies.

Methodology for haematological studies

The haemoglobin (Hb) content of the blood was estimated according to vanKampen and Zijlstra (1961), the packed cell volume (PCV) was analysed by microhaematocrit method of Strumia *et al.* (1954), while the red blood cells (RBC) and white blood cells (WBC) were counted according to the routine visual haemocytometer method (see, Dacie and Lewis, 1984). The above haematological values were also used for computing different haematological indices *i.e.*, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) by the relationships mentioned in Dacie and Lewis (1984).

RESULTS

Cypermethrin, administered as sublethal doses @ 250 mg/kg body weight (strong dose) for 40 hours (short term) duration produced many significant changes in haematology of chicks (Tables I-II). The Hb content showed 18% and 16% increase, respectively after 5 and 10 hours of cypermethrin administration, while the effects were reversed to normalization when treatment was extended upto 20 hours and decreased significantly by 11% at 40 hours cypermethrin treatment (Tables I-II). In weak dose (long term) experiment at 150 mg/kg body wt./day treatment, although 17% and 12% increase was found at 3 and 6 days which was statistically non-significant. The PCV and MCV showed 36% and 25% increase, respectively at 6 day uninterrupted insecticide feeding daily, the latter value was statistically nonsignificant (Tables III and IV).

The leukocytes (WBC count) show special sensitivity to cypermethrin in both short and long term treatments. A prominent but irregular increase was noticed in WBC count which was 75% , 48%, 86% and 62% at 5, 10, 20 and 40 hours after continuous insecticide feeding (Tables I-II). In weak dose treatment, WBC count did not show any change upto 3 day toxicant feeding, however, on increasing the duration of treatment, 28% and 20% rise was noticed at 6 and 12 day treatments, respectively (Tables IV-VI).

Amongst the haematological indices, MCHC was the only component which exhibited 13% and 14% significantly higher values when compared with control after 5 and 10 hours insecticide feeding (Tables I-II). In long term experiment MCHC was decreased by 22% following 6 day cypermethrin administration (Tables IV and VI). All other haematological parameters, such as RBC count, MCV and MCH, tested for evaluation of cypermethrin toxicity were remained undisturbed.

Table I: **Effect of cypermethrin (250 mg/kg body weight) on some haematological parameters of chick, *Gallus domesticus*, administered for a total period of 40 hours.**

Parameters[b]	Control	Cypermethrin treatment (hours)			
		5	10	20	40
	(n=4)	(n=5)	(n=5)	(n=5)	(n=5)
RBC count ($x10^6$ cells/μl)	2.397[a] ±0.154	2.434 ±0.164	2.454 ±0.147	2.492 ±0.053	2.290 ±0.137
Hb content (g/dl)	9.26 ±0.32	10.93* ±0.52	10.75** ±0.16	9.85 ±0.25	8.27* ±0.22
PCV (%)	37.77 ±1.84	39.42 ±1.97	38.52 ±1.54	38.73 ±1.03	36.94 ±1.14
MCV (fl)	158.72 ±8.90	163.68 ±9.36	158.10 ±6.34	155.90 ±6.61	163.35 ±9.92
MCH (pg)	39.07 ±2.48	45.43 ±2.68	44.28 ±2.11	39.62 ±1.50	36.50 ±1.85
MCHC (g/dl)	24.59 ±0.58	27.76** ±0.49	28.04* ±0.93	25.47 ±0.70	22.46 ±0.94
WBC count ($x10^3$ cells/μl)	21.70 ±1.70	38.00** ±2.71	32.10* ±3.46	40.30** ±3.07	35.10** ±2.49

[a]Mean ± SEM, Student's `t' test; *$P<0.05$; **$P<0.01$.

[b]**Abbreviations used:** RBC, red blood cells; PCV, packed cell volume; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; WBC, white blood cells; fl, femtolitre = 10^{-15} litre; pg, picogram = 10^{-12} gram.

Table II: Percent increase (+) or decrease (-) in different hematological parameters of chick, *Gallus domesticus*, after cypermethrin administration (250 mg/kg body weight) for a total period of 40 hours.

Parameters[b]	Cypermethrin treatment (Hours)			
	5 (n=5)	10 (n=5)	20 (n=5)	40 (n=5)
RBC	+1.54	+2.38	+3.96	-4.46
Hb	+17.98*	+16.00**	+6.30	-10.78*
PCV	+4.36	+1.98	+2.54	-2.21
MCV	+3.13	-0.39	-1.78	+2.92
MCH	+16.30	+13.35	+1.41	-6.56
MCHC	+12.86**	+14.00*	+3.57	-8.67
WBC	+75.12**	+47.93*	+85.71**	+61.75**

[a]Student's `t' test; *P<0.05; **P < 0.01.

[b]For abbreviations see Table I.

Table III: Effect of Cypermethrin (150 mg/kg body weight/day) on some hematological parameters of chick, *Gallus domesticus*, administered for a total period of 3 days.

Parameters[b]	Cypermethrin treatment (days)	
	Control (n=4)	Treated (n=6)
RBC count (x10^6 cells/μl)	1.707±0.111[a]	1.780±0.050
Hb content (g/dl)	13.10±1.96	15.36±1.82
PCV (%)	29.21±2.52	33.15±0.87
MCV (fl)	181.49±11.87	184.03±2.12
MCH (pg)	76.36±8.79	82.97±10.96
MCHC (g/dl)	44.55±4.50	44.07±5.10
WBC count (x10^3 cells/μl)	37.13±1.59	40.67±1.12

[a]Mean ± SEM, Student's `t' test.

[b]For abbreviations see Table I.

Table IV: **Effect of Cypermethrin (150 mg/kg body weight/day) on some haematological parameters of chick, *Gallus domesticus*, administered for a total period of 6 days.**

Parameters[b]	Cypermethrin treatment (days)	
	Control (n=4)	Treated (n=6)
RBC count (x10^6 cells/μl)	2.178±0.095[a]	2.358±0.185
Hb content (g/dl)	11.93±1.30	13.34±1.88
PCV (%)	27.46±2.48	37.31±2.67*
MCV (fl)	126.97±20.88	159.05±17.21
MCH (pg)	55.80±8.21	57.45±7.98
MCHC (g/dl)	46.31±3.52	36.26±2.43*
WBC count (x10^3 cells/μl)	35.25±1.45	45.10±3.01*

[a]Mean ± SEM, Student's 't' test; *$P<0.05$.

[b]For abbreviations see Table I.

Table V: **Effect of Cypermethrin (150 mg/kg body weight/day) on some hematological parameters of chick, *Gallus domesticus*, administered for a total period of 12 days.**

Parameters[b]	Cypermethrin treatment (days)	
	Control (n=4)	Treated (n=6)
RBC count (x10^6 cells/μl)	2.338±0.082[a]	2.218±0.068
Hb content (g/dl)	11.90±0.38	11.39±0.54
PCV (%)	39.39±1.92	34.64±1.00
MCV (fl)	168.38±4.44	156.33±3.21
MCH (pg)	50.92±0.68	51.70±1.25
MCHC (g/dl)	30.33±1.16	32.87±1.16
WBC count (x10^3 cells/μl)	20.88±1.48	25.97±1.08*

[a]Mean ± SEM, Student's 't' test.

[b]For abbreviations see Table I.

Table VI: **Percent increase (+) or decrease (-) in different hematological parameters of chick, *Gallus domesticus*, after cypermethrin administration (150 mg/kg body weight/day) for a total period of 12 days.**

Parameters[b]	Cypermethrin treatment (days)		
	3 (n=6)	6 (n=6)	12 (n=6)
RBC	+4.28[a]	+8.26	-5.13
Hb	+17.21	+11.76	-4.29
PCV	+13.48	+35.88*	-12.07
MCV	+1.40	+25.27	-7.16
MCH	+8.66	+2.95	+1.53
MCHC	-1.08	-21.70*	+8.36
WBC	+9.54	+27.94*	+19.76*

[a]Student's `t' test; *$P<0.05$.

[b]For abbreviations see Table I.

DISCUSSION

Following experimental ingestion or accidental exposure of animal by toxic compounds these are generally, assimilated or absorbed through the portal blood or general surface of the body. It is evident that after entry of the toxicant into the body, initially it comes incontact with the blood including its cellular components. The toxicant may induce variable toxicity in the animal systems depending upon the type of chemical, duration of exposure. and amount of material ingested.

In the present experiment, cypermethrin treatment to chicks at two sublethal levels for 40 hours and 12 days, respectively did not produce any severe abnormality in hematological parameters, except some significant increase in Hb (18% and 16%) and MCHC (13% and 14%) at 5 and 10 hours of insecticide administration respectively. Increase in both these components within initial 10 hours, without increase in RBC count, MCV and PCV, is an indication that Hb in the erythrocytes has become more condense, the condition which may be pathological for the organism. The increase in Hb and MCHC may be due to some stimulatory response of cypermethrin on Hb synthesis in the hemopoietic tissue. However, it has also been reported that in birds hypoxia and dehydration may develop due to insecticidal poisoning (Coles, 1986; Campbell, 1988) and rise in Hb during this study can be explained as a compensatory response to restore the oxygen level in the body. Ali *et al.* (1997) in another study on chicks with malathion showed rise in haemoglobin in 12 days and 4 weeks treatment at 400 and 250 mg/kg body wt./day. The findings in the present study differ from earlier studies conducted by Shakoori *et al.* (1990, 1994) and Ali and Shakoori (1994), with another pyrethroid and DDT, an organochlorine compound, bifenthrin in rabbits which showed significant decrease in Hb content, RBC count and MCHC in 30 day study with sublethal doses. Similar decrease was also observed in rats and fish with other pesticides like malathion, aldrin, gamma-BHC (Ali and Shakoori, 1981; Shakoori and Ali, 1985; Reddy and Bashamohideen, 1989). In another study, with oral administration of cypermethrin alongwith feed @ 420 mg/kg/body weight/day for 6 months in rabbits, the Hb and

MCV remained unchanged while MCHC increased just similar to the present study (Shakoori *et al.*, 1988).

The Hb content at 40 hour cypermethrin treatment showed significant decrease although significant increase (upto 18%) was found during initial 10 hours toxicant feeding with no change at 20 hours. This pattern of changes in the present study was indicative of stimulatory effect of cypermethrin during early hours of strong dose treatment (as is the case in long term experiment with comparatively weak doses) while further exposure to toxicant upto 40 hours may lead to inhibition of Hb synthesis.in hematopoietic tissue during this short term experiment. These studies suggested that the effect of cypermethrin on blood cellular components and Hb may be dose- and time-dependent. The unaltered data at 12 day cypermethrin feeding suggest that effect of this toxicant may be neutralized by the induction of drug metabolizing enzymes and other defence systems of the body (Franklin *et al.*, 1980; Hutson, 1981; Cole *et al.*, 1982; Hodgson and Levi, 1992; Hodgson *et al.*, 1995), as a result animals may have developed resistance against this pesticide under the conditions of the present experiment.

These findings were also confirmed by the increase in WBC in both treatments, which were more prominent in 250 mg dose level administered for 40 hours. Increase in WBC is a typical response of vertebrate systems against toxic insult (Ali and Shakoori, 1990). Similar rise in leukocytes was also reported by Ahmad (1988) with sublethal doses of Danitol (a pyrethroid) in fish, which may result in enhancement of detoxification process.

The study suggests that cypermethrin, under the present experimental conditions and at dose levels used in this experiment, is moderately toxic to chicks and effects are reversible on extending the treatment duration, as for as the blood morphological components are concerned.

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TOXICITY OF *BACILLUS THURINGIENSIS* STRAINS TO THE COTTON APHID, *APHIS GOSSYPII* (HOMOPTERA : APHIDIDAE)

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Abstract: Mortality bioassays were used to investigate the toxicity of *Bacillus thuringiensis* strains against cotton aphids, *Aphis gossypii*. These strains were isolated from samples collected from different localities of Pakistan. Of the total isolates screened, nine strains showed activity against cotton aphids. Two isolates viz., Fic 16.15 and Ins 2.13 gave the lowest LC_{50} values.

Key words: *Aphis gossypii, Bacillus thuringiensis*, bio-insecticide, toxicity.

INTRODUCTION

The cotton aphid, *Aphis gossypii* Glov. is a significant pest of cotton in Pakistan (Mohyuddin, 1985). Both adults and nymph imbibe liquid from host plant tissues by probing inter- and intracellularly with their maxillary and mandibular stylets (Pollard, 1973). These results in stunted growth, curled leaves and death of small plants. Heavy infestation on cotton later in the season caused shedding of curled leaves and premature opening of the bolls, thus reducing the yield and grade of lint. Black sooty mould develops on honey dew excreted by both adults and nymphs which makes cotton sticky and stained (Roy and Behvra, 1983).

The only control method available for the aphids is the foliar spray of insecticides. However, the development of resistance to chemical insecticides by many pest insects and general concern for environmental damage have shifted the trend towards biological control agents.

Subspecies of *Bacillus thuringiensis* produce insecticidal, proteinaceous and parasporal protoxins that have specific activity spectrum (Feitelson *et al.*, 1992). The inactive protoxins in the larval midgut are activated to the insecticidal toxins by solubilization in the high pH (ca 10.5) and cleavage by the specific proteases. Since *B. thuringiensis* is insecticidal, there has been great interest in its use for the control of agricultural pests and human disease vectors (Dulmage, 1981). The main objective of the present study was to evaluate the toxicity of the *B. thuringiensis* strains on the cotton aphids and to determine survival of the insects eater intoxication.

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MATERIALS AND METHODS

Bacillus thuringiensis subspecies were obtained from CAMB culture collection and grown in G. medium (pH 7.0), containing 0.2 g $MgSO_4$, 0.6 g K_2HPO_4, 0.4 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 0.08 g $CaCl_2$, 2 g yeast extract, 1 g glucose, 0.4 g caseamino acid, 10 ml Tris HCl (1M, pH 7.6) and 2.5 ml each of 1% $ZnSO_4$, 1% $CuSo_4$, 10% $MnSO_4$ and 0.04% $FeSO_4$ per litre.

All cultures were grown at 30°C with shaking (150 rpm) for three days. After three days, the bacterial culture consisting of vegetative cells, sporangia, spores and crystals were harvested by centrifugation (7,000 K, 10 min, 4°C). The pellets were washed with 0.5M NaCl, 10mM EDTA and twice with distilled H_2O. Pellets were resuspended in solubilizing buffer (50mM sodium carbonate and 10mM Dithiothretol, pH 10) and placed at 37°C for 24 h. The suspension was centrifuged (7,000 K, 10 min, 4°C) and supernatant was assayed for total protein using Bio-Rad protein assay method with bovine serum albumin as the standard. The protoxin (20 μg) was digested with 1 μg trypsin at pH 7.0, 30°C for three hours. The toxins were dialysed against distilled water (pH 7.0), using filter paper of 0.025 μm.

Bioassays were carried out on adult aphids by feeding them different dilutions of activated toxins in 18% sucrose solution of pH 7.5-8.0 (Auclair, 1965; Walters *et al.*, 1970). The diet was presented to aphids in a stretched parafilm sachets. The sachet was made on the upper rim of small plastic tube. The base of the tube had a hole covered with paper for aeration. Bioassays were performed in triplicate samples of 45 aphids per dose. The concentrations of toxin proteins were 32, 62, 125 and 250 μg/ml. The tests were repeated many times for each toxic strain. Survivorship was monitored daily by viewing through the sides of tube. The median lethal dose (LD_{50}) for each test strain was calculated after 48 hrs of toxin ingestion by probit analysis (Raymond, 1985).

RESULTS

A total of 100 *B. thuringiensis* strains, isolated from different sources and areas of Pakistan were assayed. The majority (91%) of the isolates were non-toxic to aphids. The remainder showed varying level of toxicity in the screening test. The strains of *B. thuringiensis* which caused 50% mortality in aphid population at concentration less than 300 μg toxin protein/ml were considered toxic. In total, nine strains of *B. thuringiensis*, isolated from soil, animal and bird droppings, wheat dust, pulse dust and dead insects were sufficiently toxic (Table I).

Among the active strains, Fic 16.15 showed the lowest and SHD 36.2 showed the highest LD_{50} values. The rank order of toxicity for the different strains is given in Table I. Among the toxic strains Fic 16.15 and INS 2.13 displayed the steepest slope.

All active strains, studied during the present experiments, showed a significant increase in mortality rate with the increase of dosage and exposure time of aphid population against toxin proteins. The mortality of non-toxic isolates was not significantly different from the mortality of the control aphids.

DISCUSSION

The discovery of *B. thuringiensis* isolates with toxic activity against different insect and pests (Dulmage, 1981) has initiated an interest for the search of strains which show susceptibility for cotton aphids (*Aphis gossypii*). The results of this study showed that nine *B. thuringiensis* isolates had variable level of toxicity for cotton aphids. In nature, these strains were not available to aphids that's why they did not take active part in their population control. In laboratory their toxic gene will be isolated and cloned in bacteria or plant or formulation will be prepared for spray. The strains Fic 16.15, Ins 2.13 and Fic 5.2A based on low LD_{50} values, were most active against aphids.

Table I: Median lethal concentration (LC_{50}, μg protein/ml) of *Bacillus thuringiensis* strains against cotton aphids, *Aphis gossypii*, exposed for three days.

STRAINS	SOURCE OF MATERIAL	LC_{50}	SLOPE	RELATIVE SENSITIVITY
Fic 16.15	Bird dropping	46	1.57±0.90	1.0
Ins 2.13	Dead insect	61	1.67±0.81	1.3
Fic 5.2A	Animal dropping	101	0.30±1.02	2.2
Gu 9.1	Pulse dust	113	0.94±0.86	2.5
Fic 11.2	Soil	139	1.48±0.71	3.0
CHT 17.6	Soil	140	0.77±1.17	3.0
Fic 3.16	Bird dropping	203	0.96±0.76	4.4
Hfz 24.8	Wheat dust	210	2.46±0.47	4.6
Shd 36.2	Bird dropping	296	1.87±0.55	6.4

Note: Mortality counted after 48 h.

a: LC_{50}/LC_{50} of Fic 16.5

The aphids feed on artificial diet at an estimated rate of 20 nl/h (Wright *et al.*, 1985). Assuming constant feeding an aphid took up approximately 1 μl of toxin solution in the present study. The toxin proteins required for 50% mortality of population ranged from 21 to 135 ng. These doses are higher than the activated toxin required by Lepidoptera and Coleoptera for the 50% mortality of population. It may be due to difference in food conservation time in gut of these insects. Since aphid rapidly excrete large volume of their liquid diet after ingestion, therefore midgut residence time of imbibed toxin may be low (Walters and English, 1995). At present, the activation process within the aphid gut is unknown. Aphid may have rapid brush border membrane repair system to avoid destruction induced by delta endotoxin on *B. thuringiensis* (Percy and Fast, 1983; Bauer and Pankratz, 1992).

This further suggest that these strains, mentioned in Table I, are more toxic than other strains and their effect on membrane of gut at the LD_{50} concentration is irreversible.

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SHORT COMMUNICATION

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A NOTE ON THE FISHES OF LORALAI DISTRICT, BALUCHISTAN

Abstract: The fish fauna of Baluchistan is poorly known. It is always desirable to concentrate on small areas in such cases and add whatever is discovered in the known species. The present paper is such an attempt. It deals with the fish fauna of Loralai District in North-Eastern part of Baluchistan. Three species, *viz.*, *Barilius vagra*, *Crossocheilus diplocheilus* and *Garra gotyla* are added to the known fish fauna of this district.

Key words: Freshwater fishes, *Barilius vagra*, *Crossocheilus diplocheilus*, *Garra gotyla*, Loralai, Pakistan.

INTRODUCTION

Loralai District forms the eastern part of Baluchistan. It lies between 29° 56' to 30° 37'N and 67° 43' to 69° 49'E. Its fish fauna has not been fully explored. Mirza and Naik (Fish and Fisheries of Lorali District. *Pakistan J. Scient. Res.*, **18**: 196-198, 1966), recorded only 8 species. Three more species were collected during August, 1992 by the junior author. Thus the number of species now known from Loralai District goes up to 11. The new records are marked with asterisk (*).

SYSTEMATIC ACCOUNT

ORDER: CYPRINIFORMES
FAMILY: CYPRINIDAE

1. *Barilius vagra* (Hamilton)*
2. *Crossocheilus diplocheilus* (Heckel)*
3. *Cyprinion watsoni* (Day)
4. *Garra gotyla* (Gray)*
5. *Labeo dero* (Hamilton)
6. *Racoma labiata* McClelland
7. *Schizocypris brucei* Regan
8. *Tor putitora* (Hamilton)

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FAMILY: NEMACHEILIDAE

9. *Schistura anambarensis* (Mirza and Banarescu)

10. *Schistura lindbergi* (Banarescu & Mirza)

ORDER: SILURIFORMES
FAMILY: SISORIDAE

11. *Glyptothorax naziri* (Mirza & Naik)

The species recorded as *Nemacheilus baluchinum* by Mirza and Naik (1966) is actually *Schistura anambarensis* (Mirza and Banarescu). This correction has been made in the present note. Similarly the name *Schizothoraichthys labiatus* has been changed to *Racoma labiata*.

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