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

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

Key words: Bacteria as snail controlling agent.

INTRODUCTION

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

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

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

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

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

MATERIALS AND METHODS

Collection and maintenance of snails

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

Histology of normal snails

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

Isolation and identification of bacteria

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

Treatment of snails and histology

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

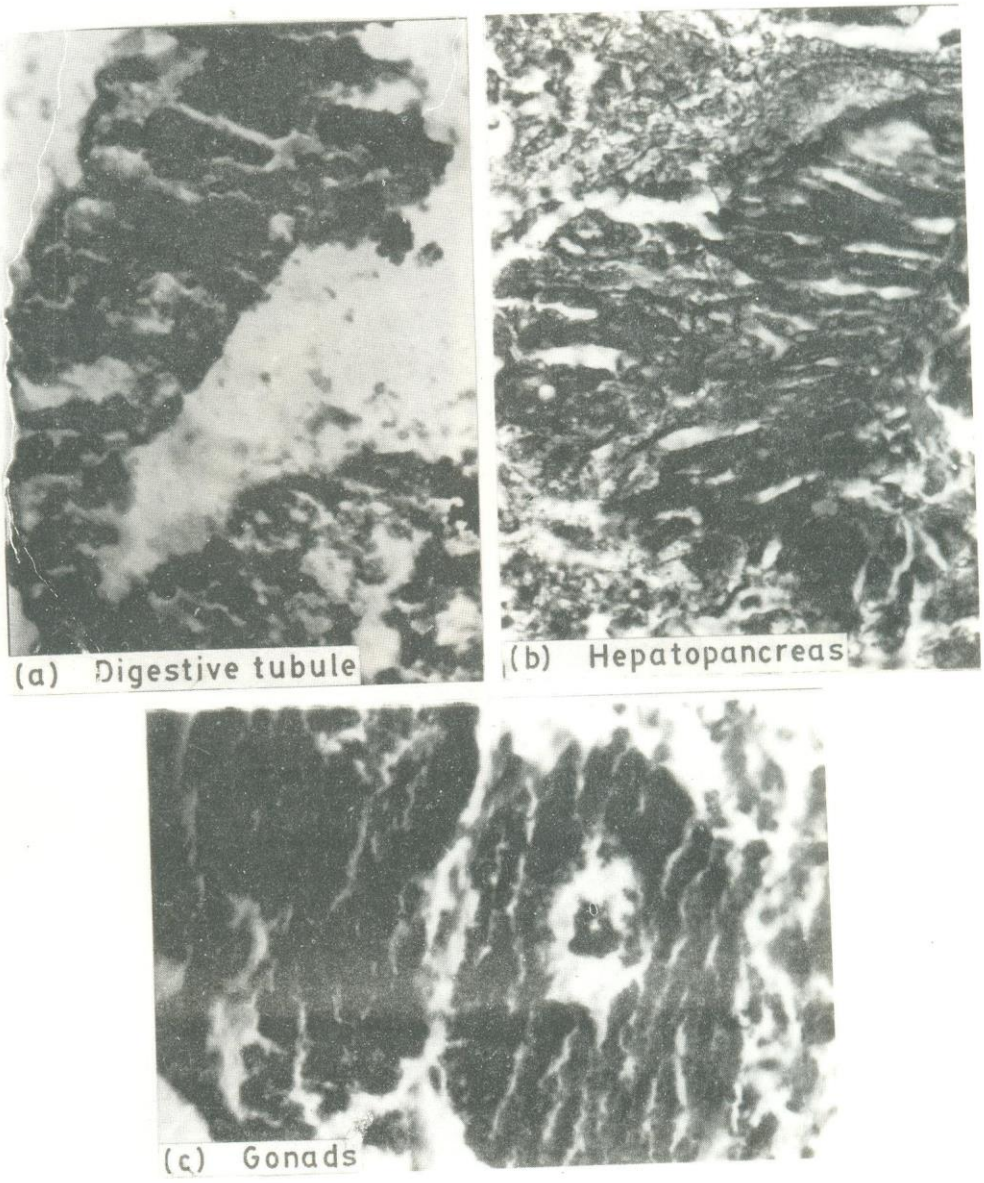

Plate I: Histology of normal *Lymnaea acuminata*.

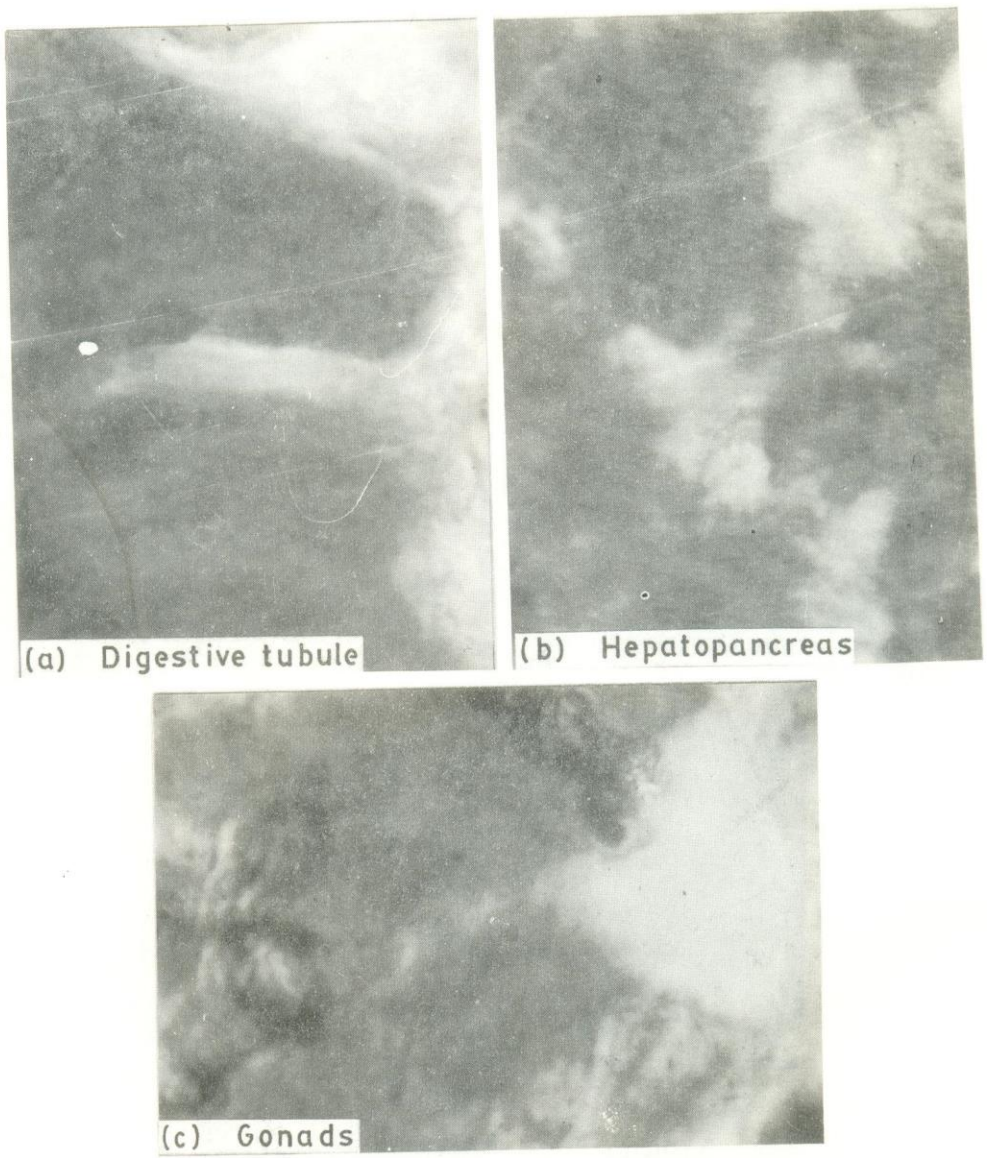

Plate II: Histology of *Lymnaea acuminata* treated with *Bacillus subtilis*.

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

RESULTS

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

Identification of isolated strain

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

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

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

Histology of normal snails

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

Digestive system

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

Hepatopancreas

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

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

Gonads

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

Histopathological studies

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

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

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

[a]Mean±S.E.; ***P<0.001.

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

DISCUSSION

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

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

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

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

Histology of uninfected digestive system

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

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

Histopathology induced by bacteria

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

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

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

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REFERENCES

AGC (AGRICULTURAL GENETICS COMPANY), 1996. Biological control of molluscs with nematodes and bacteria that support growth and pathogenicity of nematodes. US Patent 5527525.

BAUR, L.S., 1995. Resistance: A threat to insecticidal protein of *Bacillus thuringiensis*. *Folia Ent.*, **78**: 414-443.

BENTHEIN, K.E., CIMATO, T.R. AND ETTINGER, M.J., 1995. Copper binding to mouse liver S-adenosylhomocysteine hydrolase and the effects of copper on its level. *J. Bio. Chem.*, **270**: 20703-20711.

BERKLEY, R.C.W. AND GOODFELLOW, M., 1981. *Aerobic and endospore-forming bacteria*. Academic Press Inc. (Ltd.), London, pp.44-46.

BOER, H.H. AND KITS, K.S., 1990. Histochemical and ultrastructural study of the alimentary tract of the freshwater snail *Lymnaea stagnalis*. *J. Morphol.*, **205**: 97-112.

BUSH, M.S. AND MAXWELL, P., 1988. The ultrastructure and function of the intestine of *Patella vulgata*. *J. Zool. (London)*, **215**: 685-782.

CAZZANIZA, N.J., 1990. Predation of *Pomacea canaliculata* (Ampullariids) on adult *Biomphalaria peregina* (Planorbidae). *Am. Trop. Med. Parasitol.*, **84**: 97-100.

CHEESBROUGH, M., 1987. *Medical Laboratory Manual for Tropical Countries*, Vol.1. English Language Book Society, Butterworths, pp.228-339.

CHEESBROUGH, M., 1993. *Medical Laboratory Manual for Tropical Countries*, Vol.II. Lea & Febiger, Philadelphia, pp.226, 532.

CHENG, T.C., 1986. Biological control studies: Bacteria associated with moribund *Biomphalaria glabrata* (Mollusca) in the laboratory. *J. Invertebr. Path.*, **47**: 219-224.

CRICKMORE, N. AND ELLAR, D.J., 1992. Involvement of a possible chaperonin in efficient expression of the cloned cry IIA-delta-endotoxin gene in *Bacillus thuringiensis*. *Mol. Microbiol.*, **6**: 1533-1537.

DIAS, E., 1953. *Bacteriological warfare on the intermediate hosts of human schistosomes*. Fifth International Congr. Trop. Med. and Malaria. Istanbul (Mimeographic report). Republished Mem. Inst. Osw. Cruz., p.7.

EBELE, S., OLADIMEJI, A.A. AND DARAMOLA, J.A., 1990. Molluscicidal and piscicidal properties of copper(II) tetraoxosulfate(VI) on *Bacillus globosus* (Morelet) and *Clarias anguillaris* (L.). *Aquat. Toxicol.*, **17**: 231-238.

FEDERICI, B.A., 1995. The future of microbial insecticide as vector control agent. *J. Am. Mosq. Control Assoc.*, **11**: 266-268.

FEDIANINA, L.V., SHEVTSOV, V.V., ROMANENKO, N.A., BAIANDINA, D.G. AND NAINDENOVA, A.S., 1993. The efficacy of entomopathogenic Bacilli against ancyclostomidae larvae *Nippostrongylus braziliensis* Transvasson. *Med. Parazitol. Mosk.*, **1**: 16-18.

FERRE, J., REAL, M.D., VAN-RIE, J., JANSES, S. AND PEFEROEN, M., 1991. Resistance to *B.t.* insecticide in field population of *Plutella xylosella* is due to change in midgut membrane receptor. *Proc. Natl. Acad. Sci., USA*, **88**: 5119-5123.

HOFKIN, B.V., STRYKER, G.A., KOECH, D.K. AND LOKER, E.S., 1991. Consumption of *Biomphalaria glabrata* egg masses and juveniles by the ampullarid snails *Pila ovata, Lanistes carinatus* and *Marisa cormarietis*. *Acta Trop.*, **49**: 37-44.

HOLT, G.H., KREIGH, N.R., SNEATH, P.H.A., STANELY, J.T. AND WILLIAMS, S.T., 1994. *Bergey's Manual of Determinative Bacteriology*, 9th Ed. Williams & Wilkins, Baltimore, Maryland, U.S.A.

HUMASON, C.L., 1962. *Animal Tissue Techniques*, W.H. Freeman & Company, U.S.A.

HUSSAIN, T., HASHMI, H.A., KHAN, M.S., PERVAIZ, K. AND TANVEER, A., 1996. The prevalence of *Lymnaea* snails in Lahore and their eradication by molluscicide copper sulphate. *Punjab Univ. J. Zool.*, **11**: 1-6.

HYMAN, L.H., 1967. *The Invertebrate Mollusca*. McGraw-Hill Company, London, pp.153-412.

KINZIE, R.A., 1992. Predation by the introduced carnivorous snail *Euglandina rosea* (Ferrusac) on endemic aquatic lymnaeid snails in Hawaii. *Biol. Conserv.*, **60**: 149-155.

MAHILLON, J., REZSOHAZY, R., HALLETE, B. AND DELCOUR, J., 1994. IS231 and *Bacillus thuringiensis* transposon element, a review. *Genetica*, **93**: 13-26.

MALLARY, E.B., 1944. *Pathological techniques*. W.B. Saunders Company, Philadelphia, La.

METLUS, A.M. AND MACALUSO, A., 1990. Expression of *Bacillus thuringiensis* delta-endotoxin gene during vegetative growth. *Appl. Environ. Microbiol.*, **56**: 1128-1134.

MINAMI, M., HORI, H., OGIWARA, K., SATO, R., OHBA, M. AND IWAHANA, H., 1995. Deletion mutation of the gene encoding delta-endotoxin specific to scarabeid beetles. Minimum region of the gene required to express the activity. *Biosci. Biotechnol. Biochem.*, **59**: 1381-1383.

PAURT DE BACH, 1961. *Symposium on Ecological Effects of Biological and Chemical Control of Undesirable Plants and Animals* (ed. D.J. Kueun), pp.10-18.

RHODE, P.A., 1993. *BBL Manual of Products and Laboratory Procedures*, 5th edn., 5: 1973.

ROBERTS, J.K. AND KURIS, A.M., 1990. Predation and control of laboratory populations of the snail *Biomphilaria glabrata* by the freshwater prawn *Macrobrachium rosenbergii*. *Ann. Trop. Med. Parasitol.*, **84**: 401-402.

ROLAND, C. AND GARCIA-CORRALES, P., 1988. Anatomy and histology of the alimentary tract of the snail *Theba pisana* (Gastropoda : Pulmonata). *Malacologia*, **28**: 119-130.

SEELEY, H.W. AND VANDERMARK, P.J., 1962. *Microbes in action*. Freeman, San Francisco.

SHELTON, W.L., SOLIMAN, A. AND ROTHBARD, S., 1995. Experimental observations on feeding biology of black carp (*Myopharyngodon piceus*). *Isr. J. Aquacult. Banudgch.*, **47**: 59-67.

SLOOTWEG, R., VOREG, P.A. AND WIERSMA, S.J., 1993. Effect of molluscivorous freshwater quality and pond management on the development of schistosomiasis vector snails in aquaculture ponds. *Aquacult. Fish. Manage.*, **24**: 123-128.

SMITH, G.P., MERRICK, J.D., BONE, E.J. AND ELLAR, D.J., 1996. Mosquitocidal activity of CryIC-delta-endotoxin from *Bacillus thuringiensiz aizawai*. *Appl. Environ. Microbiol.*, **62**: 680-684.

STARZAK, M. AND BAJPAI, R.K., 1991. A structural model for vegetative growth and sporulation in *Bacillus thuringiensis*. *Appl. Biochem. Biotechnol.*, **28**: 699-718.

STOKES, E.J. AND RIDGEWAY, G.L., 1980. *Clinical Bacteriology*, 5th Ed., Edward Arnold, pp.109-113.

TANVEER, A. AND KHAN, D., 1991. Studies on interspecific competition between *Bellamaya bengalensis* (Lamark) and medically important Lymnaeid, Planorbid and Physid snails. *Pakistan J. Zool.*, **23**: 193-200.

TANVEER, A. AND SAMINA, M., 1992. Histopathological effects induced by trematodes on the hepatopancreas of freshwater snail *Lymnaea acuminata* Lamarck. *J. Syst. & Exp. Biol.*, **2**: 79-94.

TANVEER, A. AND SAMINA, M., 1992a. Trematode induced histopathological changes in the gonads of freshwater snail *Lymnaea acuminata* Lamarck. *J. Syst. & Exp. Biol.*, **2**: 95-106.

TANVEER, A., BANO, A. AND JABEEN, Z., 1995. Effect of copper sulphate on survival and blood cell morphology of freshwater snails *Lymnaea rufescens* Gray and *Lymnaea luteola* (Lamark) commonly found in Lahore. *Sci. Int. (Lahore)*, **7**: 509-512.

TANVEER, A., LONE, K.P. AND KHAN, D., 1990. Trophic preferences of some gastropod snails inhabiting different aquatic habitats in Lahore. *Pakistan J. Zool.*, **22**: 51-63.

WALKER, G., 1972. The digestive system of the slug *Agriolimax reticulates* (Muller): Experiments on Phagocytosis and nutrient absorption. *Proc. Malac. Soc. London*, **40**: 33.

WATKINS, B. AND SIMKISS, K., 1990. Interaction between soil bacteria and the molluscan alimentary tract. *J. Mollusc. Stud.*, **56**: 267-274.

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