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PROCEEDINGS

OF

PAKISTAN CONGRESS OF ZOOLOGY

Volume 26, 2006

All the papers in this Proceedings were refereed by experts in respective disciplines

TWENTY SIX PAKISTAN CONGRESS OF ZOOLOGY

held under auspices of

THE ZOOLOGICAL SOCIETY OF PAKISTAN

at

UNIVERSITY OF THE PUNJAB

April 4 – 6, 2006

CONTENTS

ACKNOWLEDGMENTS

University of the Punjab, New Campus, Lahore, hosted the 26th Pakistan Congress of Zoology (International).

The Zoological Society of Pakistan expresses its deep gratitude to the Vice Chancellor, University of the Punjab, Lahore and faculty members and students of the Department of Zoology and School of Biological Sciences of the University of the Punjab for extending warm hospitality.

Grants were received from University of the Punjab, ISESCO, Morocco, Pakistan Science Foundation, Islamabad, Higher Education Commission, Islamabad, Pakistan Atomic Energy Commission, Islamabad and Hamdard Foundation, Pakistan.

TWENTY SIX PAKISTAN CONGRESS OF ZOOLOGY (INTERNATIONAL)

UNIVERSITY OF THE PUNJAB, LAHORE

April 4 – 6, 2006

PROGRAMME

TUESDAY, APRIL 4, 2006

09:00 AM Registration
10:00 AM Inauguration: Recitation from the Holy Quran
10:05 AM Welcome Address by Prof. Dr. M. Akhtar, Chairman, Department of Zoology, University of the Punjab, Lahore.
10:20 AM Report by Secretary, Zoological Society of Pakistan.
10:35 AM Key Note Address by the President, Zoological Society of Pakistan, Prof. Dr. A.R. Shakoori
10:50 AM Distribution of Medals and Awards
11:10 AM Address by the Chief Guest Lt. Gen. (R) Arshad Mehmood, Vice Chancellor, University of the Punjab, Lahore.
11:25 AM Vote of Thanks by Prof. Dr. Syed Shahid Ali.
11:30 AM Refreshment

12:00 AM

JOINT SESSION I: (Plenary Lectures)

Chairperson: Prof. Dr. Mahmood H. Qazi
Vice-Chancellor, University of Lahore.

Speakers: 1. **Dr. T. Subramonian**
Department of Zoology, University of Madras, Chennai, India.
Steroid hormonal coordination of molting and female reproduction in the interdial sand crab *Emerita asiatica* (Milne Edwards).

2. **Dr. Rup Lal**
Professor of Molecular Biology, Department of Zoology, University of Delhi, Delhi, India.
Secrets of contamination of Indian environment by hexachlorocyclohexane and development of bioremediation biotechnologies.

01:00 PM Lunch and Prayer

HALL – 1

SECTION I: CELL BIOLOGY, BIOCHEMISTRY GENETICS, MOLECULAR BIOLOGY, PHYSIOLOGY, GENETICS

SESSION I

	Chairperson:	Dr. Anwar Nasim
	Co-chairperson:	Dr. Naeem Rashid
02:00 AM	Paper reading	
04:30 PM	Tea Time	

SESSION II

	Chairperson:	Prof. Dr. M. Arslan
	Co-chairperson:	Dr. Nazia Khurshid
05:00 PM	Paper reading	
06:30 PM	Prayer	

SESSION III

	Chairperson:	Prof. Dr. M. Anwar Waqar
	Co-chairperson:	Dr. Akram Shah
06:45 AM	Paper reading	
08:00 PM	Dinner	

HALL – 2

SECTION II: PEST AND PEST CONTROL

SESSION I

	Chairperson:	Dr. Sana Ullah Khan Khattak
	Co-chairperson:	Dr. Abida Butt
02:00 PM	Paper reading	
04:30 PM	Tea Time	

SESSION II

	Chairperson:	Prof. Dr. M. Suleman
	Co-chairperson:	Dr. Syed Kamaluddin
05:00 PM	Paper reading	
06:30 PM	Prayer	

SECTION III: ENTOMOLOGY

SESSION I

	Chairperson:	Prof. Dr. Imtiaz Ahmad
	Co-chairperson:	Dr. Bilquees Fatima
06:45 PM	Paper reading	
08:00 PM	Dinner	

HALL – 3

SECTION V: FISHERIES, ECOLOGY, WILDLIFE, FRESHWATER BIOLOGY, MARINE BIOLOGY

SESSION I

	Chairperson:	Prof. Dr. M. Afzal Kazmi
	Co-chairperson:	Dr. Zafar Iqbal
02:00 AM	Paper reading	
04:30 PM	Tea Time	

SESSION II

	Chairperson:	Dr. Muhammad Ayub
	Co-chairperson:	Dr. Ali Mohammad Yousuf Zai
05:00 PM	Paper reading	
06:30 PM	Prayer	

SESSION III

	Chairperson:	Dr. R.R. Ghazi
	Co-chairperson:	Dr. Abdul Rab
06:45 AM	Paper reading	
08:00 PM	Dinner	

WEDNESDAY, APRIL 5, 2006.

JOINT SESSION II: (Plenary Lectures)

Chairman: Prof. Dr. Muhammad Arslan
Department of Physiology, University of Health Sciences, Lahore

09:00 AM 1. **Meritorious Prof. Dr. A.R. Shakoori**
School of Biological Sciences, University of the Punjab, Lahore.
Role of P53 in Cancer Development.

2. **Prof. Dr. Quddusi B. Kazmi**
Marine Reference Collection and Resource Center, University of Karachi, Karachi.
Activities at MRC of importance to faunal diversity and fishery resources

HALL – 1

SECTION I: CELL BIOLOGY, BIOCHEMISTRY, GENETICS, MOLECULAR BIOLOGY, PHYSIOLOGY, GENETICS

SESSION IV

	Chairperson:	Prof. Dr. Shamsuddin Shaikh
	Co-chairperson:	Dr. Naz Abbas
10:00 AM	Paper reading	
11:00 PM	Tea Break	

SESSION V

	Chairperson:	Prof. Dr. Rup Lal
	Co-chairperson:	Dr. Shahid Nadeem
11:30 AM	Paper reading	
01:00 PM	Lunch and Prayer	

SESSION VI

	Chairperson:	Prof. Dr. Syed Shahid Ali
	Co-chairperson:	Dr. M. Saleem Haider
02:00 PM	Paper reading	
04:30 PM	Tea Break	

SESSION VII

	Chairperson:	Prof. Dr. Afsar Mian
	Co-chairperson:	Dr. Farah R. Shakoori
05:00 PM	Paper reading	
06:30 PM	Prayer	

SESSION VIII

	Chairperson:	Prof. Dr. Khalid P. Lone
	Co-chairperson:	Dr. Farah R. Shakoori
06:45 PM	Paper reading	
08:00 PM	Dinner	

HALL – 2

SECTION III: ENTOMOLOGY

SESSION II

	Chairperson:	Prof. Dr. Mushtaq A. Saleem
	Co-chairperson:	Prof. Dr. Naheed Ali
10:00 AM	Paper reading	
11:00 PM	Tea Break	

SESSION III

	Chairperson:	Prof. Dr. T. Subramoniiam
	Co-chairperson:	Prof. Dr. M.S. Wagan
11:30 AM	Paper reading	
01:00 PM	Lunch and Prayer	

SESSION IV

	Chairperson:	Prof. Dr. Shamshad Akhtar
	Co-chairperson:	Dr. Amanullah Khan
02:00 PM	Paper reading	
04:30 PM	Tea Break	

SESSION V

	Chairperson:	Prof. Dr. Nikhat Yasmeen Siddiqui
	Co-chairperson:	Dr. Sohail Ahmad
05:00 PM	Paper reading	
06:30 PM	Prayer	

SECTION V: FISHERIES, ECOLOGY, WILDLIFE, FRESHWATER BIOLOGY, MARINE BIOLOGY

SESSION IV

	Chairperson:	Prof. Dr. M. Naeem Khan
	Co-chairperson:	Dr. N.T. Narejo
05:00 PM	Paper reading	
06:30 PM	Dinner	

SESSION V

	Chairperson:	Prof. Dr. Quddusi B. Kazmi
	Co-chairperson:	Dr. Itrat Zehra
06:45 PM	Paper reading	
08:00 PM	Dinner	

THURSDAY, APRIL 6, 2006

HALL – 1

SECTION I: CELL BIOLOGY, BIOCHEMISTRY, GENETICS, MOLECULAR BIOLOGY, PHYSIOLOGY, GENETICS

SESSION IX

	Chairperson:	Dr. Nematullah
	Co-chairperson:	Dr. Javed Iqbal Qazi
09:00 AM	Paper reading	
11:00 AM	Tea Break	

SECTION V: FISHERIES, ECOLOGY, WILDLIFE, FRESHWATER BIOLOGY, MARINE BIOLOGY

SESSION IV

	Chairperson:	Prof. Dr. Muhammad Akhtar
	Co-chairperson:	Dr. Muhammad Ali
11:30 AM	Paper reading	
01:00 PM	Lunch and Prayer.	

HALL – 2

SECTION IV: PARASITOLOGY

SESSION I

	Chairperson:	Prof. Dr. F.M. Bilqees
	Co-chairperson:	Dr. Aly Khan
09:00 AM	Paper reading	
11:00 AM	Tea break	

SESSION II

	Chairperson:	Mr. Abdul Aziz Khan
	Co-chairperson:	Dr. Zahida Tasawar
11:30 AM	Paper reading	
01:00 PM	Lunch and Prayer.	
02:30 PM	General Body Meeting	
04:00 PM	Concluding Ceremony	
	Recitation	
	Congress Report by President ZSP	
	Award Ceremony	
	Concluding Remarks by the Chief Guest	
	Vote of Thanks	
05:00 PM	Refreshments	

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PROCEEDINGS OF PAKISTAN CONGRESS OF ZOOLOGY
(Proc. Pakistan Congr. Zool.)

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[Abstracted and indexed in Biological Abstracts, Chemical Abstracts, Zoological Records, Informational Retrieval Limited, London, and Service Central De Documentation De L'ORSTOM, Paris. Also listed in Index to Scientific and Technical Proceedings and ISI/ISTP & B Online Data Base of Institute for Scientific Information, Philadelphia, Pennsylvania, USA].

Website: www.zsp.org.pk

CITATIONS

RECIPIENT OF ZOOLOGIST OF THE YEAR AWARD 2006*

Dr. Sana Ullah Khan Khattak
Director, Nuclear Institute for Agriculture and Biology, Faisalabad

Dr. Sana Ullah Khan Khattak was born in village Jalozai, Nowshera, NWFP in February 1947. He obtained B.Sc. (Hons) and M.Sc. (Hons) in Entomology, with distinction, from Agriculture College, Peshawar University. He was appointed as Research Officer at Agriculture Research Department, Government of NWFP in 1968. After serving the provincial department for about seven years, he was selected as Scientific Officer and joined Nuclear Institute for Agriculture and Biology (NIAB) Faisalabad in 1975. Later he was transferred to Nuclear Institute of Food and Agriculture (NIFA), as Deputy Chief Scientist in 2002 where he is working now as a Director since 2003. He obtained his Ph.D. degree in Zoology (Entomology) from the University of the Punjab, Lahore in 1992.

Since joining the PAEC, he has worked as Group Leader, Head of various Divisions, convener of different technical committees and organized several postgraduate training courses, seminars and symposia. During his service tenure, he visited 12 counties for specialized training and scientific

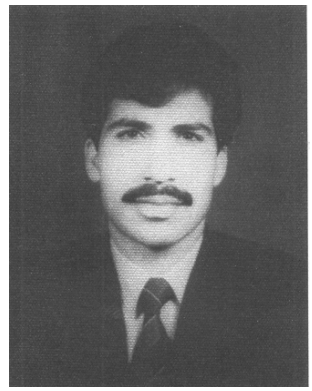
*Other nominees for this award were Dr. Muhammad Nazir Bhatti, Lahore and Dr. Aly Khan, Karachi.

visits as a subject expert. He has been Principal Investigator of various international and national sponsored projects. Dr. Khattak is actively involved in R&D activities related to agriculture and pest management for more than three decades. He was awarded 16 months specialized IAEA Fellowship in "Fruit Fly Control" in USA.

Dr. Khattak developed and applied for the first time "Male Annihilation Technique" for fruit fly eradication in NWFP. He designed a modified trap which captured 36.4% more flies than before. This technology was transferred to the farming community. Using this technique, pest incidence was reduced by 60-87% and farmer's income was increased by 58%. This technique is, pollution free and 50% more cost effective than the pesticidal control. Due to maggot-free fruit production, it fetches an economic return of Rs.120 million/year. It is now well popularized and practiced by farming community, agricultural extension, Govt. agencies, entrepreneurs and NGOs. He has supervised 4 Ph.D. and 18 M.Sc. (Hons.) students in Entomology/Plant Protection. He reviewed and edited more than 150 research papers for various journals including Pakistan Journal of Zoology. He also reviewed more than 20 research projects of HEC, PTCL and PARC. He has to his credit 140 publications in the reputed national/international journals.

On the basis of his valuable contributions in Integrated Pest Management, he was awarded "Tamgh-e-Baqa" and Honoraria by PAEC and "Star Award 2002" by South Asia Publications, and a civil award "Pride of Performance" by the President of Pakistan on August 14, 2005. Now, he is being awarded "Zoologist of the Year Award 2006" by the Zoological Society of Pakistan for his significant contribution in the filed of Zoology.

RECIPIENT OF
PROF. A.R. SHAKOORI GOLD MEDAL 2006*



Dr. Amjad Javed
Assistant Professor
Department of Cell Biology, UMass Medical School, Worcester, MA, USA

Dr. Amjad Javed obtained M.Sc. (Zoology) from University of the Punjab, Lahore in 1990 and was awarded a gold medal as the highest ranking student in the program. In 1991 he started as a Lecturer at Cadet College, Hasan Abdal, teaching under-graduate level biology and in 1994 accepted faculty position at the Crescent College, Lahore in 1995.

After obtaining his Ph.D. degree from the University of the Punjab which was completed in collaboration with University of the Massachusetts Medical School in 1999. Dr. Javed continued his post-doctoral training with Dr. Gary Stein at UMass and in 2002 was promoted as an Instructor in the Department of Cell Biology. In 2004 Dr. Javed was appointed as an Assistant Professor of Cell Biology at UMass Medical School, Worcester. He joined University of Alabama at Birmigham, School of Dentistry as an Assistant Professor in June 2005.

Dr. Javed's research focuses on molecular mechanisms involved in

*Other applicants for this award were Prof. Dr. Saifullah, Peshawar, Dr. Sohail A. Qureshi, Karachi, Dr. Shahnaz Perveen, Karachi, Dr. Imran Ali Siddiqui, Karachi, Dr. Muhammad Saeed Akhtar, Faisalabad, Dr. Rahila Tabassum, Karachi, Dr. Saida Haider, Karachi, Dr. Abid Farid, Peshawar, Dr. Sajjad-ur-Rahman, Faisalabad and Asma Saeed, Lahore.

tissue differentiation and bone synthesis and implies state of the art biochemical, genetic, cellular and molecular approaches in both *ex-vivo* and *in vivo* mouse models. Dr. Javed has over 60 publications in top-rated peer reviewed journals and over 55 published abstracts in national and international scientific meetings. His work has been selected ten times for oral presentations at the annual meeting of the American Society for Bone and Mineral Research (ASBMR) and has delivered more than 20 lectures as an invited speaker at Universities in United states, Canada, Pakistan and Chile. Dr. Javed is recipient of numerous awards including, three young investigator awards from major societies in bone and mineralized tissues.

Besides serving as reviewer for five journals, Dr. Javed is Associate Editor of Journal of Cellular Biochemistry and Critical Reviews in Eukaryotic Gene Expression. He has mentored post-doctoral fellows, Ph.D. students, MS students in Orthopedics and Oncology, resident fellows and undergraduate students. For five years he has jointly supervised NIH/NSF funded summer research program for minority and undergrad students at the University of Massachusetts Medical School. Dr. Javed has also actively participated in community based initiative to promote science education in high school and to enhance young women’s representation in biomedical research.

RECIPIENT OF
PROF. DR. MIRZA AZHAR BEG GOLD MEDAL 2006*

Dr. Naeem Tariq Narejo
Professor, Department of Fresh Water Biology and Fisheries, University of Sindh, Jamshoro

Dr. Naeem Tariq Narejo was born on 25th September 1963 in District Dadu, Sindh. He obtained M.Sc. degree in 1987 and M.Phil in 1997 from the Department of Fresh Water Biology and Fisheries, University of Sindh, Jamshoro. He has earned Ph.D. degree in 2003 in Aquaculture (Fisheries) from Bangladesh Agricultural University, Mymensingh. He has contributed some valuable information essential to enhance fish production through scientific fish culture in order to meet animal protein deficiency in the country. He has introduced Fresh Water Eel culture practices of two big sized eel species and established new benchmark values of different parameters for commercial fish farmers. He joined as Lecturer in the Department of Fresh Water Biology and Fisheries, University of Sindh, Jamshoro in the year 1987, and was appointed as Professor in 2006. He has been Principal Investigator in two research projects funded by Higher Education Commission (HEC) and Pakistan Science Foundation, Islamabad. He is supervising research work of M.Phil leading to Ph.D. students on various aspects of fish biology and fish culture. Up-til now he has produced forty seven research publications in various journals of national and international repute. He is Life-Fellow, Zoological Society of Pakistan, Life-Fellow, Pakistan Fisheries Society and Fellow, Aquaculture Society of Bangladesh.

*Other applicants for this award were Dr. Anjum Sohail, Faisalabad.

RECIPIENT OF
PROF. DR. NASIMA TIRMIZI GOLD MEDAL 2006*

Dr. Feroz Akhter Siddiqui (Mangi)
Assistant Curator (Retired), Marine Reference Collection and Resource Centre, University of Karachi, Karachi

Dr. Feroz Akhtar Siddiqui Mangi was born on January 1st, 1941. She got her Ph.D. Zoology from University of Karachi in 1980 and did Post-Doctorate from Western Washington University, United States in 1991. She worked on production of *Artemia* cysts on large scale in coastal area Karachi at Sandspit and worked on development of marine animals specialy higher crustacean *viz.*, Stenopoidea, Penaeidea, Caridea, Thalassinidea, Anomura and Brachyura, and biodiversity of marine Curstacea. She has been Senior Taxonomist, Marine Reference Collection and Resource Centre, University of Karachi during 1982-1995 and Assistant Curator, Marine Reference Collection and Resource Centre, University of Karachi during 1995-2000. Dr. Mangi has published 60 research papers in national/international journals of repute including 11 popular articles in different periodicals of Pakistan.

*Other applicants for this award were Prof. Dr. Saifullah, Peshawar and Prof. Dr. Javed Mustaquim, Karachi.

RECIPIENTS OF GOLD MEDALS AWARDED BY THE ZOOLOGICAL SOCIETY OF PAKISTAN

1. **Mujib Memorial Gold Medal 2006**
   This Gold Medal is awarded to a student of Karachi University standing first in the recent M.Sc. Zoology examination with specialization in Parasitology. Twelve Medals have already been given. This year's Mujib Memorial Gold Medal was received by Miss Sidra Rizwan.

2. **Mohd Afzal Hussain Qadri Memorial Gold Medal 2006**
   This Gold Medal is awarded to a student of Karachi University, standing first in the recent M.Sc. Zoology examination. Ten medals have already been awarded. The eighth medal was received by Miss Sidra Rizwan.

3. **Muzaffar Ahmad Gold Medal 2006**
   This Gold Medal is awarded to a student of the Punjab University, standing first in the recent M.Sc. Zoology examination. Twelve medals have already been given. Muzaffar Ahmad Gold Medal 2006 was received by Miss Uzma Rafi.

4. **Ahmad Mohiuddin Memorial Gold Medal 2006**
   This Gold Medal is awarded to M.Sc. Zoology student of University of Sindh, Jamshoro standing first in the recent M.Sc. Zoology examination. Five Gold Medals have already been given. This year Ahmed Mohiuddin Memorial Gold Medal 2006 was given to Miss Tehniyat Naz.

5. **Prof. Imtiaz Ahmad Gold Medal 2006**
   This Gold Medal is awarded to a student of Karachi University, standing first in the recent M.Sc. Zoology examination with specialization in Entomology. Five gold medals have already been given. This year Prof. Imtiaz Ahmed Gold Medal 2006 was given to Miss Hira Ayub.

6. **Prof. Dr. S.N.H. Naqvi Gold Medal 2006**
   This Gold Medal is awarded to a student of Karachi University, obtaining Ph.D. Zoology degree with specialization in the field of Toxicology. Four gold medals have already been given. This year Prof. Dr. S.N.H. Naqvi Gold Medal 2006 was given to Dr. Farzana Perveen.

7. **M.A.H. Qadri Memorial Gold Medal 2006**
   This Gold Medal is awarded to a student of Karachi University, obtaining Ph.D. Zoology degree with specialization in the field of Parasitology. Seven gold medals have already been given. This year M.A.H. Qadri Memorial Gold Medal 2006 was given to Dr. Syeda Azra Qamar.

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THE INCIDENCE OF *ENTAMOEBA HISTOLYTICA* IN HUMAN FAECES AT FFC III PRIVATE HOSPITAL, MIRPUR MATHELO (SINDH)

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Abstract.- The present study was carried out to determine the incidence of *Entamoeba histolytica* in human faeces. During the study 3317 faecal samples were examined from January 2004 to December 2004 at FFC III private hospital (Mirpur Mathelo). Out of 3317 specimens, 1691 showed the presence of *E. histolytica*. The overall rate of incidence of *E. histolytica* was 50.9%. Relationships between sex and age in respect of *E. histolytica* was also investigated. Incidence of *E. histolytica* was more in females (28.9%) as compared with males (22.0%). *E. histolytica* highest incidence was 26.2% in age group of one day to 5 years 14.3% in age group of 6 to 15 years, an 10.3% in age group of 16 to 59 years.

Key Words: *Entamoeba histolytica*, fecal contamination, dysentery.

INTRODUCTION

Amoebiasis is caused *E. histolytica*, a parasitic protozoan that infects predominantly humans. An estimated 40 million people worldwide develop the disease annually and 40,000 die due to dysentery, intestinal diseases and liver abscess. Amoebiasis is transmitted by fecal contamination of drinking water and foods and by direct contact with dirty hands or objects as well as by faecal exposure during sexual contact in which case not only cysts, but also trophozoites are infective. House flies spread the cysts and the use of human faeces as fertilizer on fruit and vegetable crops is an important mechanism of spread. The infection is worldwide.

Most people are susceptible to infection, but individuals with suppressed immunity may show severe manifestations of the disease. In industrialized countries, travelers, recent immigrants and institutionalized populations are at risk. AIDS patients are very vulnerable and the disease is common among homosexual men (Hart, 1990). Although considerable work has been done in various parts of the world and Pakistan, there are no published reports from Mirpur Mathelo.

Keeping in view importance of infection, the project was designed to study the incidence of *E. histolytica* in the human population of Mirpur Mathelo, and correlate its incidence with age and sex of the infected persons.

MATERIALS AND METHODS

The present investigation was conducted from January 2004 to December 2004. A total of 3317 human faecal samples were collected in properly labeled sterilized bottles containing 5% formalin. Temporary mounts were made according to Cable (1985).

Preserved faecal material was mixed with an applicator and a drop was placed on the slide. One drop of iodine solution was added to the faecal material, thoroughly mixed, covered with cover slip and examined under the microscope. When fresh faecal samples were examined, they were kept in normal saline solution and identification was done the same day.

RESULTS AND DISCUSSION

The prevalence of *E. histolytica* was studied in 3317 patients from FFC III, private hospital, Mirpur Mathelo, in which 1691 patients were found infected. The overall incidence of *E. histolytica* recorded during the present investigation was 50.9%. The high incidence of *E. histolytica* in humans in the present and other studies (Gonzalez *et al.*, 1995; River *et al.*, 2000; Nimri *et al.*, 2004) from the parts of the world suggests wide spread contamination of environment with cysts of the parasite.

Out of 1500 males and 1817 females examined, 730 males and 961 females were found infected. *E. histolytica* prevalence was 28.9% in females as compared with 22% with males. Cross *et al.* (1975) studied the incidence of *E. histolytica* in 695 humans, 439 were males and 256 females, from Java and Indonesia. Incidence of *E. histolytica* was 2.7% in females and 1.6% in the males. Ohnishi and Murata 1997) examined the incidence of *E. histolytica* in Tokyo. A total of 28 cases were studied. Out of which 26 males were infected with the parasite while the females were free of infection. Nimri *et al.* (2004) obtained faecal samples from humans in Taiwan. A total of 1,569 faecal samples

were examined. Out of which 11.75% were females and 6.9% were males. The results of the present study are in agreement with studies conducted by Cross *et al*. (1975) and Nimri *et al*. (2004) while the results of the present study are not in agreement with studies conducted by Ohnishi and Murata (1997). The high prevalence of the parasite in females could be due to low resistance of female hosts in the present study and the studies conducted by Cross *et al.* (1975) and Nimri *et al*. (2003).

Out of a total of 3317 patients examined, 872 were found infected in the age group 1-5 years, 477 in the age group 6-15 years and 342 in the age group 16-59 years. The incidence of *E. histolytica* was 26.2% in age group of 1 day to 5 years, 14.3% in the age group of 5 to 15 years and 10.3% in the age group 15 to 59 years. Haque and Petri (2000) examined the incidence of *E. histolytica* in Dhaka, Bangladesh. The highest incidence of parasite was 5% in age group of 2 to 5 years. Lucia *et al*. (2001) used ELISA base antigen for detection of *E. histolytica* in Brazil. A total of 735 human cases were studied. The high prevalence was 14.9% in age group 1 to 5 years. Astal (2004) determined the incidence of *E. histolytica* in the Khan Younas Government Hospital. A total of 1,370 human cases were examined. The incidence was 34.2% in the age group 6-11 years. The results of all these studies indicate that the parasitic infection is higher in younger hosts than in the older ones. This could be explained on the basis that as the age of the host increases the immunity against parasitic diseases also increases.

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ISOLATION OF FEATHER KERATIN HYDROLYSING BACTERIA AND LOW-COST PROTEIN ENRICHMENT OF POULTRY FEED

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Abstract.- Feather degrading bacteria were isolated from a soil compost sample consisting mainly of kitchen leftovers. The bacterial isolates showed a high keratinolytic activity when cultured on broth containing native feathers as sole source of carbon and nitrogen. Near to complete feather degradation was achieved within one month period. Quantitative estimation of soluble protein, and qualitative visualization of free amino acids have been worked out following the bacterial growth up to ten weeks post-inoculation on the selective medium. A bacterial isolate MH-45 yielded maximum soluble protein up to 165 mg/ml at the last sampling period. Different bacterial isolates yielded different types of amino acids. Isolate MH-62 gave eight free amino acids, while another isolate (MH-70) could yield only three amino acids in the culture fluid. These differences in the exo-products reveal metabolic diversity of the bacterial isolates. The bacteria are potentially useful for biotechnological processes involving keratin hydrolysis. The fermented feathers may be incorporated to chick feed to enrich its protein content.

Key words: keratinase; *Sporosarcina*; alkalophilic protease producer.

INTRODUCTION

Feathers are produced in large amounts as a waste by product at poultry processing plants, burgeoning millions of tons per year world wide (Williams *et al.*, 1991). Feathers comprise up to 99% of keratin. Keratin is insoluble structural protein of feathers, skin and wool and is well known for its high stability (Bradbury, 1973; Thys *et al*., 2004). Because of the high degree of cross-linkings through disulphide bonds, hydrogen bonding and hydrophobic interactions, keratin is water insoluble and shows high mechanical stability and resistance to proteolysis, in general (Bradbury, 1973; Parry and North, 1998; Lucas *et al*., 2003; Riffel *et al*., 2003). Feather protein is poorly digested by common digestive enzymes, such as trypsin and pepsin (Papadopoulos *et al*., 1986).

Concerning utilization of this huge bio-waste, feathers have been converted to feather meal, mainly by physical and chemical treatments. Apart from processes associated problems such as lesser digestibility the processing of feather meal destroys certain amino acids influencing protein quality and

digestibility. Infact feather meal, inspite of its common incorporation in animal feeds, is poorly digestible feed ingredient. Its partial digestion is even attributed to the activities of GIT microbial flora (Kim *et al.*, 2000; Riffel and Brandelli, 2002).

Amino acid imbalance of the feather meal, which appears as one of the consequences of its physio-chemical processes has been considered the more serious problem. Formation of non-nutritive amino acids, such as lysine-o-alanine, lanthionine, etc. in this process lessens the benefits (Wang and Parsons, 1997). Therefore, when this meal is incorporated into poultry (40-50g/kg), rainbow trout (150g/kg), shrimp (330g/kg) and salmon (400g/kg) feeds, it is supplemented with amino acids, especially feed grade lysine (Bureau *et al*., 2000; Brandsen *et al*., 2001; Cheng *et al*., 2002).

Both the digestibility and amino acid imbalance of feather meal might be improved by microbial activities. A number of microorganisms such as *Vibrio* sp. strain kr2 (Sangali and Brandelli, 2000), *Bacillus licheniformis* (Williams *et al*., 1990) and *Streptomyces fradiae* (Elmayrgi and Smith, 1971) have been reported to utilize feathers as fermentation substrate. Screening for non-pathogenic microorganisms with kerationolytic activity may be useful in digesting the produce in a natural way without emergence of non-nutritive amino acids. Rather microbial biomass could fortify the protein and amino acids contents of the fermented feather meal. The upgrading of the nutritional value of feathers should yield an enhanced protein feedstuff that may reduce the use of soybean and fishmeal in livestock diets. The present study was conducted to isolate feather keratin hydrolyzing bacteria from local environment to evaluate the amino acid and protein availability in the fermented products.

MATERIALS AND METHODS

Culture media

For the isolation of keratinolytic bacteria M-II medium comprising of whole chick feathers 10g, K_2HPO_4 1g, $MgSO_4$ 0.2g, $FeSO_4.7H_2O$ 0.01g, $CaCl_2.2H_2O$ 0.01g, $Cu(NO_3)$ 0.0005g, $ZnSO_4$ 0.0005g and bacteriological agar 14g l^{-1} was used. M-II medium of pH 12 and 7 were employed for the isolation of alkalophilic and neutrophilic bacteria, respectively. Feathers were obtained from a local poultry shop. They were washed with commercial detergent powder and rinsed thoroughly with tap water, and dried at 105˚C in an electric oven for overnight. The feathers were then cut into small pieces before use.

Isolation and characterization of bacteria

Seventy six feather degrading bacteria were isolated from kitchen leftover soil compost samples on the medium M-II, containing whole feather as sole carbon and nitrogen sources. Fifty six and nineteen isolates were obtained from the alkaline (pH 12) and the neutral media, respectively. All the isolates were tested for hemolytic activity employing on blood agar medium as described by Merck (1996-1997).

Screening test

All of the isolates were cultivated in 100 ml capacity bottles containing M-II broth medium at their respective pH. The medium was sterilized by autoclaving at 121°C for 15 min. Fermentation was carried out by inoculating loop full of a bacterial colony into 50 ml of a given sterilized M-II medium, at 37°C for 8 days. At the end of experimental period aliquot of samples was used for quantitative estimation of soluble protein and qualitative visualization of free amino acids.

Cell-free culture fluids were used for the determination of soluble protein by the UV spectrophotometric method as described by Darbre (1986) using bovine serum albumin as standard. Qualitative estimation of free amino acids was done by paper chromatographic method described by Jayaraman (1988).

Batch assay

Based upon higher yields of proteins (up to 5.70 mg/ml) nineteen bacterial isolates were selected for batch assay for up to 10 weeks. All the incubations were performed at 37˚C. The cultures were sampled at every fortnight period and evaluated for feather degradation, yield of soluble protein, diversity of amino acids and change in pH. Of these 7 bacterial isolates were characterized and classified up to genera levels as described by Holt *et al.* (1994).

RESULTS

Out of the 75 bacterial isolates, 19 were selected on the basis of maximum feather hydrolysis, production of soluble protein and obtaining maximum diversity of amino acids. Values for the soluble protein ranged from 2 to 5.6 mg/ml, while up to 7 types of amino acids were obtained. The alkalophiles, in general, did not alter initial pH level (12) of the M-II medium. Whilst the broths of the neutrophils attained alkaline pH with the passage of time (Table I; Fig. 1

B, C, D). Twenty one isolates were found positive for hemolytic activity.

TABLE I.- SOLUBLE PROTEIN CONTENTS AND ACCOMPANYING pH CHANGES OF 1% WHOLE FEATHER MEDIA FERMENTED BY DIFFERENT BACTERIA.

Isolate no.	Fermentation time				
	2 week	4 weeks	6 weeks	8 weeks	10 weeks
MH-45 (12)[a]	7.34* (7.76)[b]	19.72 (8.21)	11.45 (7.81)	50.963 (7.30)	165.38 (6.67)
MH-62 (7)	0.94 (7.48)	1.43 (7.40)	10.205 (7.93)	55.829 (8.40)	153.95 (7.43)
MH-36 (12)	9.08 (8.19)	19.519 (8.00)	11.22 (7.68)	51.95 (7.68)	145.72 (7.02)
MH-73 (7)	10.07 (7.40)	21.91 (7.22)	27.46 (8.14)	59.23 (8.09)	142.16 (7.47)
MH-68 (7)	0.99 (7.48)	2.75 (7.53)	4.64 (7.83)	29.30 (8.26)	137.06 (7.76)
MH-70 (7)	0.56 (7.88)	6.34 (8.23)	11.46 (7.98)	24.93 (8.06)	130.90 (7.41)
MH-59 (7)	2.38 (7.59)	2.76 (7.81)	16.49 (8.03)	46.79 (8.09)	129.03 (7.40)
MH-74 (7)	4.35 (7.73)	16.20 (7.40)	20.30 (7.87)	43.11 (7.84)	119.86 (7.51)
MH-58 (7)	5.29 (8.08)	10.20 (7.99)	13.29 (7.95)	25.88 (7.99)	75.34 (7.67)
MH-63 (7)	2.21 (7.91)	2.64 (7.71)	3.83 (7.80)	9.28 (8.21)	66.66 (8.12)
MH-75 (7)	1.165 (7.61)	3.45 (7.77)	8.747 (8.15)	21.62 (8.16)	34.54 (7.55)
MH-72 (7)	0.545 (7.52)	2.787 (7.20)	4.712 (7.92)	10.72 (8.04)	32.63 (8.00)
MH-67 (7)	0.20 (7.19)	0.35 (7.19)	0.95 (7.70)	10.18 (8.30)	29.34 (7.99)
MH-57 (7)	0.98 (7.05)	1.47 (7.00)	3.48 (8.00)	23.81 (8.20)	28.11 (7.42)
MH-60 (7)	0.14 (7.11)	0.69 (7.31)	0.85 (7.71)	4.79 (7.82)	26.16 (8.36)
MH-12 (12)	1.28 (7.47)	1.36 (7.52)	1.83 (7.53)	5.73 (7.91)	23.55 (7.50)
MH-39 (12)	2.162 (7.73)	2.44 (7.55)	2.95 (7.65)	6.10 (7.50)	19.72 (7.21)
MH-69 (7)	1.032 (7.58)	1.530 (7.15)	2.63 (7.64)	4.682 (7.58)	6.276 (7.51)
MH-65 (7)	0.205 (7.56)	1.365 (7.36)	2.077 (8.01)	2.770 (7.58)	5.647 (7.43)

*Values represent soluble protein (mg/ml). a: Initial pH values b: final pH values.

During the batch assay the 19 isolates hydrolyzed the whole feather pieces to varying degree and consequently showed increase in protein level throughout the study period. The intact shafts of the feather (Fig. 1A) were attacked by the bacterial keratinase and the nodes were observed being detached from the shaft possibly by the dissolution of keratin based cementing substance at 6^{th} week of incubation (Fig. 1B). This process of hydrolysis continued in the succeeding periods of study (Fig. 1C, 1D) so that at 8^{th} and 10^{th} weeks of the incubations excessive free spaces were evident within the lightly stained feather shafts (Fig. 1E-J). Maximum soluble protein was obtained by the isolate MH-73 on 2^{nd}, 4^{th}, 6^{th} and 8^{th} week measuring up to 10.07, 21.91, 27.46 and 59.23 mg/ml, respectively. While on the 10^{th} week maximum protein was yielded by the bacterial isolate, MH-45 yielding up to 165.38 mg/ml (Table I, Fig. 2). At the last sampling period 153.95, 145.72, 142.16, 137.06, 130.90, 129.03, 119.86 mg/ml of protein were obtained from isolates MH-62, 36, 73, 68, 70, 59 and 74, respectively (Table I, Fig. 2).

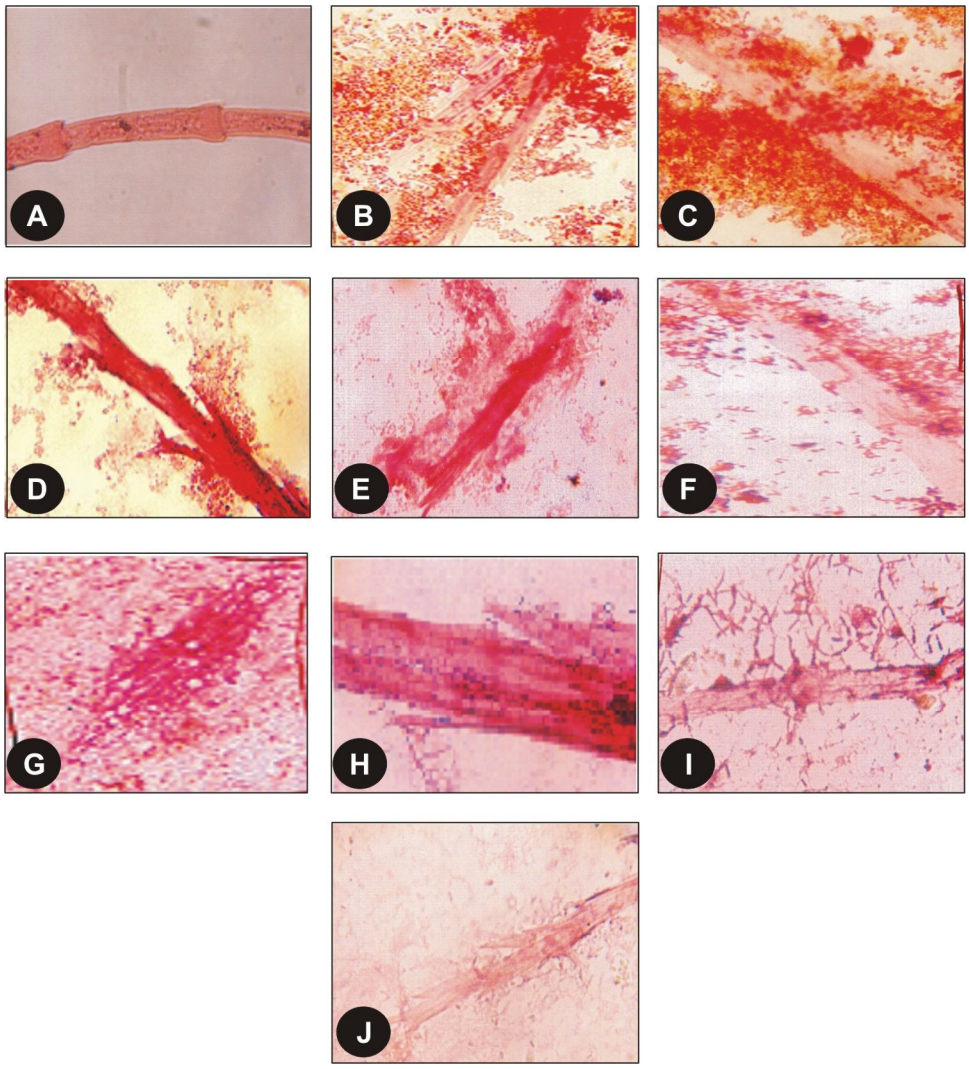

Fig. 1. Microscopic appearance of the chicken feather **A:** Control (intact). Compact nature of the feather barbule and its nodes are evident **B**: semi-hydrolysed feather bar exposed to the culture of MH-73 for 6^{th} weeks **C**: 8^{th} week **D:** 10^{th} week **E:** semi-hydrolysed feather barbules exposed to the culture of MH-68 for 4^{th} week **F:** 6^{th} week **G**: Advance stage of digestion of a feather barbule on 10^{th} week **H**: Semi-hydrolysed feather barbs exposed to the culture of MH-36 for 4^{th} week **I**: 6^{th} week **J:** 10^{th} week. Note: In all the photomicrographs bacterial cells of the respective strain are visible. All photomicrographs were taken on the same magnification. Gram's staining × 1000.

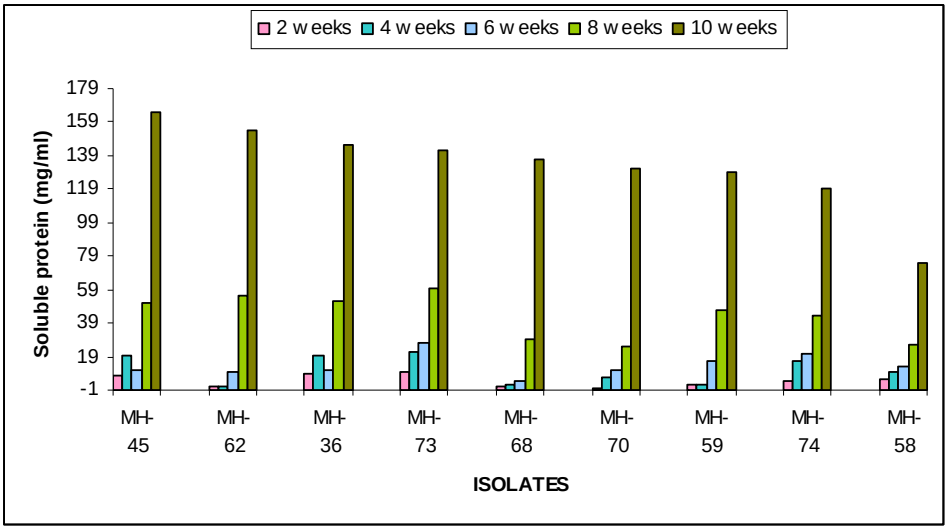

Fig. 2. Soluble protein profiles of different bacterial isolates at different weeks post-cultivation in M-II.

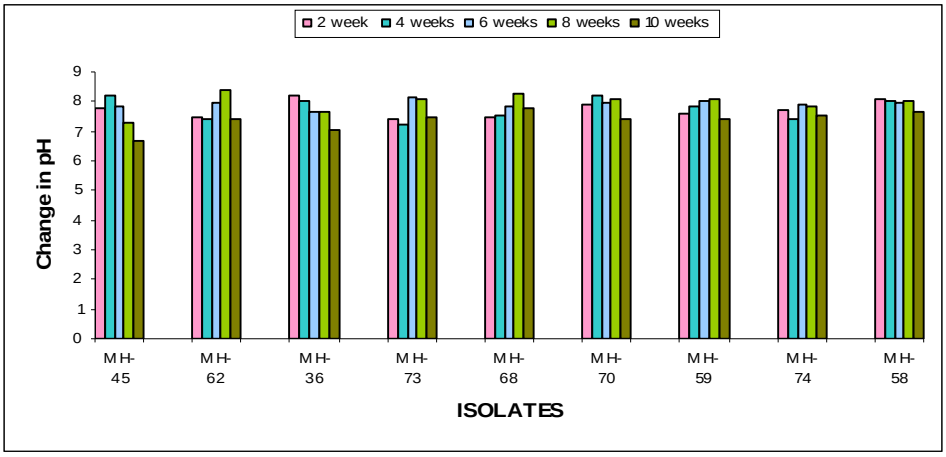

Fig. 3. Change in pH profiles of different bacterial isolates at different weeks post-cultivation in M-II.

Regarding diversity of amino acids, maximum diversity was obtained at 2nd week from the isolate MH-73, the amino acids obtained were lys, arg, glu, met and ile while lys, met and ile (Table II; Fig. 4). On the 4th week maximum

TABLE II.- FREE AMINO ACID PROFILES OF 1% WHOLE FEATHER MEDIA FERMENTED BY DIFFERENT BACTERIA AT DIFFERENT TIME POST INOCULATIONS.

Isolate no.	Free amino acid profiles from fermentation time				
	2 week	4 weeks	6 weeks	8 weeks	10 weeks
Control	none	none	none	none	none
MH-75	none	unkown	Unknown unknown	Lysine Arginine methionine	Lysine Arginine methionine
MH-74	arginine	unknown	Methionine Iso-leucine	Lysine Arginine methionine	Lysine Cystine Glutamine α-alanine methionine
MH-73	Lysine Arginine Glutamine Methionine Iso-leucine	Unknown Arginine α-alanine Methionine iso-leucine	Praline Methionine Iso-leucine	Lysine methionine	Lysine Cystine Serine Arginine Glutamine Tryptophan Phe-alanine β-alanine leucine
MH-72	none	none	none	none	Lysine Unknown Unknown
MH-70	none	none	none	none	Lysine Glutamine Arginine
MH-68	None	none	none	Lysine Threonine L-proline Methionine Tryptophan Iso-leucine	Lysine Cystine Arginine Glutamine Proline Phe-alanine β-alanine
MH-67	none	none	none	Lysine arginine Methionine proline iso-leucine	unknown
MH-65	None	none	none	none	unknown
MH-63	None	none	none	lysine	lysine

Continued

Isolate no.	Free amino acid profiles from fermentation time				
	2 week	**4 weeks**	**6 weeks**	**8 weeks**	**10 weeks**
MH-62	none	none	Unknown Unknown Methionine Iso-leucine	Lysine Unknown Unknown Methionine Tryptophan Iso-leucine	Lysine Cystine Serine Glutamine Tryptophan Methionine Phe-alanine Iso-leucine
MH-60	None	none	none	none	Lysine Threonine Methionine Iso-leucine
MH-59	none	none	Asparagine Arginine a-alanine methionine iso-leucine	Serine a-alanine proline unknown methionine tryptophan leucine	Lysine Arginine a-alanine praline methionine tryptophan leucine
MH-58	Unknown Methionine Iso-leucine	none	none	lysine	unknown
MH-45	Unknown Threonine Methionine Iso-leucine	Unknown Methionine Iso-leucine	Asparagines Unknown Arginine a-alanine phe-alanine	Asparagine Unknown unknown	Cystine Serine Proline tryptophan
MH-36	Lysine Arginine Methionine Iso-leucine	none	unknown	Lysine Phe-alanine	Lysine Threonine Proline tryptophan
MH-57	None	none	Arginine Phe-alanine	Asparagines Unknown Unknown leucine	Cystine threonine

Bold words indicate intense chromatographic spots.

diversity was again yielded by the MH-73 naming lys, arg, α-ala, met and ile. On sixth week, 5 types of amino acids were obtained from the hydrolysates of MH-59 and MH-45 (Table II). On 8^{th} week maximum diversity of amino acids was given by the isolate MH-59 yielding up to 7 types of amino acids naming ser, ala, pro, val, met, trp and leu. Nine types of amino acids were obtained on 10^{th} week from MH-73 naming lys, cys, ser, arg, glu, trp, phe, ala and leu. At this study

period isolate MH-62 yielded 8, while MH-68 and MH-59 both yielded 7 types of amino acids.

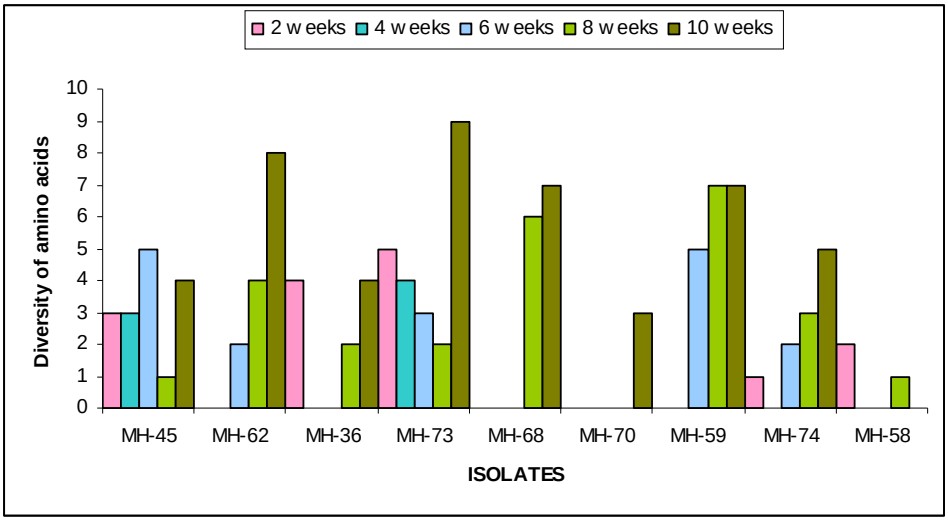

Fig. 4. Diversity of free amino acids of different bacterial isolates at different weeks post-cultivation in M-II.

The bacterial isolates MH-45, MH-62, MH-36, MH-73, MH-68, MH-59 and MH-74 were characterized and all of belonged to genus *Sporosarcina.*

DISCUSSION

In the present study bacteria were isolated from a compost material. Compost material is well known for the isolation of keratinolytic bacteria Lin *et al.* (1999) and Zerdani *et al.* (2004). The hydrolysis most likely occur through the proteolytic enzymes produced by the bacteria, which degrade the β-keratin and other proteins found in feathers and produce usable carbon, sulphur and energy for their growth maintenance (Hansen *et al.*, 1993; Burtt and Ichida, 1999).

The isolates possessing high keratinolytic activities were confined to gram positive: genus *Sporosarcina*. Gram-positive bacteria have been shown to represent an important part of the soil microbial communities (Sessitsch *et al.*, 2001). Keratinolytic activity has been reported by many bacteria, e.g. *Bacillus*

(Williams, *et al*., 1990), *Streptomyces* (Garcia- Kirchner *et al*., 1998), *Thermoactinomyces* (Ignatova *et al.,* 1999), *Vibrio* (Sangali and Brandelli, 2000) and *Microbacterium* species (Thys *et al*., 2004). Up to the best knowledge of the authors, the genus *Sporosarcina* had never been associated with keratinolytic activity. The present bacterial isolates were not studied for the effect of differing temperatures on their keratinolytic activities. Riffel *et al*. (2003) have reported bacterial isolates with maximum keratinolytic activity at mesophilic temperatures, as expected from its environmental origin. Whilst, previously described keratinolytic bacteria had been reported with optimum growth and feather degrading activity at high temperatures (Willaims *et al*., 1990; Atalo and Gashe, 1993).

Maximum keratinolytic activity was observed for the bacterial isolates MH-59, MH-62, MH-68, MH-73, MH-70 and MH-74 at 7 pH. Thys *et al.* (2004) have also reported bacterial keratinolytic activity of comparable levels. pH 12 was found suitable for the isolates MH-45 and MH-36. Many workers have reported alkalophiles keratinolytic bacteria. (Williams *et al.,* 1990; Atalo and Gasho, 1993).

A change in pH of the medium has been regarded as a sensitive parameter indicating keratinolysis. Marked alkalinization of the culture medium of the neutrophiles was obtained in this study. This parameter has been considered indicative of keratinolysis by the isolates cultured at 7pH (Lal *et al*., 1999). In vitro breakdown of keratin into proteins, peptides and amino acids results in marked alkalinization of the culture medium because of the reduction of cystine bridges (-S-S-) into cysteine (-SH) residues. The increase in pH revealed the preferential utilization of nonkeratinious peptidic compounds as a source of nitrogen and a consumption of keratin as a source of carbon. Mathison (1963) considered the deamination of amino acids and peptides with the production of ammonia, a key reaction that leads to a gradual denaturation of keratin in the alkaline environment.

Hydrothermal and chemical treatment has few limitations such as poor digestibility, loss of nutritionally essential amino acids like methionine, lysine and tryptophan and formation of non nutritive amino acids viz lysinoalanine and lanthionine (Dalev *et al*., 1997; Kim *et al.,* 2000; Gousterova *et al*., 2005). In this scenario keratin degradation by strains has been reported to yield intact free amino acids such as asn, pro, lys and met.

Nam *et al*. (2002) and Gousterova *et al*. (2005) reported proteolysis of keratin by suitable microorganisms is an alternative method for improvement of the nutritional value of feather waste and for avoidance of the destruction of certain amino acids (Steiner *et al*., 1983; Papadopoulos, 1985; Papadopoulos *et al.,* 1985,1986). Onifade *et al.* (1998) had described several limitations of using feather keratin because of poor digestibility and nutritional limitation (low content of essential amino acids) has been perceived the major ones. The newly isolated isolates of the genus *Sporosarcina* can be expected to overcome such limitations as the isolates have yielded appreciable amounts of soluble protein and diversity of essential amino acids in the culture broth. Many of them produced essential amino acids such as met, trp, his and lys for whom the feather keratin is deficient from native feather keratin (Harrap and Woods, 1964; Williams *et al.*, 1991; Sangali and Brandelli, 2001). In addition to the nutritional availability of essential amino acids and soluble protein Shih (2005) reported that keratinase is able to digest prions that cause transmissible sponiform encephalopathies (TSE) and other diseases, including mad cow disease, sheep scrapie deer chronic waste disease and human Creutz feldt Jakob disease (CJD).

Regarding industrial applications, microbial conversion of feathers waste is a potential technique for degradation and utilization of feathers as a feed stuff. Feather protein has been considered as an excellent source of metrabolizble protein (Klemesrud *et al*., 1998) and bacterial feather lysate has similar nutritional features to soya bean meal (Willaims *et al.,* 1991). The isolates reported here posses' high keratinolytic activity and proved effective in feather degradation. They present potential for developing biotechnological processes involving keratin hydrolysis.

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RECOVERY OF INTEGRONS FROM MANMADE AND NATURAL ENVIRONMENTS

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Abstract.- The integron system is remarkably versatile in its ability to recognize highly variable target recombination sequences and its apparently limitless capacity to exchange and stockpile cassettes. Such flexibility permits rapid adaptation to the unpredictable flux of environmental niches by allowing bacteria to scavenge foreign genes that may ultimately endow increased fitness to the host. Likewise genes that fail to provide a meaningful function may be readily eliminated. Integrons are genetic elements capable of the acquisition, rearrangement and expression of genes within gene cassettes. Such acquisition is mediated by an integron-encoded integrases, which capture the gene associated with gene cassettes. Genomic DNA from five environmental isolates was subjected to amplification using integron specific primers HS298 and HS286, which were designed to conserved regions of the integrase gene and the gene cassette recombination site respectively. PCR product was cloned and then sequenced. BLAST search was performed to determine nucleotide and protein matches to the world databases. The recovered genes had no significant matches with existing sequences in the databases. So the genes recovered from clones of environmental samples were novel with no identifiable ORF homologues in the databases. The protein alignment of isolated clones with known integrases gene was sufficient to classify them within new class of integrons. This study indicates that integron recovery represents a new opportunity to prospect for genes of biotechnological significance by culture independent means. Notably, identification of gene boundaries and location in a sequence fragment is greatly simplified. The orientation of open reading frames is predictable and genes are pre-packaged in a form amenable to manipulation by site specific recombination. Moreover, integron driven gene acquisition is likely to be an important factor in the more general process of horizontal gene transfer in the evolution of microbial genomes and proteomes.

Key Words: Integrons, gene cassettes, site-specific recombination, diverse environments.

INTRODUCTION

Integrons are genetic elements commonly found in multidrug–resistant and pathogenic bacteria (Hall *et al*., 1996) and are characterized by their ability to

integrate and excise gene cassettes by site-specific recombination (Nield *et al*., 2001). The integrons consist of two parts: (i) The stationary integron platform, including the integrase gene (*Int*I), a strong promoter (P_{ant}), and a recombination site (*attI*). (ii) The mobile gene cassettes, which are promoter less open reading frames (ORFs) with a recombination site (*attC*) (Nemergut *et al*., 2004).

The integron encoded (*IntI*) integrase are part of a large and diverse family of site-specific recombinases (Nield *et al*., 2001). Tyrosine recombinases are used in a number of processes that involve integration and excision or inversion of discrete DNA segments. All members of this large tyrosine family of recombinases nonetheless have characteristic features including the presence of conserved regions for *IntI*, designated as *IntI* motif, within which are located very highly conserved residues, not found in other tyrosine recombinases and the members of *intI* family can be identified on the basis of this motif (Nunes-Duby *et al*., 1998; Collis *et al*., 2002).

Integrons are the natural reservoirs for members of a very large family of small mobile elements called gene cassettes, which, comprises normally an antibiotic resistance gene, and a recombination site that is recognized by *IntI* (Hall and Collis, 1995). Gene cassettes generally do not include a promoter and transcription of cassette-associated genes is from a common promoter, P_{ant}, found in the integron (Collis and Hall, 1995).

An essential feature of gene cassette is the presence of a recombination site known as 59-base element that is located downstream of the gene coding region (Hall *et al*., 1991). The recombination sites associated with cassettes are variable in terms of both their sequence and length. Each 59-be site includes two potential simple sites that are imperfect inverted repeats of one another, but only 8bp are conserved in all 59-be sites (Stokes *et al*., 1997).

The *attI* site includes only one simple site structure, together with two further directly repeated integrase-binding domains designated strong and weak that are in the same orientation (Collis *et al*., 2002, Gravel *et al*., 1998). Furthermore, neither the *attI* site nor the 59-be sites represent a single sequence, rather, member of each group often share only limited sequence identity (Nield *et al*, 2001).

Several features of the integrons might provide a means by which intact novel genes could be recovered directly from environmental DNA by PCR without prior sequence data. Integrons appear to be a feature of many and diverse

bacterial species containing large cassette arrays associated with chromosomal integrons. The structure of multiple cassette arrays means that individual genes are flanked by conserved sequences (59-be sites) that are potential targets for PCR primers. In this study we show that the use of PCR primers HS298 and HS286 targeting integrase gene and 59-be sites respectively are used which allow the recovery of full length of integrons with associated genes which may encode products that have no orthologs in protein databases.

MATERIALS AND METHODS

Acquisition of samples

Samples were collected from manmade (20 tonnes coal heap set at NIBGE campus, Faisalabad for biodesulphurization and 300 tonnes coal heap at Askari Cement Factory, Nizampur) and natural (root soil from rice, marigold and mung bean plants) environments.

DNA template isolation

Microbial pellets were used for the extraction of DNA by employing protocol described by Nemerget *et al*. (2004).

PCR amplification

Primers for regions corresponding to a conserved C-terminal sequence in *IntI* integrases (HS298, 5′ TGGATCCCACRTGNGTRTADATCATNGT 3′) and to conserved sequences in 59-be (HS286, 5′ GGGATCCTCSGCTKGARCGAMTTGTTAGVC 3′) were used (Nield *et al*., 2001). Linkers that include a *BamHI* site are underlined. Reaction mixtures consisted of 5ng of template DNA, 100pmol of each of the primer, 200nM deoxynucleotide triphosphate (dNTP) mix, 2mM $MgCl_2$ and 5U of Taq DNA polymerase.

The PCR amplification was carried out using standard techniques with the cycling programme as follows: 94°C for 2 minutes and 30 seconds for 1 cycle, 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 2 minutes and 30 seconds for 35 cycles, and 72°C for 5 minutes for 1 cycle.

Cloning of PCR amplified products

PCR products were ligated into the pTZ57R/T Vector using Fermentas DNA ligation kit following the manufacturer's instructions. The ligation mixture was transformed by heat shock into DH5α *Escherichia coli* competent cells.

Plasmid from clones containing insert was isolated from 3 ml overnight grown cultures using the BIO-RAD Quantum prep® plasmid miniprep kit. Then restriction digestion was carried out by *Sal1* and *Sac1* enzymes. The insert size was checked on 1.5% agarose gel.

Sequence retrieval and analyses

Sequence analyses were performed using programmes available through the National Center for Biotechnology Information (USA) programme (www.ncbi.nlm.nih.gov). The attachment site *attI* and 59-be site was observed visually.

ORFs showing sequence relationship to *IntI* were aligned to a representative set of tyrosine recombinase proteins using Clustal X and GeneDoc software's.

Nucleotide sequences isolated from NP and R clones were deposited in GenBank of National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Accession numbers obtained for Np (Nizampur) and R (Rice) clones were DQ417594 and DQ417595 respectively.

RESULTS

To recover integrons from environmental DNA by PCR, two primers were used (i) HS298, is specific for the coding region of a conserved domain located 14-22 amino acids from the carboxy-termini of *IntI*1-4 (Nunes-Du´by *et al.*, 1998). (ii) HS286, targets the left-hand simple site of 59-be site. The use of these primers in a PCR should allow the recovery of at least one near complete gene cassette, the *attI* site, and all but about the last 20 codons of *intI* (Nield *et al.*, 2001).

PCR amplification was performed with 5 DNA samples derived from coal heap and soil. Out of five isolated DNA samples two (Np and R) showed PCR amplification. Amplified products showed the presence of integrons in these

samples, which were proceed for further analysis. Multiple products with fragment size ranging from 200–1018 bp were recovered (Fig. 1).

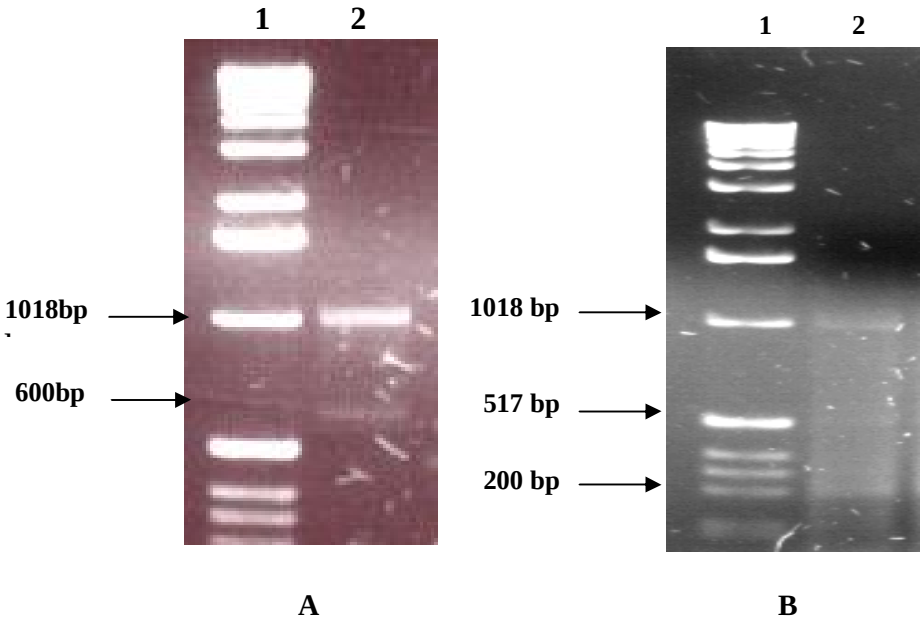

Fig. 1. PCR amplification of integrons; A, Lane: 1. 1kb DNA ladder, 2. Nizampur (Np); B, Lane: 1. 1kb DNA ladder, 2. Rice (R)

After commercial sequencing the database search was done. BLAST N gave top hits for Np sample with quite less significant similarity to *Mus Musculus* Strain (source: house mouse) C57BL6/J chromosome 5 BAC, RP23-383N15, with an E-value of 0.77 while, BLAST X top hits showed significant homology with Histone deacetylase/ AcuC/AphA family protein (*Methylococcus capsulatus* str. Bath) with an E-value 1e-14. BLAST N top hits for R sample showed less significant similarity to PREDICTED: *Pan troglo-dytes* (source: chimpanzee) similar to kielin-like (LOC463714), mRNA with an E-value of 0.20 while BLAST X top hits showed significant homology with Acetoin utilization protein (uncultured archaeon, GZfos9D1) with an E-value of 2e-28.

The predicted integrase protein encoded by Np and R samples were aligned with, and compared, to known *IntIs* (1-10). It was found that pair wise comparison between the environmental integrases (Np and R) and known

integrases showed much significant amino acid homology. The data suggest that the *IntI* genes encoded by Np and R may each be part of an integron. This notion is supported by the fact that both clones showed *IntI* motif that is distinct feature of *IntI* integrases. It was observed that this *IntI* motif was quite conserved with 5 of 16 invarient residues, across the twelve *IntI* proteins (Fig. 2). Different shaded regions specifying the presence of residues, in all the *IntI* classes or those, which fell in functionally equivalent groups, while highly conserved regions were present in white on black strips. Point variations were also observed in highly conserved regions. It was also observed that both the isolated sequences showed very significant similarity with each other in *IntI* motif.

Putative sequences suggested for *attI* and 59-be sites were identified visually in isolated cloned sequences. Although the location of primer exclude the recovery of a complete cassette, but the presence of cassette could be inferred by the presence of an ORF extending up to, or through, the primer sequence. On examination of the sequences, both clones showed six ORFs with sense and antisense orientations having standard start and stop codons. Neither ORF matched within COG database except one ORF of Rice clone, which showed significant match with deacetylases, including yeast histone deacetylase and acetoin utilization protein (Table I).

Presence of novel *IntI* gene together with putative 59-be and *attI* sites strongly implies to designate the recovered integrons from Rice and Nizampur samples within class 11 integron due to significant similarity with each other.

DISCUSSION

The recovery of new environmental integrons sampled from two of the five locations is consistent with the hypothesis that integrons are a common feature of bacterial populations and that they are not restricted to pathogenic and multidrug- resistant bacteria. The two environments from which the new integron class was recovered are quite contrasting. The core samples were collected from coal heaps at Askari cement factory, Nizampur (Np) and NIBGE (Nb) (National Institute for Biotechnology and Genetic Engineering) Faisalabad, Pakistan. These heaps were set for biodesulfurization of coal. The samples were collected at the time when studies for biodesulfurization were already concluded. The soil samples were acquired from the roots of Marigold (Mg), Rice (R) and Mung bean (Mb) plants at NIBGE, Faisalabad, Pakistan.

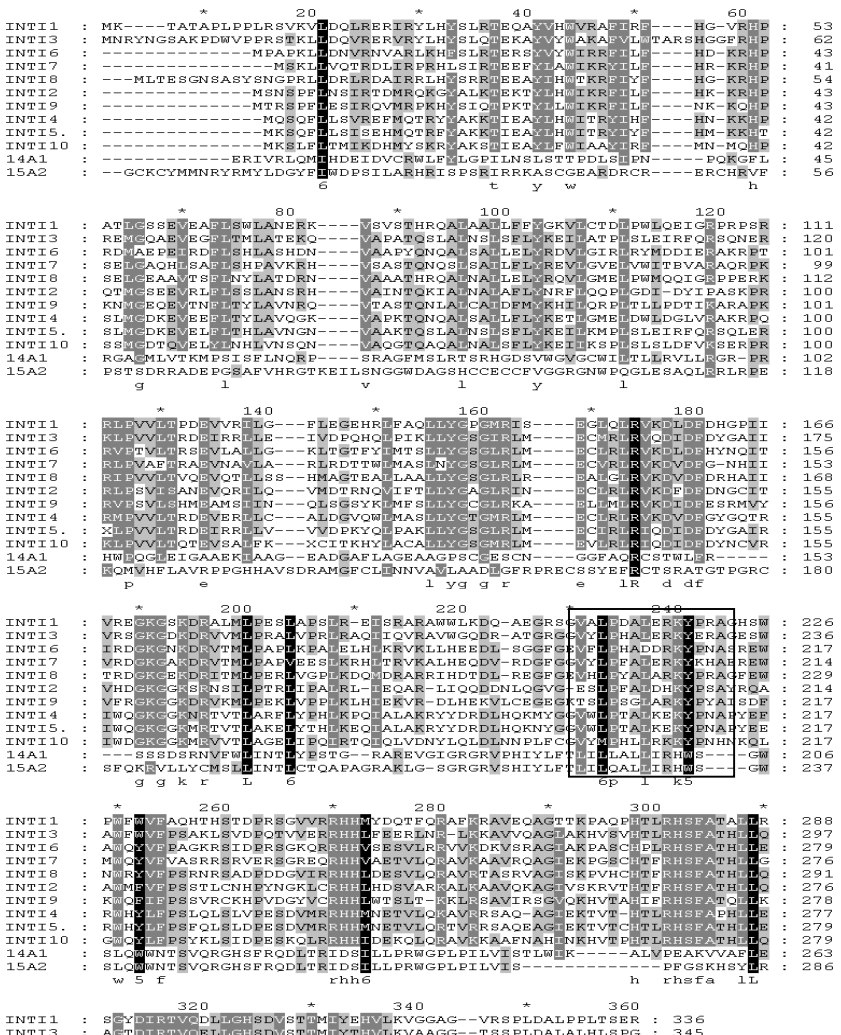

Fig. 2. Protein alignment of environmental integrases A1 (Np) & A2 (R) with known integron-encoded integrases (1-10). Residues present in all integron-encoded integrases or that fall into functionally equivalent groups are shaded. The highly conserved residues from across the tyrosine family of recombinases are shown white on black strips. *IntI* motif is inticated in box.

Diverse species hosting integrons provide a very suitable and efficient platform for the storage, acquisition, rearrangement, and expression of gene cassettes thus resulting in a great biodiversity. This study strength this view by recording the recovery of novel integrons from genomic DNA extracted from coal heap and soil samples.

The stretch of about 16 amino acids located between boxed region (Nunes-Du´by *et al*., 1998), is relatively conserved with five invariant residues. As this motif appeared to be unique to the *IntI* integrases so, was designated as integrase specific patch (*IntI* patch). Residues present in all integron-encoded integrases or that fall into functionally equivalent groups were shaded, while the highly conserved residues were white on black strips. Total nine highly conserved regions were found. While the remaining showed variable residues within the same strip having non-significant similarity. Point mutations were observed even in highly conserved areas, which validates their novelty. As these environmental integrons are significantly different from those previously described, they are designated as class11.

From a sampling of two environments, we have been able to recover DNA fragments that include features characteristic of known integrons. In both cases, this includes evidence of an inserted gene cassette at a site possessing the architecture of known *attI* sites. Consequently, we conclude that these two recovered sequences are derived from complete integrons.

The observation arising from this study is that the recovered integron are novel and cassettes include a diverse range of genes, the vast majority of which have no known homologues in the databases. Since integrons are widespread features of bacterial populations, it is clear that this pool of novel mobile genes represents a previously unrecognized genomic resource for bacteria. Collectively these data give cause to reconsider our ideas of bacterial genome flexibility and the diversity of proteins likely to be found in even well known bacterial species. In addition to providing a means of tracking integron in the environment, the PCR strategy presented here represents a unique opportunity to prospect for new genes of biotechnological importance by culture-independent means. It is also apparent that, this system may has a general role in the transfer of genes between species, this approach may be a practical one for the recovery of new genes that are unlikely to be identified by whole genome sequencing strategies given that such `floating' genes may not reside in the chromosome of a defined organism.

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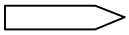
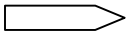
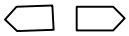
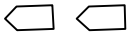
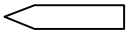
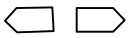
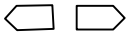
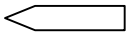
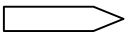
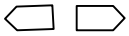
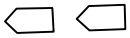
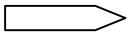
TABLE I. - SCREENING OF OPEN READING FRAMES IN INTEGRONS.

Sr. No.	Clone code	No. of ORF with frame no.	Orientation of ORFs	Amino acid no. in ORFs	ORF Cognitor Results
1	Np	1, +1		147	
		1, +2		231	
		2, +3		315,153	
		2, -1		174,201	
		1, -2		177	
		2, -3		171,261	
2	R	2, +1		114.111	Deacetylases, including yeast histone deacetylase and acetoin utilization protein
		1, +2		315	
		1, +3		506	
		2, -1		171,276	
		2, -2		132,201	
		1, -3		117	

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PERIPHYTIC COLIFORM CONTENTS OF A COMMONLY USED SALAD VEGETABLES, *BRASSICA OLERACEA*

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Abstract.- Incidence of food borne bacterial diseases occur due to contaminated irrigation to vegetables and crops besides many other reasons. In this study, three cabbage samples collected from two cities were processed for the detection and enumeration of coliforms. All the samples were found to harbour coliform bacteria including *E. coli.* Many of the isolates were found haemolytic and showed antibiotic resistance to different drugs. Total sixty-eight strains of coliforms were isolated from three samples. The isolates were represented by *Bacillus, Enterococcus, Bordetella, Clostridium, Enterobacter* and *Acetobacter* genera. The salad vegetable leaves showed upto 80.1×10^5 c.f.u./cm^2. Thirty-three of them showed α and β-hemolytic activities on blood agar. Of these hemolytic bacterial isolates 38.4% showed resistance to Piperacillin, 69.2% showed resistance to Cephalexin, 15.3% were found resistant to Streptomycin and 69.2% isolates expressed resistance to Trimethoprim.

Keywords: Bacteria on vegetable leaves, *E.coli* on cabbage leaves, Bacilli on cabbage leaves.

INTRODUCTION

Diverse types of vegetables are cultivated throughout the year in Pakistan that provide a large share of basic food to majority of the population. Some of these are consumed uncooked or in a minimally processed way. In many areas of developing countries untreated domestic wastewaters flow through channels towards rivers. Meanwhile their flow is diverted by subsistence farmers to small plots where, in addition to other vegetables, salad crops including carrots, lettuce, tomatoes, pepper, cabbage, garden egg etc. are grown. A large variety of human pathogens including bacteria, viruses, protozoa and helminth eggs are frequently present in such wastewaters. Public health risks associated with the consumption of such vegetables are obvious (Lund, 1992; Abdul-Raouf *et al*., 1993; Beuchat, 1996, 2002; Okafo *et al*., 2003).

Bovine manure, a good source of macro- and micronutrients for plants, is used as fertilizer without any treatment. It is well-known source of food-borne pathogenic bacteria, and contributes to prevalence of epiphytic pathogenic bacteria on vegetables (Jones, 1980; Zschock *et al*., 2000; Wells *et al*., 2001).

Cabbage *(Brassica oleracea*) is a major, salad vegetable crop in Pakistan. It is mostly consumed raw in salad bars and various other fast foods. It is not even washed and its whole and chopped leaves are served as such. Doubtless, it represents a threat to the public health. Presence of coliform bacteria on the surfaces of leaves of *B.oleracea* may be associated with incidence of enteric infections. Further the contaminated vegetables may be carrying agents for viral infections such as hepatitis C etc. The aim of this study was to observe epiphytic coliform contents on the leaves of a few cabbage samples. Three leaves representing the outer, central and inner locations of a sample were processed for the analysis of their coliform contents. These information express concern for using contaminated water for irrigation purpose and proper processing of the raw vegetables as well.

MATERIALS AND METHODS

Sampling

Samples of cabbage were collected from Qaboola (Arifwala) and Lahore In sterile polythene air-tighten bags. Whole cabbage from field was sampled in the sterile polythene bags. The samples were brought to the laboratory and processed for the microbial contents.

Viable counts

Under aseptic conditions three leaves from outer, central and inner parts of a sample were obtained. Their outlines were traced under aseptic conditions to measure surface area (cm^2). Each leaf was chopped into small pieces with the help of sterilized scissors and forceps and the pieces immediately immersed into 100ml of sterile water in a culture bottle. The culture bottles covered with aluminum foil and then kept on an orbital shaker at 100 rpm for 20 minutes. From a given suspension two more dilutions (1:100 and 1:10,000) were prepared in sterilized water. Viable counts of coliforms were obtained by spread plate technique from each dilution on sterilized Eosin Methylene Blue agar (Oxoid). Plates after incubation at 37°C for 24 hours, total C.F.U./leaf was estimated. The parameter was then calibrated as C.F.U./cm^2 of a leaf's surface area.

Bacterial characteristics and identification

Representative colonies were processed for pure culturing. Size, shape, color, configuration, elevation, margin, consistency and opacity of the colonies of bacterial isolates were noticed.

Their morphological and physiochemical characteristics viz. Gram staining, motility, catalase and indole tests were determined as described by (Benson, 1994). Based upon their colonial and physiobiochemical characteristics the isolates were identified mainly upto genus level after Holt *et al.* (1994). Bacterial isolates were also cultured on blood agar to observe their haemolytic activity as described by Merck (1996-1997). And haemolytic isolates were subjected to antibiotic sensitivity test (Oxoid).

TABLE I.- SOME ATTRIBUTES OF THE *B. OLERACEA* LEAF AND THEIR CORRESPONDING COLIFORM CONTENTS.

Sample	Locality	Leaf	Surface Area $(cm)^2$	C.F.U./ 100 ml	C.F.U./cm2 of leaf
1	Arifwala	Outermost	130.5	58×10^6	44.4×10^5
		Central	83.4	50×10^6	59.9×10^5
		Innermost	27.3	20×10^5	73.2×10^5
2	Lahore	Outermost	104.2	40×10^4	0.38×10^5
		Central	64.9	52×10^6	80.1×10^5
		Innermost	13.0	20×10^5	15.38×10^5
3	Lahore	Outermost	105.9	31×10^6	29.2×10^5
		Central	59.5	90×10^3	0.15×10^5
		Innermost	27.0	15×10^5	5.5×10^5

RESULTS AND DISCUSSION

Colony forming units (C.F.U.) of coliform bacteria obtained from leaves of *Brassica oleracea* on Eosin methylene blue agar by processing a given leaf / 100ml of water and the corresponding C.F.U./cm^2 of leaf surface are given in Table I as can be seen from this table that leaf surface are reduced while going from the periphery towards center in a diagrammatic way. However, C.F.U. of the coliform/cm^2 of the leave surfaces did not show such pattern. Rather the middle leaves of the samples yielded highest C.F.U./cm^2. This may indicate viability of the coliforms on the surfaces of the leaves to be more which are protected from direct sunlight exposures and are experiencing less uniform moisture content. From the sample No.1 (Table III) 9, 6, 1, 8, 1, 2 and 4 strains of the genus *Bacillus, Enterococcus, Bordetella, E. coli, Clostridium, Enterobacter* and *Acetobacter*, respectively were recorded. While from sample No.2 (Table IV) 10, 2, 5, 1 and 8 strains of the genus *Enterococcus*, *E. coli, Bacillus,*

Acetobacter and Bordetella, respectively were recorded. Similarly from sample No.3 (Table V) 1, 2, 1 and 7 strains of the genus *E. coli, Bacillus, Bordetella and Enterococcus,* respectively were recorded.

TABLE II.- TOTAL NUMBER AND % REPRESENT OF HEMOLYTIC ISOLATES POOLED FOR THE THREE LEAVES OF THE SAMPLE.

Sample No.	Isolates (hemolytic)	Outer	Central	Inner	% hemolytic
1	31 (19)	3	8	8	61.29
2	26 (7)	3	3	1	26.92
3	12 (7)	2	2	3	58.33

When the isolates were screened for their hemolytic activities, 61.29, 26.42 and 58.33% appeared positive for the samples 1, 2 and 3, respectively. Frequency of presumptive pathogen's (hemolytic) appeared, in general, higher for the middle and inner leaves as compared to the outer leaves (Table II). This once again indicates lesser viability of the bacteria for the surfaces directly exposed to sunlight as reported by Anesio *et al*. (2005) and Hockberger (2000).

Some representative isolates from each samples were tested for their antibiotic susceptibilities against Piperacillin (100µg/disc), Cephalexin (30µg/disc), Streptomycin (10µg/disc) and Trimethoprim (5µg/disc) (Table VI). Many of the isolates indicated resistance to multidrugs, this nature also indicates their anthropogenic origin or association.

Vegetable may become contaminate when are irrigated with sewage water and/or the soil is fertilized with animal manure In fact, the sanitary quality of water is based on the profiles of indicator organisms present in it. According to WHO (1975) guidelines used for irrigation of vegetables and salad crops must have bacterial counts not exceeding 2.0 log10 C.F.U./ml in 80% of the samples. Of all possible different pathogens that might survive and travel along the sewage effluents coliform for their survival value, ease of culturing and intimate association with fecal contamination is universally studied as indicator organisms (Pelczar *et al*., 1993.). Results of the present study bring support to those earlier reports. Further for irrigating water no guidelines such as those of WHO are followed in this country. In fact a direct relationship of such enteric infections to contaminated environment has been established (Mosupye and Von Holy, 1999, 2000).

TABLE VI.- ANTIBIOTIC SENSITIVITY PATTERN OF SOME COLIFORM BACTERIAL ISOLATES FROM DIFFERENT CABBAGE SAMPLES.

Sample	Isolates	Antibiotics			
		Piperacillin (100)a	**Cephalexin (30)**	**Streptomycin (10)**	**Trimethoprim (5)**
1	Enterococci (O-2)	6.00[b]	R	1.00	1.00
	Clostridium (M-1)	*R	1.00	7.00	14.00
	Enterococci (M-8)	R	R	3.00	R
2	*Acetobacter* (O-4)	R	1.00	5.00	R
	Enterococci (O-5)	4.00	R	1.00	R
	Bacillus (M-8)	R	R	1.00	R
	Bordetella (I-8)	R	4.00	R	5.00
3	*E. coli* (O-1)	7.00	R	6.00	R
	Enterococci (M-1)	3.00	R	10.00	R
	E.coli (M-2)	2.00	R	R	R
	Enterococci (I-2)	6.00	1.00	1.00	R
	Bacillus (I-3)	10.00	R	6.00	R
	Enterococci (I-4)	5.00	R	4.00	5.00

*R= represent resistance. a: µg of the drug/disc. b: diameter (mm) of growth inhibition zone.

Many of these bacterial strains were α and β-hemolytic which indicate their pathogenecity. Its highly alarming that the salad vegetables routinely used as raw uncooked at home as well as at street food levels might have been involved in the spread of a number of enteric infections.

The situation appears highly dangerous when one considers that the sewage effluents are not treated in this country at all. Moreover, many viral diseases such as hepatitis etc. are common in the population. Although this study did not directly worked on this aspect but presence of high C.F.U. values of coliform bacteria on the leaves of cabbage is suggestive to speculate prevalence of such viral pathogens that might be spreading in connection to handling and consumption of the cabbage.

It is conclusive to warn the public health authorities to educate public not to use sewage water, for irrigating vegetables especially salads. Ingham, *et al.* (2004) has correlated level of coliform present in the irrigation water to C.F.U. value obtaining for salad vegetables. This present and other studies clearly demonstrate that incidence of enteric infectious diseases especially Typhoid, Hepatitis and Bacillus dysentery etc. happen to occur and spread via fecally contaminated water and food. And vegetables which are cultivated in

contaminated areas and are irrigated with contaminated water of varying degrees including raw untreated sewage which frequently harbor enteric bacterial, viral and protozoan pathogens. Of such vegetables those which are served uncooked and poorly processed are posing health risks. The vegetable, *Brassica oleracea* is frequently consumed raw in salad both at house hold as well as restaurant levels. And the three samples tested in this study alarm that the vegetables must be properly monitored for its contaminated nature and methods to disinfect it are needed to be established which might allow its safer use as raw salad.

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TABLE III.- COLONIAL AND BIOCHEMICAL CHARACTERIZATION OF COLIFORM BACTERIA ISOLATED FROM SAMPLE NO. 1 ON EMB AGAR

Bacterial Isolate	Strain	Colonial morphology				Biochemical characterization					
		Colour	Configu-ration (margin)	Eleva-tion (mm)	Opacity (consistency)	catalase (oxidase)	Grams staining (morphology)	Motility test (Endospore staining)	MR (VP)	Indole Test (Citrate test)	Haemo-lytic test
Bacillus	O-1	Whitish purple	Round (Smooth)	convex (2)	opaque Gummy	+ve -ve	+ve/bacilli	+ve +ve	+ve +ve	-ve +ve	α
Enterococcus	O-2	Violet	Round (Smooth)	convex (3)	translucent Gummy	+ve -ve	+ve/cocci	+ve +ve	+ve -ve	+ve +ve	α
Bordetella	0-3	Off-white	Round (Smooth)	Drop like (1)	translucent Mucoid	+ve +ve	-ve/cocci	+ve -ve	+ve -ve	+ve +ve	-ve
Bacillus	O-4	violet	filamentous (smooth)	convex (3)	opaque Gummy	+ve -ve	+ve/bacilli	+ve +ve	-ve -ve	-ve +ve	A
Bacillus	O-5	Light violet	L-form (smooth)	convex (2)	opaque Mucoid	+ve -ve	+ve/bacilli	+ve +ve	+ve -ve	-ve +ve	-ve
E.coli	O-6	Green metallic sheen	Filiform (lobate)	raised (1)	opaque Gummy	+ve -ve	-ve/diplo bacilli	+ve +ve	-ve +ve	-ve +ve	-ve
Bacillus	O-7	Light violet	Round (Smooth)	convex (3)	opaque Gummy	+ve +ve	+ve/diplo bacilli	+ve -ve	-ve -ve	-ve +ve	-ve
Clostridium	M-1	grey	Round (Scalloped)	convex (3)	translucent Mucoid	-ve -ve	+ve/bacilli	+ve +ve	+ve -ve	-ve +ve	-ve
E. coli	M-2	Green metallic sheen	Irregular & spreading (Wavy)	raised (1)	transparent Mucoid	+ve +ve	-ve/bacilli	-ve -ve	+ve -ve	-ve +ve	B
Enterococcus	M-3	Dark purple	L-form Smooth	Drop-like 2	opaque Mucoid	+ve -ve	+ve/cocci	+ve +ve	-ve +ve	-ve +ve	-ve

Continued

Bacterial Isolate	Strain	Colonial morphology				Biochemical characterization					
		Colour	Configu-ration (margin)	Eleva-tion (mm)	Opacity (consistency)	catalase (oxidase)	Grams staining (morphology)	Motility test (Endospore staining)	MR (VP)	Indole Test (Citrate test)	Haemo-lytic test
Enterococcus	M-4	grey	Round Smooth	convex 1	opaque Mucoid	+ve -ve	+ve/cocci	+ve +ve	+ve -ve	-ve +ve	-ve
Enterobacter	M-5	Whitish purple	L-form Smooth	convex 1	opaque Mucoid	+ve -ve	+ve/diplo bacilli	+ve +ve	+ve -ve	-ve +ve	-ve
Enterobacter	M-6	Grey	Round Smooth	convex 1	opaque Mucoid	+ve -ve	+ve/ diplo bacilli	+ve +ve	-ve -ve	-ve +ve	α
E. coli	M-7	Green metallic sheen	Irregular & spreading lobate	flat 1	opaque Mucoid	+ve +ve	-ve/cocci	-ve -ve	-ve -ve	-ve -ve	-ve
Enterococcus	M-8	Whitish purple	L-form wavy	convex 2	opaque Gummy	+ve +ve	+ve/cocci	-ve -ve	-ve -ve	-ve -ve	α
Bacillus	M-9	purple	Round smooth	convex 2	translucent Gummy	+ve -ve	+ve/ diplo bacilli	+ve +ve	+ve -ve	-ve -ve	α
Enterococcus	M-10	grey	Round smooth	convex 1	opaque Gummy	-ve +ve	+ve/cocci	+ve -ve	+ve -ve	-ve -ve	α
E.coli	M-11	Green metallic sheen	Irregular & spreading wavy	flat 1	translucent Gummy	-ve +ve	+ve/bacilli	+ve +ve	+ve -ve	-ve -ve	α
Acetobacter	M-12	Dark purple	Round smooth	convex 3	opaque Gummy	+ve -ve	-ve/ diplo bacilli	+ve +ve	-ve -ve	-ve +ve	β
Acetobacter	M-13	Off-white	Round smooth	convex 1	opaque Mucoid	+ve -ve	-ve/ diplo bacilli	+ve +ve	-ve -ve	-ve +ve	-ve
E.coli	M-14	Green metallic sheen	Round smooth	Drop-like 3	Opaque Gummy	+ve +ve	+ve/bacilli	-ve +ve	+ve +ve	-ve -ve	B
Bacillus	M-15	Grayish pink	Round scalloped	wavy 3	translucent Gummy	+ve -ve	+ve/bacilli	+ve -ve	+ve -ve	+ve +ve	-ve

Continued

Bacterial Isolate	Strain	Colonial morphology				Biochemical characterization					
		Colour	Configu-ration (margin)	Eleva-tion (mm)	Opacity (consistency)	catalase (oxidase)	Grams staining (morphology)	Motility test (Endospore staining)	MR (VP)	Indole Test (Citrate test)	Haemo-lytic test
Bacillus	I-1	grey	Round wooly	raised 3	translucent Gummy	+ve -ve	+ve/diplo bacilli	-ve +ve	-ve +ve	-ve +ve	α
E.coli	I-2	Green metallic sheen	Round smooth	flat 1	translucent Mucoid	+ve +ve	-ve/ diplo bacilli	+ve +ve	-ve -ve	-ve +ve	α
Enterococcus	I-3	Grey	Round wavy	flat 1	translucent Gummy	+ve -ve	+ve/cocci	ve+ +ve	-ve +ve	-ve +ve	β
E.coli	I-4	Green metallic sheen	Round Raised	Raised 1	translucent Gummy	+ve -ve	+ve/ diplo bacilli	+ve +ve	-ve -ve	-ve +ve	α
Acetobacter	I-5	Blackish pink	Round Raised	Flat 3	translucent Mucoid	+ve -ve	-ve/ diplo bacilli	+ve +ve	-ve +ve	-ve +ve	-ve
Acetobacter	I-6	Grey	Round Raised	Convex 1	Opaque gummy	+ve -ve	-ve/ diplo bacilli	+ve +ve	-ve +ve	-ve +ve	β
E.coli	I-7	Green metallic sheen	Round Scalloped	Raised 1	Translucent Mucoid	+ve +ve	-ve/ diplococci	-ve +ve	-ve +ve	+ve +ve	α
Bacillus	I-8	Grey	Round Smooth	Convex 3	Opaque Gummy	+ve -ve	+ve/ diplobacilli	+ve +ve	+ve +ve	-ve +ve	α
Bacillus	I-9	Light purple	L-form wooly	convex 3	Opaque Mucoid	+ve -ve	+ve/ diplobacilli	+ve +ve	+ve -ve	-ve +ve	β

TABLE IV.- COLONIAL AND BIOCHEMICAL CHARACTERIZATION OF COLIFORM BACTERIA ISOLATED FROM SAMPLE NO.2ON EMB AGAR

Bacterial Isolate	Strain	Colonial morphology				Biochemical characterization					
		Colour	Configu-ration (margin)	Eleva-tion (mm)	Opacity (consistency)	catalase (oxidase)	Grams staining (morphology)	Motility test (Endospore staining)	MR (VP)	Indole Test (Citrate test)	Haemo-lytic test
Enterococcus	O-1	Dirty black	Round Raised	Flat 2	Opaque Mucoid	+ -	+ve/cocci	+ +	- -	- +	-
E. coli	O-2	Grey	Round smooth	Raised 2	Transparent Mucoid	+ +	-ve/ diplobacilli	- +	+ -	- +	-
Bacillus	O-3	Dark purple	Round smooth	Raised 1	Opaque Mucoid	+ -	+ve/ diplococci	+ +	- -	- +	-
Acetobacter	O-4	Milky	Wrinkled smooth	Convex 1	Opaque Mucoid	+ -	-ve/ diplococci	+ +	- +	- +	α
Enterococcus	O-5	Off white	Round smooth	Convex 3	Opaque Mucoid	+ -	+ve/cocci	+ +	+ +	- -	β
Bordetella	O-6	Dark purple	Filamentous smooth	Flat 2	translucent Mucoid	+ +	-ve/cocci	+ +	- +	- +	-
Bacillus	O-7	Bluish black	Round smooth	Convex 1	translucent Mucoid	+ -	+ve/ diplobacilli	+ +	- +	- +	α
Enterococcus	O-8	Dark purple	L-form smooth	Raised 1	translucent Mucoid	+ +	-ve/ diplococci	- +	+ -	- +	-
Enterococcus	M-1	Grey	Round smooth	Convex 2	translucent Mucoid	+ +	+ve/cocci	+ +	- +	- +	β
Bordetella	M-2	Dark purple	round smooth	convex 2	opaque Mucoid	+ -	-ve/ diplococci	+ +	- -	- +	β
Bordetella	M-3	Milky black	Round Smooth	convex 2	opaque Mucoid	+ -	-ve/cocci	+ +	- -	- +	-
Bordetella	M-4	Green metallic sheen	Concentric Wavy	convex 2	opaque Mucoid	+ +	-ve/diplococci	+ +	- -	- +	-

Continued

Bacterial Isolate	Strain	Colonial morphology				Biochemical characterization					
		Colour	Configu-ration (margin)	Eleva-tion (mm)	Opacity (consistency)	catalase (oxidase)	Grams staining (morphology)	Motility test (Endospore staining)	MR (VP)	Indole Test (Citrate test)	Haemo-lytic test
Enterococcus	M-5	Smoky black	Round smooth	convex 2	opaque Mucoid	+ +	+ve/cocci	+ +	- -	- +	-
Enterococcus	M-6	Purple	Round Smooth	convex 2	translucent Mucoid	+ -	+ve/ cocci	+ +	- +	- +	-
Enterococcus	M-7	Smoky	Round Smooth	Raised 2	opaque Mucoid	+ +	+ve/cocci	+ +	- -	- -	-
Bacillus	M-8	Grey	Round Smooth	convex 1	opaque Mucoid	+ -	+ve/ diplobacilli	+ +	+ +	- -	α
Bacillus	M-9	Green metallic sheen	Concentric wavy	flat 3	opaque Mucoid	+ -	+ve/ diplobacilli	+ +	- +	+ +	-
Enterococcus	I-1	Smoky black	round Smooth	convex 2	opaque gummy	+ +	+ve/cocci	+ +	- +	+ +	-
Enterococcus	I-2	Purple	Round Smooth	convex 1	opaque gummy	+ -	+ve/diplococci	+ +	- +	- +	-
Bacillus	I-3	Green metallic sheen	Round	raised 1	translucent gummy	+ +	+ve/bacilli	+ +	- -	- +	-
E.coli	I-4	Grey	Round	raised 1	translucent mucoid	+ +	-ve/diplobacilli	+ +	- -	- +	-
Bordetella	I-5	Milky	Round Smooth	raised 1	translucent mucoid	+ +	-ve/diplococci	+ +	+ -	- +	-
Bordetella	I-6	Smoky black	Round Smooth	raised 1	translucent mucoid	+ -	-ve/diplococci	+ +	- +	- +	-
Bordetella	I-7	Light black	Round Smooth	convex 2	opaque mucoid	+ +	-ve/diplococci	- +	- +	+ +	-
Bordetella	I-8	Light black	Round Smooth	raised 1	translucent mucoid	+ -	-ve/cocci	+ +	- +	- +	α
Enterococcus	I-9	milky	Round Smooth	raised 2	translucent mucoid	+ -	+ve/cocci	+ +	- -	- +	-

TABLE V.- COLONIAL AND BIOCHEMICAL CHARACTERIZATION OF COLIFORM BACTERIA ISOLATED FROM SAMPLE NO.3 ON EMB AGAR

Bacterial Isolate	Strain	Colonial morphology				Biochemical characterization					
		Colour	Configu-ration (margin)	Eleva-tion (mm)	Opacity (consistency)	catalase (oxidase)	Grams staining (morphology)	Motility test (Endospore staining)	MR (VP)	Indole Test (Citrate test)	Haemo-lytic test
E.coli	O-1	Pink	Round (Raised)	Convex 1	opaque mucoid	+ +	-ve/bacilli	+ +	- -	- +	-
Bacillus	O-2	Purple	L-form Smooth	raised 1	translucent mucoid	+ +	+ve/diplobacilli	+ +	+ -	- +	α
Bordetella	O-3	Pink	Round Raised	raised 1	translucent mucoid	+ -	-ve/cocci	+ +	- -	+ +	-
Enterococcus	O-4	Dark purple	Round Smooth	raised 1	translucent mucoid	+ -	+ve/cocci	+ +	- +	- +	-
Enterococcus	O-5	Blue	Round Smooth	raised 1	opaque mucoid	+ -	+ve/cocci	+ +	+ -	- +	α
Enterococcus	M-1	blue	Round Smooth	raised 1	opaque mucoid	+ -	+ve/cocci	+ -	+ +	- +	α
E. coli	M-2	Pink	L-form Smooth	Convex 1	Translucent mucoid	+ -	-ve/bacilli	+ +	- -	+ +	α
Enterococcus	M-3	Grey	Round Smooth	Flat 1	Opaque mucoid	+ -	+ve/cocci	+ -	- -	- +	-
Enterococcus	I-1	Pink	Round Smooth	Raised 1	Translucent mucoid	+ -	+ve/cocci	+ +	- -	- +	α
Enterococcus	I-2	Dark purple	Round Smooth	Raised 2	Translucent mucoid	+ -	+ve/diplococci	+ +	+ -	+ +	α
Bacillus	I-3	Light pink	Round lobate	Convex 1	Opaque mucoid	+ +	+ve/bacilli	+ +	- -	- +	β
Enterococcus	I-4	Light pink	L-form Smooth	raised 2	translucent mucoid	+ -	+ve/cocci	+ -	- +	+ +	-

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MICROBIOLOGICAL UPGRADATION OF FORMULATED FISH FEED

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Abstract.- An artificial fish feed was fermented by bacteria representing *Pseudomonas*, lactic acid bacteria and amino acids producers. Solid state fermentations of the fish feed indicated significant increases within five days in total protein, total carbohydrates, total lipids, glucose and amino acid contents than the corresponding values of non fermented control feed. *Bacillus cereus* AsCh-A9 enhanced total protein content of the fermented feed up to 97.29% on day 3 when incubated optimally. *Pseudomonas pseudoalcaligenes* AsCh-A4 elevated total carbohydrates up to 131.78%; glucose upto 105.29%; free amino acids up to 99.26% and total lipids up to 99.26% on day 7. These bacterial isolates caused elevation in different contents with 10% inocula sizes and 70% moisture contents. CFU/g and accompanying pH levels of the fermented feeds employing the bacterial isolates are also reported. These results are suggestive to design commercial level solid state fermentation facilities for improving further development of aquaculture in this country.

Key Words: Solid state fermentation; solid substrate fermentation; *Bacillus cereus*; *Pseudomonas*; protein increment.

INTRODUCTION

One of the major expenses of raising a fish culture involves the cost of feed. Use of supplementary feed has become inevitable for the success of contemporary fish culturing. Supplementary feeding is known to increase the carrying capacity of culture systems and enhance fish production by many folds (Hepher, 1975; Devaraj *et al*., 1986; DeSilva and Gunasekera, 1991; Balogu *et al*., 1993; Mahboob *et al*., 1997; Ali *et al*., 2003). Artificial diets are available for growing out of fingerlings and adults of most cultured fish species. But they may be less than optimal and consequently result in lowered growth rates and excessive waste, either by excessive fecal material, excessive urinary nitrogen, or uneaten food. Thus, less than optimum diets are wasteful in terms of money spent on feed. The key challenge of producing productive feeds is the maximization of fish growth. Development of high-quality artificial diets could potentially ameliorate water quality and disease problems, as well as reduce the high cost of live feed (Bengston, 1993).

While considering economical aspects of aquaculture fermented feeds had been hypothesized to further assimilation efficiency of supplementary feeds (Mukhopadhayay and Ray, 1999; Skrede *et al*., 2001; Skrede *et al*., 2003). Fermentation has been suggested as one of the means to improve the nutritional value of protein sources for fish feeds (Refstie *et al.,* 2005). Solid state fermentation has been attempted as a mean of elevating total protein content of different substrates by many workers (Reade and Gregory, 1975; Rodriguez *et al*., 1985; Balagopalan and Padjama 1988; Iyayi *et al*., 2004).

Fermented feeds are obtained from normal substrates that are invaded by microorganisms. Whose enzymes, particularly amylases, proteases, lipases hydrolyze the polysaccharides, proteins, and lipids, respectively (Steinkraus, 1996a). Bacteria, yeast and fungi can be grown on solid substrates and find applications in solid-state fermentation (SSF) processes. In the last decades, there has been increasing interest in the use of SSF processes as alternatives to submerged fermentation because it lowers energy requirements, produces less waste water and partly because of environmental concerns regarding the disposal of solid waste (Lonesane and Ramash, 1990).

SSF involves the growth of microorganisms on moist solid substrates in the absence of free flowing water (Doelle *et al*., 1992; Tengerdy, 1996; Soccol and Vandenberghe, 2003; Suryanarayan, 2003). Bacterial fermentation such as by *Lactobacillus* sp. has been used with success to reduce the levels of non-starch carbohydrates in wheat and barley whole meals (Skrede *et al*., 2001, 2002, 2003).

In the present study seven bacteria were isolated from raw milk and yogurt samples and cultured in formulated fish feed. The bacterial isolates were optimized for growth as well as accompanying nutrient levels *viz*. total protein, carbohydrates, lipids, glucose, amino acids while, fermenting the formulated fish feed in self designed SSF chambers at laboratory scale. All the bacterial isolates significantly increased the fish feed nutritional levels in terms of the parameters described from 50-100 %. The fermented feeds bear a high potential for enhancing fish growth.

MATERIALS AND METHODS

Test organisms

Seven bacterial isolates representing Lactic-acid bacteria, Pseudomonas and amino acid producers were employed in this study. For isolation of lactic

acid bacteria and *Pseudomonas sp.*, raw milk and yogurt samples were obtained from different sale points in sterilized containers from different areas of Lahore. Lactic acid bacteria were isolated on MRS medium (DeMan *et al.*, 1960) while Pseudomonads on *Pseudomonas* agar F base (Merck, 1996-1997). Amino acids producing bacteria were isolated from soil samples collected from the vicinity of Quaid-e-Azam Campus, University of the Punjab, Lahore on (M-I) medium that comprised of dipotassium hydrogen phosphate 0.5, potassium dihydrogen phosphate 0.5, $MgSO_4.7H_2O$ 2.5, calcium carbonate 20.38, ammonium sulphate 20.0, D (+) glucose 20.0 and Agar agar 20 (g/l). pH of the medium was adjusted at 7.5-8.0. All the samples were serially diluted in sterilized water [1:10 (v/v or w/v)] and spread on respective selective media.

Formulation of fish feed

Feed ingredients were purchased from local market and passed through a sieve to remove dust and large aggregates. All the ingredients were ground separately to powder form. The feed was then prepared by mixing thoroughly fish meal 5.0%, rice polishing 34.3%, ground nut oil cake 53.7%, molasses 4.0%, diclacium phosphate 1.0%, table salt 1.0% and vitamin premix 1.0%.

Screening of the bacterial isolates on 2% fish feed

Fish feed and agar agar (2% each) were sterilized by autoclaving and poured in petriplates. All the selected bacterial isolates were streaked on the solidified preparation and the plates were incubated at 37°C. Appearance and magnitude of growth of each bacterial isolate was recorded at daily basis up to 72 hours post incubation. The bacterial isolates yielding maximum solid growth were selected for further studies.

Inoculum preparation

Forty-one screened bacterial isolates were grown in nutrient broth. A given fresh bacterial culture (overnight incubated) was centrifuged at 5000 rpm for 10 minutes. Supernatant was discarded and the pellet was washed twice with sterile saline solution (0.89%). Finally the pellet was suspended in the sterile saline solution and the cell concentration was adjusted to 0.5 ± 0.05 at 600nm. To estimate C.F.U./ml., the suspension was diluted serially and was spread on nutrient agar medium as described by Nikoskelainen *et al.* (2001). The C.F.U. defined inocula of the 7 bacterial isolates were then used for SSF experiments.

Solid state fermentation (SSF) of the formulated fish feed

For solid state fermentation, an apparatus was designed and installed according to Hofrichter *et al.* (1999). SSF was carried out in screw capped sterile containers measuring 12 and 06 centimeters for length and diameter, respectively. Twenty g of the formulated fish feed, sterilized by autoclaving at 121°C for 30 minutes was introduced in each of the container and height of the substrate remained less than 2 cm. The feed was inoculated with already prepared inoculum (2ml) alongwith 12 ml of sterilized distilled water. The containers were closed with lids fitted with inlet and outlet tubes for aeration and incubated at a temperature range of 37–40°C for 5 days, with a constant flow of filtered-sterilized air. Autoclaved distilled water was introduced daily in each fermentation jar in an amount to replenish its 70% (v/w) content. The fermentation jars were stirred twice while keeping the whole assembly within laminar air-flow cabinet. Control jars were processed similarly but without bacterial inoculation. All the experiments were performed in triplicates. Half gram of a given fermented feed was sampled at day 1, 3, 5 and processed for the determination of various biochemical contents *viz.* total protein, total carbohydrates, glucose, total lipids, amino acids according to the methods described below.

Optimization of the solid state fermentations (SSF) process

Fish feed SSF was optimized for moisture contents, incubation time and inoculum size. To keep the fish feed moist differing amounts of water were added once daily to the feeds undergoing the process of SSF as well as to the control to maintain 50%, 70% and 100% (v/w) moisture contents. The sampling day at which highest biochemical parameters were recorded for a given fermented feed sample was considered optimum incubation period. SSFs of the feed were initiated with 10%, 20% and 30% (v/w) inocula to determine the optimum inoculum size. In all the experiments a given bacterial isolate was performed in triplicates. The control and the experimental feeds were aerated at the same rate.

Biochemical analysis of the feed

From each container 1.5g of a feed was sampled and the samples were kept for drying at 105°C in an electric oven till consistent weight. From the dried samples, 0.5 g was suspended in 4 ml of 0.89% saline solution, homogenized

with the help of a motor driven fitted with Taflon-coated probe homogenizer at 8000 rpm for one minute and then centrifuged at 4900 rpm, in a refrigerated centrifuge (4°C) for 45 minutes. The supernatant (feed extract) was collected in glass vials and freezed till further use for the biochemical analyses. Total protein contents were estimated by Biuret method (Gornall *et al.*, 1949), total carbohydrates by phenol sulphuric acid assay (Dubois *et al.,* 1956), glucose content by O' toluidine method (Hartel *et al.,* 1969) and free amino acids by ninhydrin reagent (Jayarraman, 1981).

For total lipids' estimation, 0.5g from a dried sample was suspended in 3 ml of 95% ethyl alcohol. It was homogenized at 8000 rpm for one minute, covered by aluminum foil caps, kept at room temperature for 24 hours and then centrifuged at 18000 rpm for 15 minutes. Total lipids were then estimated in the supernatant by the method of Zöllner and Kirsch (1962).

C.F.U. determination of SSF feed

During the solid state fermentations under optimum conditions, 0.5g of the sampled fermented substrate was suspended in 10 ml of 0.89% sterile saline solution. This suspension was diluted serially to represent 1:100, 1:1000 and 1:10000 of the original suspension. From each dilution 0.1 ml was spread on nutrient agar and the plates were incubated at 37^{o}C in inverted position for 24 hours. The bacterial colonies were then counted with the help of a colony counter and C.F.U. /g of fermented feed was calculated by the following formula:

$$\text{C.F.U./g of wet. feed} = \frac{\text{No. of colonies} \times \text{dilution factor} \times \text{vol. spread} \times 10}{\text{weight of feed (0.5g)}}$$

In the same way, pH was recorded by immersing 0.5 g of a feed sample in 4 ml of water at the start and end of each experiment. The fluid portion of the processed sample was separated by centrifugation and its pH was measured with the help of a pH meter.

RESULTS

All the selected 7 bacterial isolates grew well on 2% formulated fish feed agar medium within 24 hours except *Aeromonas caviae*AsCh-L14 that manifested its growth after 48 hours. Growth was accompanied with a change in color of the solid medium, from light to dark for the isolate *Pseudomonas pseudoalcaligenes* AsCh-A4 only (Table I).

TABLE I.- APPEARANCE OF GROWTH ON 2% FORMULATED FISH FEED AGAR MEDIUM USED FOR SELECTION OF BACTERIAL ISOLATES TO BE EMPLOYED IN SOLID STATE FERMENTATION (SSF) OF THE FEED.

Bacterial isolates	Appearance of growth (Hours)	Color change
Sporolactobacillus inilunis AsCh-L6	24	−
Aeromonas caviae AsCh-L14	48	−
Edwardsiella hoshinae AsCh-P8	24	−
Bacillus cereus AsCh-A2	24	−
Listeria murnyi AsCh-A3	24	−
Pseudomonas pseudoalcaligenes AsCh-A4	24	+
Bacillus cereus AsCh-A9	24	−

Solid state fermentation (SSF) on fish feed

SSF of the fish feed by the 7 potential probiotic bacterial isolates caused in general significant elevations in the total proteins, total carbohydrates, glucose, amino acids and total lipid contents, as compared to sterile feed processed as control on various days of experiment (Table II, Fig. 1). Regarding total protein contents of the SSF feeds increased 51 to 69, 60 to 70, 69 to 80%. Significantly on day 1, 3 and 5 considering total carbohydrates increased 60-97, 60-92 and 60-100%, the glucose content increased 47-56, 50-65, 60-70%, amino acid contents increased up to 60-80, 55-92, and 60-93%; and lipid contents increased 50-78, 50-92, 60-115%, respectively on day 1, 3 and 5, when compared with the control values (Table II, Fig.1). All the elevations appeared statistically significantly higher from the control values except amino acid contents and total lipids of SSF feeds raised by employing the isolates *Aeromonas caviae* AsCh-L14 and *Sporolactobacillus inilunis* AsCh-L6, respectively for which the increases of the parameters turned out statistically to be non significant.

Optimization of growth conditions of the bacterial isolates

The bacterial isolates differed in growth optimization conditions as well as days at which they yielded higher biochemical contents. For instance *Sporolactobacillus inilunis* AsCh-L6 caused 78.20, 11.20, 109.20, 87.54 and 119.51% increases over the control values in the total protein, total carbohydrates, glucose, amino acids and lipids contents respectively at day 3 with inoculum size of 10% that was found optimum (Fig. 2).

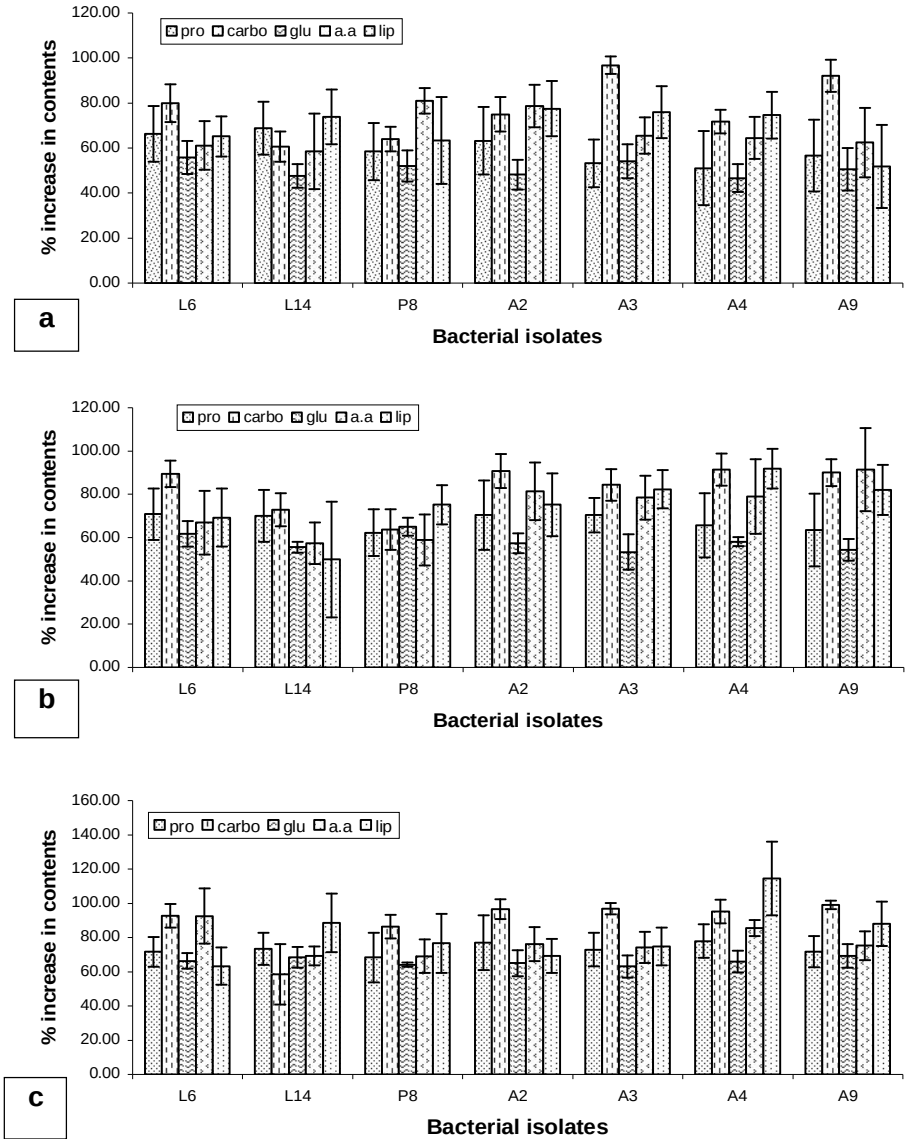

Fig. 1. Effect of SSF on percent change on different contents of the formulated fish feed employing different bacterial isolates on different days of incubation; a, day 1; b, day 3; c, day 5. Bars represents mean±SEM. Bars represent S.E.M.

pro, protein; carbo, carbohydrates; glu, glucose, a.a. amino acids; lip, lipids.

Fig. 2. Effect of different inocula sizes of *Sporolactobacillus inilunis* AsCh-L6 on different biochemical contents (% change) of fish feed at different days of incubation (SSF). Bars represent S.E.M.
a, protein contents; b, carbohydrate contents; c, glucose contents; d, amino acids; e, lipids.

The bacterial isolate *Aeromonas caviae* AsCh-L14 caused optimally 89.86, 116.85, 78.17, 63.82 and 98.70% elevations in the total protein, total carbohydrates, glucose, amino acids and lipids contents respectively at day 5 with inoculum size of 10% (Fig. 3). Optimum increases of 90.93, 89.65, 83.25, 68.04 and 109.21% for total protein, total carbohydrates, glucose, amino acids and lipid contents, respectively were recorded at day 3 with inoculum size of 10% for the isolate *Edwardsiella hoshinae*AsCh-P8. Again all these values show significant difference statistically from sterile control feed (Fig. 4). The isolate *Bacillus cereus*AsCh-A2 caused 90.93, 107.77, 75.45, 69.51 and 122.49% increases in the total protein, total carbohydrates, glucose, amino acids and lipid contents respectively as compared to control values at day 3 with inoculum size of 10% (Fig. 5).

The bacterial isolate *Listeria murnyi* AsCh-A3 caused 79.11, 94.11, 68.16, 63.10 and 102.71% elevations in the total protein, total carbohydrates, glucose, amino acids and lipids contents respectively at day 3 with inoculum size of 10% (Fig. 6). The isolate *Pseudomonas pseudoalcaligenes* AsCh-A4 caused 93.79, 131.78, 93.21, 95.17 and 130.59% elevations in the total protein, total carbohydrates, glucose, amino acids and lipids contents respectively at day 7 with inoculum size of 10% (Fig. 7). The isolate *Bacillus cereus* AsCh-A9 caused 97.29, 90.64, 73.00, 89.66 and 91.05% increases in the total protein, total carbohydrates, glucose, amino acids and lipids contents respectively at day 3 with inoculum size of 10% (Fig. 8).

Apart from these optimum elevations of the parameters, which were found statistically higher from the control values, the SSF feeds raised on other conditions and days also showed, in general, higher values of parameters than control figures.

Optimization for moisture content for the SSF of the fish feed

Majority of the bacterial isolates yielded higher biochemical parameters with 70% water content (v/w) at third day of SSF.

Sporolactobacillus inilunis AsCh-L6 caused 80, 103.56, 112.33, 81.77 and 102.42% increases in the total protein, total carbohydrates, glucose, amino acids and lipid contents, respectively at day 3 at a moisture content of 70%. Except for the total protein all the other parameters in the described moisture conditions and incubation period were found statistically significantly higher from the respective control values (Fig. 9). The bacterial isolate *Aeromonas caviae* AsCh-L14 caused

Fig. 3. Effect of different inocula sizes of *Aeromonas caviae* AsCh-L14 on different biochemical contents (% change) of fish feed at different days of incubation (SSF). Bars represent S.E.M.
a, protein contents; b, carbohydrate contents; c, glucose contents; d, amino acids; e, lipids.

Fig. 4. Effect of different inocula sizes of *Edwardsiella hoshinae* AsCh-P8 on different biochemical contents (% change) of fish feed at different days of incubation (SSF). Bars represent S.E.M.
a, protein contents; b, carbohydrate contents; c, glucose contents; d, amino acids; e, lipids.

Fig. 5. Effect of different inocula sizes of *Bacillus cereus* AsCh-A2 on different biochemical contents (% change) of fish feed at different days of incubation (SSF). Bars represent S.E.M.
a, protein contents; b, carbohydrate contents; c, glucose contents; d, amino acids; e, lipids.

Fig. 6. Effect of different inocula sizes of *Listeria murnyi* AsCh-A3 on different biochemical contents (% change) of fish feed at different days of incubation (SSF). Bars represent S.E.M.
a, protein contents; b, carbohydrate contents; c, glucose contents; d, amino acids; e, lipids.

Fig. 7. Effect of different inocula sizes of *Pseudomonas pseudoalcaligenes* AsCh-A4 on different biochemical contents (% change) of fish feed at different days of incubation (SSF). Bars represent S.E.M.
a, protein contents; b, carbohydrate contents; c, glucose contents; d, amino acids; e, lipids.

Fig. 8. Effect of different inocula sizes of *Bacillus cereus* AsCh-A9 on different biochemical contents (% change) of fish feed at different days of incubation (SSF). Bars represent S.E.M. a, protein contents; b, carbohydrate contents; c, glucose contents; d, amino acids; e, lipids.

Fig. 9. Effect of different moisture contents of *Sporolactobacillus inilunis* AsCh-L6 n different biochemical contents (% change) of fish feed at different days of incubation (SSF). Bars represent S.E.M. a, protein contents; b, carbohydrate contents; c, glucose contents; d, amino acids; e, lipids.

73.45, 92.95, 76.68, 82.06 and 121.42% increases in the total protein, total carbohydrates, glucose, amino acids and lipid contents, respectively at day 5 at moisture content of 70% that was found optimum. Except for the total protein and glucose contents all the other parameters in the described moisture conditions and incubation period were found statistically significantly higher from the respective control values (Fig.10).

*Edwardsiella hoshinae*AsCh-P8 caused 90.02, 111.05, 75.97, 81.27 and 99.91% increases in the total protein, total carbohydrates, glucose, amino acids and lipid contents respectively at day 3 at the moisture content of 70%. Except for the glucose all the other parameters in the described moisture conditions and incubation period were found statistically significantly higher from the respective control values (Fig. 11). *Bacillus cereus*AsCh-A2 caused 73.65, 120.13, 92.08, 86.82 and 100.80% increases in the total protein, total carbohydrates, glucose, amino acids and lipid contents, respectively at day 3 at moisture content of 70%. Except for the total protein all the other parameters in the described moisture conditions and incubation period were found statistically significantly higher from the respective control values (Fig. 12).

Listeria murnyi AsCh-A3 caused 70.92, 92.75, 83.90, 77.46 and 77.69% increases in the total protein, total carbohydrates, glucose, amino acids and lipid contents respectively at day 3 at moisture content of 70%. Total carbohydrates and glucose in the described moisture conditions and incubation period were found statistically significantly higher from the respective control values (Fig. 13). *Pseudomonas pseudoalcaligenes* AsCh-A4 caused 93.79, 127.38, 105.29, 90.55 and 129.31% increases in the total protein, total carbohydrates, glucose, amino acids and lipid contents respectively at day 5 at moisture content of 70%. All the parameters in the described moisture conditions and incubation period were found statistically significantly higher from the respective control values (Fig. 14).

Bacillus cereus AsCh-A9 caused 93.65, 66.84, 69.63, 99.26 and 102.15% increases in the total protein, total carbohydrates, glucose, amino acids and lipid contents respectively at day 3 at moisture content of 70%. Only total protein and amino acid contents in the described moisture conditions and incubation period were found statistically significant higher from the respective control values (Fig. 15).

Fig. 10. Effect of different moisture contents of *Aeromonas caviae* AsCh-L14 on different biochemical contents (% change) of fish feed at different days of incubation (SSF). Bars represent S.E.M.
a, protein contents; b, carbohydrate contents; c, glucose contents; d, amino acids; e, lipids.

Fig. 11. Effect of different moisture contents of *Edwardsiella hoshinae* AsCh-P8 on different biochemical contents (% change) of fish feed at different days of incubation (SSF). Bars represent S.E.M.
a, protein contents; b, carbohydrate contents; c, glucose contents; d, amino acids; e, lipids.

Fig. 12. Effect of different moisture contents of *Bacillus cereus* AsCh-A2 on different biochemical contents (% change) of fish feed at different days of incubation (SSF). Bars represent S.E.M.
a, protein contents; b, carbohydrate contents; c, glucose contents; d, amino acids; e, lipids.

Fig. 13. Effect of different moisture contents of *Listeria murnyi* AsCh-A3 on different biochemical contents (% change) of fish feed at different days of incubation (SSF). Bars represent S.E.M.
a, protein contents; b, carbohydrate contents; c, glucose contents; d, amino acids; e, lipids.

Fig. 14. Effect of different moisture contents of *Pseudomonas pseudoalcaligenes* AsCh-A4 on different biochemical contents (% change) of fish feed at different days of incubation (SSF). Bars represent S.E.M.
a, protein contents; b, carbohydrate contents; c, glucose contents; d, amino acids; e, lipids.

Fig. 15. Effect of different moisture contents of *Bacillus cereus* AsCh-A9 on different biochemical contents (% change) of fish feed at different days of incubation (SSF). Bars represent S.E.M.
a, protein contents; b, carbohydrate contents; c, glucose contents; d, amino acids; e, lipids.

Viable counts of the fermented fish feed and accompanying pH levels

When the formulated fish feed was inoculated with optimum inocula sizes and moisture levels and subjected to SSF processes it appeared that all the seven selected bacterial isolates showed moderate decrease in C.F.U. from day 1 to day 3 followed by a drastic decline at day 5. Thereafter, the C.F.U. contents of the bacterial isolates more or less stabilized at day 7 (Table III, Fig. 16). Regarding the pH level except for the bacterial isolates *Aeromonas caviae* AsCh-L14, which caused an elevation approaching from 5.65 to 5.94 at day 7 of the fermentation. Other isolates, in general, did not change the pH throughout the experimental period of SSF (Table IV).

TABLE III.- VIABLE COUNTS OF THE SELECTED BACTERIAL ISOLATES IN FORMULATED FISH FEED AT DIFFERENT DAYS POST SSF CARRIED AT THEIR RESPECTIVE OPTIMUM GROWTH CONDITIONS.

Bacterial isolate	Inoculum CFU/ml	Day-1 CFU/g	Day-3 CFU/g	Day-5 CFU/g	Day-7 CFU/g
AsCh-L6	28×10^5	173×10^6	36×10^6	90×10^4	52×10^4
AsCh-L14	228×10^5	184×10^6	57×10^6	82×10^4	44×10^4
AsCh-P8	72×10^5	157×10^6	41×10^6	150×10^4	56×10^4
AsChA2	37×10^5	108×10^6	28×10^6	64×10^4	36×10^4
AsCh-A3	217×10^5	127×10^6	35×10^6	128×10^4	62×10^4
AsCh-A4	288×10^5	204×10^6	142×10^6	276×10^4	104×10^4
AsCh-A9	207×10^5	114×10^6	50×10^6	115×10^4	68×10^4

TABLE IV.- pH OF THE SELECTED BACTERIAL ISOLATES IN FORMULATED FISH FEED AT DIFFERENT DAYS POST SSF CARRIED AT THEIR RESPECTIVE OPTIMUM GROWTH CONDITIONS.

Bacterial isolate	Day-1	Day-3	Day-5	Day-7
Control	5.60	5.50	5.43	5.47
AsCh-L6	5.58	5.53	5.44	5.53
AsCh-L14	5.65	5.82	5.93	5.94
AsCh-P8	5.55	5.73	5.47	5.51
AsChA2	5.64	5.58	5.55	5.57
AsCh-A3	5.56	5.55	5.73	5.62
AsCh-A4	5.58	5.56	5.58	5.50
AsCh-A9	5.55	5.49	5.45	5.62

pH was determined by mixing 0.5 g fermented or control fish feed/ 4 ml of water

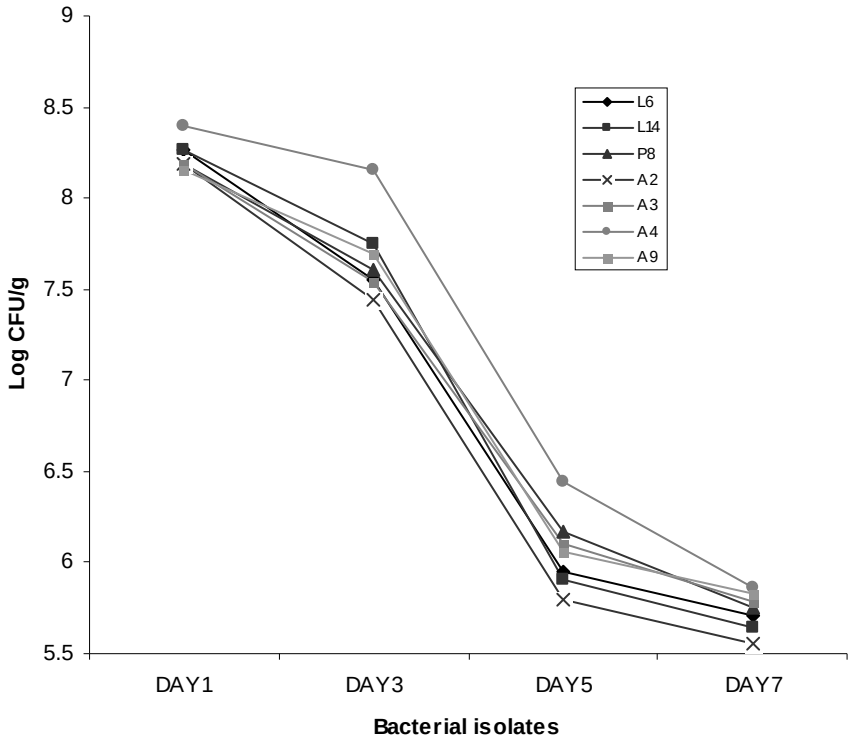

Fig. 16. Viable counts of the selected bacterial isolates during SSF in the formulated fish feed at different days of incubations.

DISCUSSION

Solid-state fermentation (SSF) is a process used for the production of fermented feed. Animal feed industry has been utilizing several products of microbial fermentations as feed additives with appealing growth promoting efforts (Twomey *et al*., 2003; Soccol and Vandenberghe, 2003; Suryanarayan, 2003; Macey and Coyne, 2005). This study represents the earlier report on the improvement of nutritive values in this country of formulated fish feed through bacterial (locally isolated) fermentation.

Bacterial isolates being reported in this study enhanced various important

nutritional contents of fermented fish feed from 50-100% in five days. The nutritional values approached 80% for total protein, 100% for total carbohydrates, 70% for glucose contents, 93% for free amino acids and 115% for total lipids elevations over the control non fermented feed within five days of fermentations. Microbes are well known for their abilities to produce a variety of enzymes such as amylases, polysaccharidases, cellulases, proteases, lipases and lignocellulases (Ofuya and Nwanjiuba, 1990; Balagopalan, 1996; Hamlyn, 1998; Iyayi and Losel, 2001). These enzymes help to degrade the non starch polysaccharides and other polymeric form of the molecules in the substrate to soluble monomers with a beneficial increase in energy and protein. Further bioconversion of starch and sugars to proteins including single cell protein leads to the protein enrichment of various natural substrates which then become promising to be used for animal feeds (Mikami *et al*., 1982; Manilal *et al*., 1985; Balagopalan and Padjama, 1988).

Significantly several folds increases of protein contents of SSF feeds over the control values are highly appealing to speculate the growth promoting effects on the fish by increasing assimilation efficiencies of the consumers. *Bacillus cereus* AsCh-A9 enhanced total protein contents up to 97.29% on 3rd day of incubation. Comparable 185% increase in the protein was reported by Ofuya and Nwanjiuba (1990) when they cultured for cassava peels with *Rhizopus sp*. Such protein enrichment of solid substrates has been carried out on barley, wheat, cassava and dehydrated beet pulp by many workers (Rodriguez *et al*., 1985; Mathot and Brakel, 1991; Soccol *et al*., 1994; Balagopalan, 1996; Duru and Uma, 2003).

Maximum elevation in total carbohydrate was obtained by *Pseudomonas pseudoalcaligenes* AsCh-A4 upto 131.78% on day 7. Glucose contents increased up to 105.29% on 7th day of incubation by the same bacterial isolate. Such increases in soluble sugars has been noticed by many workers while upgrading different agroindustrial byproducts (Habijanic and Berovic, 2000; Iyayi and Losel, 2001; Iyayi and Aderolu, 2004).

Protein rich fish feed is growth promoting in fish. Thus nutritional value of feed is raised by amino acids supplementation (Mambrini *et al*., 1999; Refstei *et al*., 2001). *Pseudomonas pseudoalcaligenes* AsCh-A4 caused 99.26% elevation in amino acid contents of formulated fish feed on 7th day of incubation. It may be hypothesized that free amino acids elevation in feed will help in fish growth because crystalline amino acids supplemented feed had earlier been reported due

to faster uptake and subsequent catabolism (Murai and Andrews, 1976). Fermentation of formulated fish feed by bacterial isolates also caused increase in its lipids contents. *Pseudomonas pseudoalcaligenes* AsCh-A4 elevated the lipids contents up to 130.59% at day 7. According to Hamre *et al*. (2004) adequate lipid level in fish diet is important for the growth of fish and product quality.

The results of this study have shown that solid state fermentation by bacterial isolates is an effective and economically feasible tool for enhancing the nutritive values of formulated fish feed. This *in vitro* assay provide base for practical application of SSF. Regarding the effects of probiotics in the SSF feed, actual *in vivo* experimental evidences would suggest best of the organisms in term of yielding higher growth. And that probiotics may not be necessarily the one yielding highest increases in the nutritional parameters of the formulated fish feed. Actually experimental data from this laboratory was expected to throw light on this aspect in forth coming papers.

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TABLE II.- EFFECTS OF SSF ON DIFFERENT BIOCHEMICAL CONTENTS (mg/g of dry weight) OF THE FORMULATED FISH FEED EMPLOYING THE SELECTED BACTERIAL ISOLATES AT DIFFERENT TIME INTERVALS OF INCUBATION.

Days post incubation	Contents	Bacterial Isolates							
		Control	*Sporolactobacillus inilunis* AsCh-L6	*Aeromonas caviae* AsCh-L14	*Edwardsiella hoshinae* AsCh-P8	*Bacillus cereus* AsCh-A2	*Listeria murnyi* AsCh-A3	*Pseudomonas pseudoalcaligenes* AsCh-A4	*Bacillus cereus* AsCh-A9
1	Total Protein	63.91±2.20	106.24±7.92^{*a}	107.90±7.54^{*a}	101.26±8.15^{a}	104.30±9.59^{*a}	97.94±9.59^{a}	96.55±10.55^{a}	100.15±10.17^{a}
	Total Sugars	39.65±0.58	71.37±3.33^{*a}	63.72±2.66^{*c}	65.02±2.18^{*a}	69.35±3.03^{*a}	78.00±1.83^{*b}	68.09±2.12^{*a}	76.18±2.83^{*a}
	Glucose	28.96±0.20	45.13±2.13^{*a}	42.75±1.50^{*a}	44.02±2.01^{*a}	42.90±1.94^{*a}	44.63±2.28^{*a}	42.46±1.79^{*a}	43.58±2.74^{*a}
	Amino acids	0.651±0.039	1.047±0.07^{*a}	1.03±0.11^{a}	1.176±0.03^{*b}	1.161±0.06^{*b}	1.076±0.05^{*a}	1.069±0.06^{*a}	1.055±0.10^{*a}
	Total Lipids	173.55±7.57	286.68±15.44^{*a}	305.31±19.88^{*a}	283.50±33.53^{a}	303.04±22.20^{*a}	305.31±19.88^{*a}	303.04±17.90^{*a}	308.94±21.58^{a}
3	Total Protein	64.46±2.46	110.11±7.68^{*a}	109.56±7.72^{*a}	104.58±6.97^{a}	109.83±10.31^{*a}	109.83±7.43^{*a}	106.79±9.61^{*a}	105.41±10.84^{a}
	Total Sugars	38.63±2.20	75.13±2.41^{*a}	68.55±3.01^{*c}	64.89±3.69^{*b}	75.62±3.09^{*a}	73.08±2.12^{*a}	75.87±2.93^{*a}	75.34±2.47^{*a}
	Glucose	30.89±0.50	49.96±1.79^{*a}	48.05±0.78^{*a}	50.97±1.29^{*a}	48.62±1.40^{*a}	47.33±1.79^{*a}	48.84±0.64^{*a}	47.66±1.56^{*a}
	Amino acids	0.663±0.09	1.101±0.09^{*a}	1.038±0.06^{a}	1.049±0.07^{a}	1.197±0.08^{*a}	1.178±0.06^{*a}	1.181±0.11^{*a}	1.093±0.17^{*a}
	Total Lipids	166.74±15.84	282.14±22.43^{a}	293.50±15.86^{a}	292.13±14.99^{a}	292.13±24.16^{a}	303.95±14.76^{*a}	297.13±12.38^{*a}	303.49±19.36^{*a}
5	Total Protein	66.4±1.73	113.98±5.76^{*a}	115.09±6.24^{*a}	111.77±9.64^{*a}	117.58±10.61^{*a}	114.81±9.35^{*a}	118.13±6.53^{*a}	113.98±6.07^{*a}
	Total Sugars	39.32±1.23	75.77±2.67^{*a}	62.36±6.93^{*b}	73.32±2.72^{*a}	77.31±2.28^{*a}	77.41±0.97^{*a}	76.74±2.70^{*a}	78.26±0.95^{*a}
	Glucose	29.75±0.54	49.48±1.36^{*a}	50.11±1.82^{*a}	48.81±0.36^{*a}	49.08±2.23^{*a}	48.53±1.39^{*a}	49.36±1.87^{*a}	50.31±2.05^{*a}
	Amino acids	0.686±0.05	1.156±0.05^{*a}	1.151±0.03^{*a}	1.149±0.06^{*a}	1.198±0.06^{*b}	1.185±0.06^{*a}	1.261±0.03^{*b}	1.192±0.05^{*a}
	Total Lipids	174.46±6.30	284.86±19.10^{a}	328.93±29.83^{*b}	308.04±29.93^{*a}	295.31±17.34^{*a}	304.85±19.36^{*a}	315.31±26.06^{*a}	328.03±22.64^{*b}

All values represent means of three replicates ±S.E.M. significant difference of a given value from its respective control is indicated by asterisk.

Two values within a respective row not sharing a common alphabet differ significantly from each other.

- Significantly different at $p \leq 0.5$ at single factor analysis of variance.

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BIOCHRONOLOGY OF HASNOT, THE MIDDLE SIWALIKS, PAKISTAN

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Abstract.- Deposits near Hasnot, Potwar Plateau, northern Pakistan yield a diverse mammal fossil assemblage, which is frequently more productive and taxonomically rich. Biochronology of Hasnot is based on the palaeontological expeditions in 2003 to 2005. An account of the ruminant fossils collected in the expeditions is given and the collection is housed in 'Abu Bakr Fossil Display and Research Centre', Zoology Department, Punjab University, Lahore, Pakistan. The fossil localities, H15-H22, northeastern side of the Hasnot, contain particularly various specimens of the cervids and other elements of the fauna argue for an age younger than that of the Dhok Pathan Formation. The deposits at west, north and northwest of the Hasnot are considered older than its northeastern deposits. Temporal ranges of the Hasnot fauna, as presently known, confine Hasnot from 7 to 5 Ma. The revised localities and the deposits are mentioned in the text and the importance of the deposits for future Pliocene research is emphasized.

Key words: Biochronology, Hasnot, Siwaliks, Dhok Pathan Formation.

INTRODUCTION

Siwalik Group is part of southern Himalayan mountains extending about 1689 km (1050 miles) from southwest Kashmir through India into southern Nepal. The hills are noted for their extensive fossil remains. The Siwalik sediments are found in widely separated areas all along the foothills of Himalayas (Fig. 1). The name Siwalik was introduced for the sub Himalayan rocks by Medlicott (1864), and this term is derived from the Siwalik Hills in Dehradun (India). It is commonly used for the molasse-type Neogene sediments of the Himalayan foothill zone. The best record of the fossiliferous layers for the Siwaliks can be found in Pakistan (Potwar Plateau). The Potwar Plateau of the Punjab Province (72°30′ E, 33°00′ N) is an elevated area of some 20,000 km^2 bound by the Kala Chitta and Margala Hills in the north, the Salt Range in the south, Jhelum River in the east and Indus River in the west.

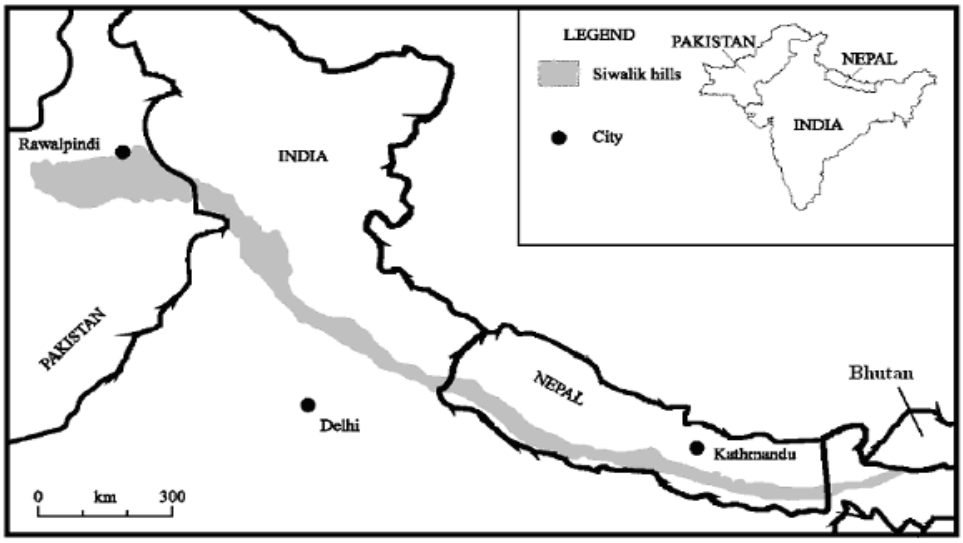

Fig. 1. Distribution of the Siwalik sediments along the foothills of Himalayas.

STUDY AREA

The Hasnot village (Lat. 32° 49′ N: Long. 73° 18′ E) is situated at about 70 km west of the Jhelum city in the Potwar Plateau of the northern Pakistan. The village is surrounded by the extensive Neogene freshwater sedimentary rocks. The region of the Hasnot exposes the most complete sequence of the Siwalik Group and yields a diversified assemblage of the Middle Siwalik Formation. The Hasnot village is situated on the east bank of the river Bunha, surrounded by number of highly fossiliferous localities at an altitude of about 326 meters. The average thickness of the sequence around this area is about 180 m. Sites surrounding the village have an abundance of the vertebrate fossils that represent almost all the major Eutherian groups (Fig. 2).

RESULTS AND DISCUSSION

The Dhok Pathan Formation faunas are mainly very similar to those of the underlying sediments; thus, the suids, the carnivores and the bovids continue virtually unchanged. *Miotragocerus* and other bovids apparently become extinct; Reduncini make their first appearance (known elsewhere from Kenya in the Mpesida Beds and Lukeino Formation of the Baringo Basin dated at 7 and 6.5 Ma respectively), together with a species very similar to *Prostrepsiceros*

houtumschindleri from Maragha in Iran, and a species of *Sus* (Pickford, 1975). The Hasnot, where a substantial fauna is known, is found in a relatively isolated part of the southern Potwar Plateau, 90 km from the Dhok Pathan, surrounded by number of fossiliferous localities.

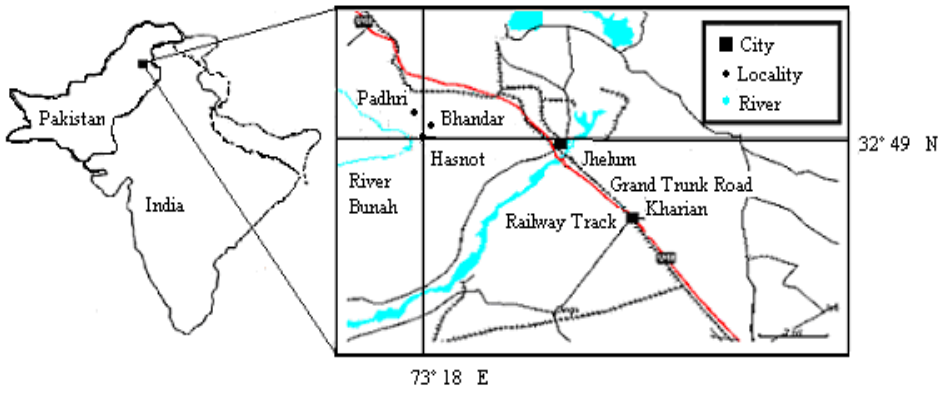

Fig. 2. Location of the study section; the Hasnot and the surrounding localities of the study section.

A close inspection of the works of Pilgrim and in particular of Colbert (1935) shows that, although a number of species are common to both the Dhok Pathan and the Hasnot areas, most of the species of the 'Dhok Pathan fauna' that differ from those from the upper parts of the Nagri Formation (at Nagri and near Khaur), are in fact known mainly or exclusively from the localities around the Hasnot.

The Middle Siwalik deposits at the Hasnot have yielded several species of Reduncini, cervids, several suids including *Sivachoerus, Hippohyus, Sivahyus* and *Sus*, the oldest Siwalik cercopithecoid, *Presbytis sivalensis*, and a rodent fauna clearly distinct from that found in the upper part of the Nagri Formation and its equivalents. The deposits at the Hasnot include a succession of rocks of similar lithology with those at the Dhok Pathan. They have the same age (or younger) as those of the type Dhok Pathan Formation but the deposits towards the Bhandar side, which is the northeast of the Hasnot, are younger than the Dhok Pathan Formation. Rocks of similar age, so far unexplored, are probably present in the area east of Kaulial (Pilbeam *et al.*, 1977).

Pilbeam *et al.* (1977) suggested that the age of the Hasnot was around 7 Ma but the cervids, the bovids, the giraffids, and the suids suggest a late Miocene to early Pliocene age. The suid *Propotamochoerus* sp. (10.2-6.5 Ma), the sivathere *Bramatherium* sp. (10.3-7.1 Ma), *Selenoportax* (7.4-ca 4.0 Ma) and the *antilopini* (8.6-7.4 Ma) suggest an age of 7-5 Ma for the localities H5-H14 (north west) of the Hasnot (Fig. 3). The cervids are very common in the Hasnot and the cervids entered the Siwaliks in the early Pliocene. For *Cervus simplicidense* and *Cervus tripledense*, Pilgrim (1910) mentioned the Horizon as the Middle Siwaliks while Brown (1926) mentioned the Horizon as the Upper Siwaliks, but in the present work, all the collected specimens come from the upper portion of the Middle Siwaliks, close to the Bhandar bed that is based on the northeast of the Hasnot. According to Colbert (1935) the Bhandar bed fauna is more close to the Tatrot (Upper Siwaliks) fauna, supporting the Horizon mentioned by Brown (1926) and showing that the fossil ratio of *C. simplicidense* is more in the upper portion of the Middle Siwaliks than that of the Upper Siwaliks.

An interpretation of the fauna (cervids) at the localities H15-H22 (Hasnot 15- Hasnot 22) suggests that at least part of the fauna is younger than the bulk of that from the Dhok Pathan; an age of 5 Ma can be suggested for the localities situated at the northeastern side of the Hasnot near the Bhandar bed. The localities H1-H4 is located south of the Hasnot. Its age is less certain, but it can be approximately correlated to the upper part of the Andar Kas magnetostratigraphic section of Johnson *et al*. (1982) and is known to be within the *Selenoportax lydekkeri* Interval-Zone (Barry *et al*., 1982). It is most likely between 6.5 and 5.3 Ma old (Barry, 1987). Overall, the Dhok Pathan Formation faunas resemble those from the Turolian Land Mammal 'Age' as defined in Europe, North Africa and West Asia (Fig. 3).

A suggestion of absolute age can be obtained from radiometric dates on the Samos faunas of around 9 Ma (Van and Miller, 1971) and of around 8 to 9 Ma (or perhaps less) on similar Turkish faunas (Sickenberg, 1975). Of interest is the overall similarity between the faunas of the upper Nagri Formation and its equivalents and those from the Dhok Pathan Formation. There probably, the rather restricted time range seems to fall close to the boundary between Vallesian and Turolian Land Mammal 'Age'. The magnetostratigraphy and biostratigraphy (Pilbeam *et al*., 1977; Johnson *et al*., 1982; Barry *et al*., 1982) of the Hasnot indicates that the outcrops in the immediate vicinity of the Hasnot and particularly to the north near Bhandar are younger than the sediments lie some distance to the west and southeast (Colbert, 1935, p. 46, Fig. 22). Specimens

from a wider area than the immediate vicinity of the village could therefore be old as 12 or 13 Ma (Johnson *et al.*, 1982; Barry *et al.*, 1982).

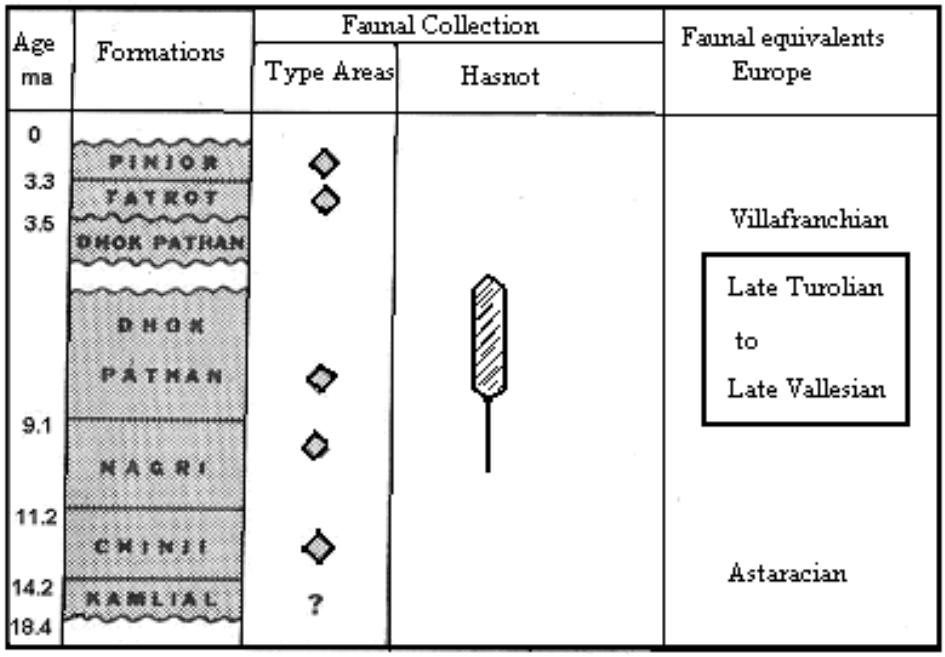

Fig. 3. Modified tentative stratigraphic chart showing the Siwalik formations, main faunal collection and faunal comparison (Source Pilbeam *et al.*, 1977), Boundary dates from Barry *et al.*, 2002.

The age of the deposits at the west, the north and the northwest of the Hasnot is considered as 7-6.3 Ma which is found in close proximity to the Dhok Pathan, whereas the deposits towards the northeastern side is respectively 5 Ma and deposits south of the village Hasnot is most likely between 6.5 and 5.3 Ma old based on their correlation of the Kaulial section to the geomagnetic reversal time scale of Mankinen and Dalrymple (1979). Therefore, the age of the Hasnot localities ranges from 7 to 5 Ma (Late Turolian) (Fig. 3).

The localities of the Hasnot through the Dhok Pathan Formation of the Middle Siwaliks have the potential of producing one of the most complete fossiliferous Neogene records of vertebrate fauna in South Asia. Although,

present faunal collections are merely snapshots of a few time periods, these results suggest that continued palaeontologic survey will not only add to the known 'Siwalik faunas' but will also fill the existing gaps.

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A SURVEY OF APHID FAUNA OF ABBOTTABAD, NWFP, PAKISTAN

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Abstract.- A survey of aphid fauna was conducted in Abbottabad from thirty two-host plants. Fourteen species were identified belonging to subfamilies Aphidinae, Lachninae and Callipterinae. Lachninae and Callipterinae were represented by one species each *viz. Maculolachnus rubi* and *Periphylus californiensis darjeelingsis,* respectively. While twelve species belonging to subfamily Aphidinae *viz. Acyrthosiphon pisum, Aphis nasturtii, Brevicoryne brassicae, Macrosiphum rosae, Macrosiphum sikkimensis, Phorodon cannabis, Me Ian aphis sacchari, Melanaphis bambusae, Myzus persicae, Rhopalosiphum maidis, Rhopalosiphum yoksumi* and *Shizaphis graminum* were collected from plants including trees, crops, ornamental plants, shrubs and weeds. Out of the fourteen species *Maculolachnus rubi, Periphyllus californiensis darjeelingsis* and *Phorodon cannabis* were recorded for the first time in N.WF.P.

Key words: Aphids, Abbottabad, NWFP, Pakistan

INTRODUCTION

Aphids are probably the most important family of crop pests on a world scale (Van-Emden, 1972). They suck the juices mostly from soft parts of the plants and may be monophagus or polyphagus. They are frequently found throughout the world on every type of plant in different climatic conditions. There is scarcely a kind of plant, cultivated or wild, but what supports from one to several species of aphids, and a large percentage of individual plant will be found infested each summer (Metcalf, 1951).

The sucking of plant sap by aphids leads to wilting of the plant due to plant sap deficiency along with phenoloxidase, pectinase and peroxidase activities caused by the salivary juices. In cases of heavy infestations, flowering and fruiting is adversely affected (Cherqui and Tjallingii, 2000). Transmission of virus is another affect of aphid infestation. The circulative mode of transmission can be utilized to identify different species of aphids (van-den-Heuvel *et al.,* 1994).

Lefroy (1909) was the first to report *Macrosiphum granarium* Kirby, 1798, from wheat. Das (1918) gave a consolidated account of the Aphididae of Lahore.

He described forty species belonging to eighteen genera out of which five genera and thirteen species were new. Hasib (1957) surveyed in Chittagong district and found eight species of aphids, infesting various crops. Raychaudhuri (1980) classified 378 aphid species of North India and Bhutan in 150 genera.

The record of Aphididae of N.W.F.P. can be traced up to Ayyar (1939), he considered wooly aphis as a serious pest in N.W.F.P and Punjab. Pruthi and Batra (1940) recorded *Pterochlorus persicae* Cholodkvosky, as a serious pest of fruit trees in Peshawar. Mannan (1955) in "Fruit Pests and Diseases" while recommending control measures, mentioned seven species of aphids. Shahjehan (1991) carried out a preliminary survey of Aphids of Peshawar and presented a checklist of twenty-seven species of aphids.

Ali (1981) identified ten aphid species among other insect pests of flowering ornamental plants of Peshawar region. Mohammad (1982) carried out the survey in different Districts of N.W.F.P, and identified and recorded thirty-two species. Karim (1990) recorded twenty species during his survey for aphids of Dir. Hameed (1977) studied aphids of only graminaceous plants from Abbottabad and the rest of Pakistan, described in their unpublished results.

The present work describes aphid species found in Abbottabad which is at 34°, 08', 42" latitude and 73°, 12', 06" longitude on 4124 feet elevation. It is located North-east of Peshawar with hilly terrain and moderate temperature in summer and very cool in winter.

MATERIALS AND METHODS

The collection of aphids was carried out from June to November 2004 in Abbottabad. Aphids were collected from fields, woodlands and well-kept flowerbeds. The plants included vegetables, fruits, trees, shrubs and weeds. The aphids were collected, preserved and treated by methods adapted from Van-Emden (1972).

A pair of scissors was used to cut the plant parts containing the aphid infestation. The detached parts were placed in polythene bags and stapled. Later, aphids were tapped or brushed off the plant part in a large Petri dish and transferred to 70% alcohol and 3-4 drops of acetic acid in a vial for storage. The aphids were later transferred to 10% KOH for 24 hours in a small beaker, and then subsequently transferred to 70%, 90% and absolute alcohol for thirty minutes each. The cleared aphids were mounted on glass slides using Berlese

medium as mounting material according to Raychaudhuri (1980). The slides were observed under LeitzDialux 20 EB microscope and measurements taken by ocular micrometer.

Aphids were identified using keys of Palmer (1952), Bodenheimer and Swirski (1957) and Raychaudhuri (1980). The identified slides have been deposited in the Entomology section of Department of Zoology, University of Peshawar.

RESULTS AND DISCUSSION

This survey resulted in 14 species of aphids from 32 different host plants (Table I). Most of the fields or other areas from where the aphids were collected contained many varieties of host plants growing together. This may have resulted in collection of an aphid species from a plant that may not be its preferred or main host. However, the fourteen identified species with their respective host plants and location are listed as follows.

1. *Acyrthosiphon pisum* Harris, 1776.
   Synonymy:
   Macrosiphum pisum (Harris, 1776). *Macrosiphum pisi* (Kaltenbach, 1843). *Acyrthosiphon spartii nigricantis* (Borner, 1952).
   Nectarophora destructor (Johnson, 1990)

Material examined : Aptera.

Locality : Malak pura

Host plants : *Rosa indica, Phaseolus vulgaris.*

Acyrthosiphon pisum Harris, was collected from *Rosa indica* and its typical host *Phaseolus vulgaris.* It was reported by Klingauf (1970) and Cook (1955) on beans and alfalfa plants, respectively. Shahjehan (1991) recorded it on *Peganum hermala,* while Ali (1981, unpublished results) recorded it on *Lathyrus odoratus.* This indicates the polyphagous nature of the species. *A. pisum* is the main host of parasitoid *Aphidius smithi,* which can therefore be used in its biological control (Mackauer, 1971).

TABLE I.- LIST OF APHID SPECIES AND THEIR HOST PLANTS RECORDED DURING SURVEY OF ABBOTTABAD.

Sr.No.	Aphid species	Host plant
1.	*Acyrthosiphon pisum* Harris, 1776.	*Rosa indica, Phaseolus vulgaris*
2	*Aphis nasturtii* Kaltenbach, 1843	*Capsicum annum, Pyrus communis, Mentha sylvestris, Zinnia elegans*
3.	*Brevicoryne brassicae* Linnaeus, 1758	*Brassicae oleraceae*
4.	*Macrosiphum sikkimensis* Ghosh and Raychaudhuri, 1968	*Lycopersicum esculentum, Helianthus annus, Solanum tuberosum*
5.	*Macrosiphum rosae* Linnaeus, 1758	*Rosa indica*
6.	*Maculolachnus rubi* Ghosh and Raychaudhuri, 1968	*Prunus bokhariensis*
7.	*Melanaphis bambusae* Fullaway, 1910	*Brusonettia papyrifera,Brassica napus, Eugenia jambolana*
8.	*Melanaphis sacchari* Zehntner, 1897	*Hibiscus esculentus, Mentha sylvestris, Brusonettia papyrifera, Raphanus sativus, Brassica napus, Citrus medica, Cosmos bipinnata*
9.	*Myzus persicae* Sulzer, 1776	*Nicotina tobacum, Eugenia jambolana*
10.	*Periphyllus californiensis* David, 1980	*Populus euphratica*
11.	*Phorodon cannabis* Passereni, 1860	*Cannabis sativa*
12.	*Rhopalosiphum maidis* Fitch, 1856	*Zea mays, Ammi visniga, Lycopersicum esculentum, Cosmos bipinnata*
13.	*Rhopalosiphum yoksumi* Ghosh, Banerjee and Raychaudhuri, 1971	*Solanum nigrum, Rumex* sp., *Ficus palmata, Morus alba, Lycopersicum esculentum, Phaseolus vulgaris, Cosmos bipinnata*
14.	*Shizaphis graminum* Rondani, 1852.	*Impatiens balsamina*

2. *Aphis nasturtii* Kaltenbach, 1843

Material examined : Aptera.

Locality : Malak pura, Dadehal

Host plants : *Capsicum annum, Pyrus communis, Mentha sylvestris, Zinnia elegans*

Aphis nasturtii Kaltenbach, was found on four host plants. This species has been recorded by Nassar (2003, unpublished results) on *Convolvulus* sp. and

Raphanus ratinus from Zhob, Baluchistan. Similarly Karim (1990, unpublished results) found this species on *Calotropis procera.* Its preferred host cannot be deduced however its polyphagous nature can be assumed.

3. *Brevicoryne brassicae* Linnaeus, 1758.
Synonymy:
Aphis brassicae Linnaeus, 1758.
Brevicoryne brassicae Van der Goot, 1915.

Material examined : Aptera, Alate Locality: Dadehal

Host plants : *Brassicae oleraceae.*

Brevicoryne brassicae Linnaeus, easily recognizable because of its triangular cauda and barrel-shaped siphunculi was recorded from *Brassica oleraceae.* This aphid feeds on all cultivated and wild cruciferous plants. Major economic hosts include broccoli, Brussels sprouts, cauliflower, and head cabbage. It attacks carrot, celery, Chinese broccoli, Chinese cabbage, daikon, radish, kale, rape and most other members of the genus Brassica, however, damage is usually less severe than on cabbage (Metcalf and. Flint, 1963).

4. *Macrosiphum sikkimensis* Ghosh and Raychaudhuri, 1968.

Material examined : Aptera and alate.

Locality : Raashan and Nawanshehar

Host plants *: Lycopersicum esculentum, Helianthus annus, Solanum tuberosum*

5. *Macrosiphum rosae* Linnaeus, 1758.
Synonymy:
Aphis rosae Linnaeus,1758.
Siphonophora rosae Buckton, 1876. *Macrosiphum rosae* Gillette and Palmer, 1934.

Material examined : Aptera.

Locality : Kaanchi pura, Mirpur,

Host plants : *Rosa indica.*

Genus *Macrosiphum* was represented by *Macrosiphum rosae* Linnaeus, and *Macrosiphum sikkimensis* Ghosh and Raychaudhuri. *M rosae* was found on its preferred host *Rosa indica* while *M sikkimensis* was reported from three host plants. Shahjehan (1991) recorded it on *Rosa indica* and *Triticum vulgare,* while Nassar (2003, unpublished results) found it on *Pelargonium zonale* and *Morus* species from Zhob, Baluchistan

6. *Maculolachnus rubi* Ghosh and Raychaudhuri, 1968.

Material examined : Aptera

Locality : Malak pura.

Host plants : *Prunus bokhariensis*

7. *Periphyllus californiensis darjeelingsis* David, 1980.

Material examined : Aptera

Locality : Kakul.

Host plants : *Populus euphratica.*

8. *Phorodon cannabis* Passereni, 1860.

Material examined : Aptera

Locality : Kakul.

Host plants : *Cannabis sativa.*

These three species *viz. Maculolachnus rubi* Ghosh and Raychaudhuri, *Periphyllus californiensis darjeelingsis* David and *Phorodon cannabis* Passereni, were recorded for the first time in N.W.F.P., on *Prunus bokhariensis, Populus euphratica* and *Cannabis sativa.* The reason for their record for the first time may be related to the climatic and floral differences between Abbottabad and the areas in which the surveys were conducted before.

9. *Melanaphis bambusae* Fullaway, 1910.

Material examined : Alate.

Locality : Kakul, Rashaan.

Host plants : *Brusonettia papyrifera,Brassica napus, Eugenia jambolana*

10. *Melanaphis sacchari* Zehntner, 1897.

Material examined : Alate.

Locality : Kakul, Rashaan Malakpura, Mirpur.

Host plants : *Hibiscus esculentus, Mentha sylvestris, Brusonettia papyrifera, Raphanus sativus, Brassica napus, Citrus medica, Cosmos bipinnata.*

The genera *Melanaphis* and *Rhopalosiphum* were found to be the most wide spread in this survey. *Melanaphis bambusae* Fullaway was found on three host plants while *M sacchari* Zehntner on seven host plants. Karim (1990, unpublished results) recorded *M bambusae* from *Prunus bokhariensis* only. Nassar (2003, unpublished results) recorded it from *Cucurbita pepo, Daucus carota* and *Ipomoea sp.* while *M sacchari* from *Capsicum annum* and *Datura metel.*

11. *Rhopalosiphum maidis* Fitch, 1856
Synonymy:
Aphis maidis Fitch, 1856.
Rhopalosiphum maidis Webster, 1887.

Material examined : Alate and Aptera.

Locality : Rashaan, Dadehal, Mirpur.

Host plants : *Zea mays, Ammi visniga, Lycopersicum esculentum, Cosmos bipinnata.*

12. *Rhopalosiphum yoksumi* Ghosh, Banerjee and Raychaudhuri, 1971.

Material examined : Alate and Aptera.

Locality : Rashaan, Malak Pura, Mirpur.

Host plants : *Solanum nigrum, Rumex* sp., *Ficus palmata, Morus alba, Lycopersicum esculentum, Phaseolus vulgaris, Cosmos bipinnata.*

Genus *Rhopalosiphum* in this survey consisted *of* two species *viz. R. maidis and R.. yoksumi. R. maidis* Fitch, reported from *Zea mays, Ammi visniga, Cosmos bipinnata,* and *Lycopersicum esculentum.* Previously it was recorded on *Zea mays,* by Bodenheimer and Swirski (1957), Raychaudhuri (1980), and Shahjehan (1991). This makes *Zea mays* a favorite *of R. maidis.* Whereas *R.. yoksumi* Ghosh, Banerjee and Raychaudhri, was found on eight host plants (Table I) among which *Cucurbeta pepo* was also recorded by Shahjehan (1991).

13. *Myzus persicae* Sulzer, 1776
Synonymy:
Aphis persicae Sulzer, 1776.
Aphis dianthi Schrank, 180 1
Myzus maivae Oestlund, 1886.

Material examined : Aptera, Alate.

Locality : Dadehal, Raashan.

Host plants : *Nicotina tobacum, Eugeniajambolana*

Genus Myzus was represented by *Myzus persicae Sulzer,* found on *Nicotiana tabbacum* and *Eugeniajambolana.* While Ali (1981, unpublished results) recorded it on *Antirhinum majus* and *Tagetes patula* flowering ornamental plants. *M. persicae* is considered a serious pest because of its role in transmission of viral particles.

14. *Shizaphis graminum* Rondani, 1852.

Material examined : Aptera.

Locality : Shimla Pahari.

Host plants : *Impatiens balsamina.*

Shizaphis graminum Rondani, was found on an ornamental plant *Impatiens balsamina.* It was recorded previously by Nassar (2003, unpublished results) on *Morus sp.* while in this survey *Morus alba* hosted *Rhopalosiphum yoksumi.*

In conclusion, despite the different climatic and geographical conditions Abbottabad has a rich aphid fauna, most of the fauna was found to be common with that found in corresponding surveys, however species such as *Maculolachnus rubi*, *Periphyllus californiensis darjeelingsis* and *Phorodon cannabis* were reported in Abbottabad for the first time.

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A COMPARATIVE STUDY ON THE GROWTH PERFORMANCE OF MAJOR CARPS AND CHINESE CARPS

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Abstract.- The problem of quality protein deficiency in Pakistan can be over come by intensive fish farming because fish is considered to be the most promising source of animal protein. The chinese carps (*Hypophthalmichthys molitrix, Ctenopharyngodon idella* and *Cyprinus carpio*) and major carps (*Catla catla, Labeo rohita* and C*irrhinus mrigala*) were offered artificial feed at the rate of 2% of fish body weight on daily basis. Statistically significant growth difference were observed between major carps and chinese carps. Chinese carps gave three times more production than major carps on body weight basis. So, chinese carps may be bred in our country to fulfill the protein deficiency.

Key words: Fish production, *Hypophthalmichthys molitrix, Ctenopharyngodon idella, Cyprinus carpio, Catla catla, Labeo rohita,* C*irrhinus mrigala.*

INTRODUCTION

Fish is the most promising, in expensive, alternate source of animal protein. It offers relatively high amount of essential amino acids, minerals and fatty acids as compared with livestock (Ling, 1977; Huisman, 1989). The problem of quality protein deficiency in the country can be overcome by intensive fish farming. Fish culture system requires a relatively less amount of energy for protein production than any other farming system (Werner, 1991), although stocking density exerts significant effect on its growth (Passmore and Eastwood, 1987; Varghese *et al.*, 1990; Chakrabarty and Jana, 1991; Kumar, 1992; Trzebiatowski and Filipiak, 1992; Lorenzen, 1995). Besides stocking density, artificial feed and fertilizers are of considerable importance in intensive fish culture practices. In exotic species there is an inherent vigor and potential to grow fast, while in our indigenous species the condition is different. Due to the continuous inbreeding, the genetic make up of local species has been effected. The present work is undertaken to compare the growth performance of major carps and Chinese carps in polyculture.

MATERIALS AND METHODS

The present experiment was conducted to determine the comparison of growth performance of major carps and Chinese carps, in two earthen ponds, situated at Fisheries Research Farms, University of Agriculture, Faisalabad, each pond having dimensions of 33 m x 16 m x 1.5 m. After preliminary preparations, these ponds were stocked with Chinese and major carps at the rate of 750/acre for period of 12 months. The water level was maintained upto 1.5 m through out the experimental period.

The growth parameters viz., increase in body weight, standard length, fork length and total length of fish were monitored on fortnightly basis. After obtaining the data, fish was released back into their respective ponds. Water samples were also taken from these ponds after fifteen days for the physicochemical characteristics *viz*. water temperature, light penetration, pH, dissolved oxygen, total alkalinity, carbonates, bicarbonates, total hardness, calcium, magnesium, total solids, total dissolved solids and planktonic biomass following APHA (1996).

In pond 1 (P_1) 38 *Hypophthalmichthys molitrix*, 27 *Ctenopharyngodon idella* and 27 *Cyprinus carpio* having average weight of 81.4 g, 193.4 g, 87.8 g and in pond 2 (P_2), 38 *Catla catla*, 27 *Labeo rohita* and 27 *Cirrhinus mrigala* having average weight of 116.2g, 39.0 g and 55.8 g, respectively were stocked.

Artificial feed (Maize gluten) was added at the rate of 2% of fish body weight daily and quantity of feed was calculated on the basis of fresh body weight of fish/day. All the ponds were sampled fortnightly to monitor the growth performance and after recording the data the fishes were released back in their respective ponds. Statistical analysis was applied following Steel *et al.* (1996).

RESULTS AND DISCUSSION

The initial average body weight of *H. molitrix*, *C. idella* and *C. carpio* were observed to be 81.4 g, 193.4 g and 87.8 g, respectively. The final average body weights were 860 g, 936 g, 700 g. The initial average body weights of *C. catla*, *L. rohita* and *C. mrigala* on the other hand, were 116.2 gm, 39 gm and 55.8 gm and their final average body weights were observed as 350 gm, 376.2 gm and 280 gm, respectively (Fig. 1).

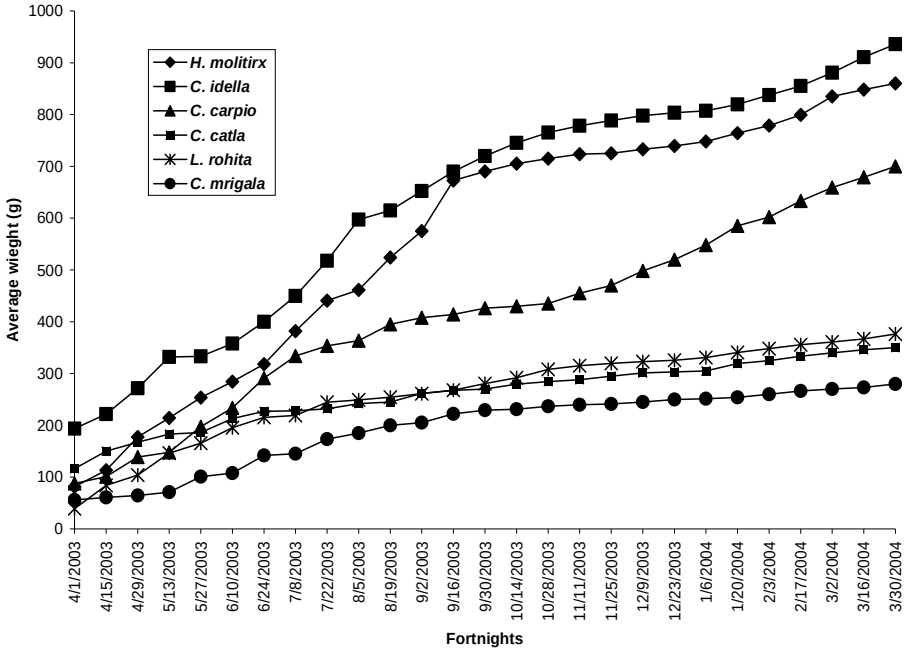

Fig. 1. Fortnightly growth performance of six carp species for one year.

H. molitrix gained maximum body weight of 97.6 g during the month of September, *C. idella* gained maximum body weight of 79.4 g during the month of August and *C. carpio* gained the maximum weight of 57.4 g during the month of June. On the other hand, the maximum increase in body weight of *C. catla* was observed as 33.8 g during the month of April, that of *L. rohita* was 45.2 g during the month of April, and in *C. mrigala* it was 30.2 g during the month of June.

Similarly the minimum weight gain by *H. molitrix* was observed to be 1.5 g during the month of November, that of *C. idella* was 1.4 g during the month of June and by *C. carpio* it was 3.8 g, during October. The minimum weights gained by *C. catla*, *L. rohita* and *C. mrigala* were 1.20 g, 2.7 g and 1.5 g during July, December and November, respectively.

Analysis of variance showed that fortnightly changes of weight were highly significant, while the interaction between fortnight and species were also significant. Analysis of variance for standard, fork length and total length showed that fortnight and species wise differences were also highly significant (Table I).

TABLE I.- ANALYSIS OF VARIANCE FOR WEIGHT, STANDARD LENGTH, FORK LENGTH AND TOTAL LENGTH.

Source	DF	F. value (average weight)	F. value (average Standard length)	F. value (average fork length)	F. value (average total length)
Fortnights	26	17.8219**	49.2767**	39.1286**	54.5801**
Carps	5	119.6981**	223.4321**	175.4427**	182.8303**
Errors	130				
Total	161				

** = Highly significant (P<0.01)

The minimum production was found for major carps while the maximum production and maximum weight gain was found with Chinese carps. Net production/hectare/year was calculated as 1253.516 kg and 455.477 kg for major carps and chinese carps, respectively. Similar results were reported by Hassan *et al*. (1997) making a comparison of monoculture of Nile tilapia (*Oreochromis niloticus*) and polyculture of carps (*H. molitrix*, *L. rohita* and *C. mrigala*) in 200 m^2 two earthen ponds fertilized with inorganic manure at 3 kg nitrogen and 15 kg phosphorus/ha/day. A control treatment of tilapia monoculture without fertilizer was included to assess the effect of pond basal fertility. Net yield of 3.88 t/ha/year was obtained in tilapia monoculture ponds and it was 31 t/ha/year in carp polyculture. In the carp polyculture, silver carp was dominant species, contributing 73% of the total net fish production compare to 9% and 19% by *L. rohita* and *C. mrigala*, respectively.

Maximum weight gain in major carps was noted during September which was due to appropriate water temperature, while the lowest weight gain was observed during November. The maximum weight gain in chinese carps was observed during April and minimum weight gain during December. The similar results were reported by Villaluz and Unggui (1983).

In major carps the gain in body weight was recorded as 860 g, 936 g and 700 g for *H. molitrix*, *C. idella* and *C. carpio*, respectively. Whereas the gain in body weight were 350 g, 376.2 g and 280 g for *C. catla, L. rohita* and *C. mrigala*, respectively.

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EFFECT OF URBANIZATION ON LAHORE CANAL WATER POLLUTION

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Abstract.- The water samples from Lahore canal were processed for the measurements of some physicochemical parameters and bacterial coliform contents. It was found that the canal water enters the city Lahore with relatively healthier conditions as assessed by the lower total organic suspended matter (TOSM), pH and the absence of total minerals (TM). These parameters showed gradual increase along the passage of the canal water through the city, suggesting a negative urbanization impact on the canal water. pH, biological oxygen demand (BODs), total suspended matter{TSM),TM, TOSM and coliform contents ranged upto 9, 2.3 mg/l, 9.7 g/l, 0.5 g/l, 9.4 g/l and 5.8 x 10^7 C.F.D/ml, respectively for different samples. Reduction in some of the parameters downstream is an indicative of the potential of the running water to recover in terms of its contamination levels regarding particulate matter as wel1 as coliform bacterial profile. This survey concludes that the canal receives heavy urban pol1utant loads of diverse nature. Besides the fact that the canal water has a potential to decompose/respire the urban inputs to a greater extent, the water appeared unsuitable for human consumption and for other recreational purposes.

Key words: Canal water, pol1ution, BODs, suspended matter in canal water.

INTRODUCTION

Growing populations, increased economic activity and industrialization have resulted in increase demand for fresh water (Verma and Agarwal, 1994). No source of water however that is intended for human consumption can be assumed to be free from pollution. All sources have different microbial qualities and may be subject to normal or man made sources of pollution which may result in the deterioration of water quality to a point where treatment is no longer effective in removing the contamination (HMSO, 1994; Momba and Kaleni, 2002). Discharging untreated sewage and chemical wastes directly into water bodies *i.e.*, rivers, lakes, streams and drains have become a traditional habit in developing countries. The pollutants may includes pesticides, domestic sewage, infectious agents, detergents, radioactive substances and various petroleum based products etc (Purdom *et al.*, 1994).

An 82 Km long irrigation canal with an average discharge of 350 cusecs/sec passing through provincial metropolis Lahore has for all practical purposes been converted into a drain or a dumping pit as it is regularly receiving discharge of industrial waste of 16 factories, sewage of 26 housing societies, municipal solid waste, edible residues and dead animals (Gilani, 2005). This canal water is source of various skin diseases, contamination of crops and hazardous to indigenous birds and livestock (Ahmad, 2005).

The present survey was conducted to see the gradient of urbanization impacts on quality of the canal water. Levels of some physicochemical parameters and coliform content of the canal water as it passes through the city are being reported for the first time.

MATERIALS AND METHODS

Study locations

The present study was conducted on the Lahore canal that passes through different localities of Lahore. The canal water flows from the Bumbain wala Ravi Badian Depalpur Link (B.R.B.D. link) near Jallo village and ends at Bhaipheru in Lumbe Jagir. The area of study started from B.R.B.D. link to 15 km down stream *i.e.* Towards Thokhar Niaz Baig. Speed of the canal water was measured by noting the time taken by thrown floats, while covering a distance of 0.8 km. The water had a speed of 2.16Km/hr. Thus 2:31hrs were required by the canal water to travel a distance of 5 Km.

Sampling and analysis of canal water

Water samples collected in plastic containers *i.e.* from B.R.B.D. Link(I), Dayal House (II), Fatah Garah (III) and Dharmpura (IV) at 11:30 AM, 1:50 PM, 4:10 PM and 6:30 PM. were immediately processed for measurements of temperature, pH, conductivity and dissolved Oxygen (D.O.) by using a portable water quality measuring meter (OSK 14806, water quality checker). A portion of the water sample was taken in two sterilized glass amber bottles of 500 ml capacity, which were later processed for BOD measurements, and for determination of different physicochemical parameters and bacteriological parameters *viz.* total suspended matter (TSM), total minerals (TM) and total organic suspended matters (TOSM) by the standard methods (Csuros, 1997; Hussain, 1998). The water samples were also processed for enumeration of

coliform bacterial contents employing the standard dilutions and spreading technique.

RESULTS AND DISCUSSION

Table I shows the physiochemical characteristics of canal water at different sampling stations. During sampling period of 7 hours, there was a gradual increase in canal water temperature from 16°C (11:30 AM) to 17.5°C (4:10 PM), which slightly decreased to 16.9°C (6.30 P.M). The water was found alkaline (pH 8), which increased to pH 9 probably because of inflow of household detergents and municipal effluent entering the canal (Ahmad, 2005). In this context, coliform contents of the present samples yielded a pictorial pattern. The sample I through IV were found to yield $5.8x10^7$, $4.82x10^7$, $2.89x10^7$ and $0.lx10^7$ C.F.U./ml.

TABLE I.- SOME PHYSICOCHEMICAL ATTRIBUTES OF THE LAHORE CANAL WATER, PASSING THROUGH DIFFERENT SAMPLING SITES .

Sample No	Time of sampling	pH	Temp °C	Conductivity μΩ	D.O mg/l (BOD_5)	TSM g/l (BOD_5)	TM g/l (BOD_5)	TOSM g/l (BOD_5)
I	11:30A.M	8	16	22.5	6.1 (N.D)	0.4 (0.2)	0.0 (0.1)	0.4 (0.1)
II	1:50 P.M	9	17	20	6.6 (0.7)	0.6 (0.4)	0.5 (0.1)	0.1 (0.3)
III	4:10 P.M	8.9	17.5	25	7.1 (2.3)	9.7 (0.4)	0.3 (0.0)	9.4 (0.4)
IV	6:30 P.M	9	16.9	25	6.7 (1.0)	0.8 (0.4)	0.3 (0.0)	0.5 (0.4)

Abbreviations used: D.O, Dissolved Oxygen; TSM, Total Suspended Matter; TM, Total Minerals; TOSM, Total Organic Suspended Matter; N.D, Non detectable.

Values within the parentheses indicate the corresponding values found for the water samples after five days of respiration (BOD_5)

The conductivity of water at station I was recorded as 22.5 μ Ω, which decreased to 20μ Ω at station 2 followed by considerable increase (25 μ Ω) for the other two stations (Table I). Water conductivity is affected by presence of inorganic dissolve solids anions Cr^-, NO^-_3, SO^{-2}_4, PO^{-2}_4 and cations Na^+, Mg^{+2}, Ca^{+2}, Fe^{+2} and AI^{+3} and organic compounds including oils, which are non-

conductor of electric current. Therefore, this increase in conductivity may be because of contaminants including chemicals used in washing of plastic bags at Lal Pul in Mughalpura and at Dharampura bridge (Ahmad, 2005).

Dissolved oxygen contents of the canal water increased at each successive sampling station. More oxygen can be dissolved in running water and is introduced through photosynthetic plants and atmosphere as compare to still water (Dutka, 1973). So decrease in oxygen contents of last sample might be related to photosynthesis inhibition after sunset. BOD_5 of the samples water showed a progressive increase from non-detectable level to a value of 2.3 obtained for the third sample. So 2.3 fold decrease was observed in last sample as compared with third sample showed highest value. The pattern of BOD_5 values in the present study appeared well correlated with coliform contents, which in turn indicate the bacterial load capable to respire the organic matter.

Total Suspended Matter (TSM), were measured as to be 0.4, 0.6 and 9.7g/l for 1^{st}, 2^{nd} and 3^{rd} localities, respectively while in the case of last station a drastic decrease in TSM was observed (Table I). Total Minerals (TM) trend ranged from non-detectable value at 1^{st} station to 0.5 g/l and 0.3 g/l for 2^{nd}, 3^{rd} and last sampling station. Highest total organic suspended Matter (TOSM) was found to be 9.4g/l, the highest among the other sampling station, which were noticed as 0.4g/l, 0.1g/l and 0.5g/l for 1^{st}, 2^{nd} and 4^{th} locations, respectively (Table I).

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MUTATIONS IN THE HEPATITIS B VIRUS CORE GENE AND ITS EFFICACY AS A VACCINE: A REVIEW

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Contents

Abstract.- Human HBV virus is the prototype for a family of viruses, referred to as Hepadnaviridae. Currently the hepatitis B virus (HBV) is categorized into eight genotypes (A to H). HBV has a partially double-stranded DNA. The circular genome of approximately 3200 bp comprises four open reading frames encoding the polymerase, surface antigen, nucleocapsid, and X proteins. The hepatitis B virus core gene is divided into the precore region of 29 amino acid codons and the core region of 181 codons and has two in-frame initiating ATG codons. The nucleocapsid gene possesses two in-frame initiation ATG codons, but it is accountable for at least four different polypeptides *viz.*, p25, p22, p21 and p17. The hepatitis B virus core antigen (HBcAg) is a highly immunogenic subviral particle with at least four known epitopes. HBcAg is a virus-like particle easy to work with because of its high-level expression and efficient particle formation in many expression systems, including bacteria. HBcAg-specific $CD4^+$ T-cell responses play an important role in the control of human HBV infection.

Keywords: Hepatitis B virus, HBV core gene, HBcAg, mutations in core gene, HbeAg.

INTRODUCTION

Hepatitis B is a worldwide disease with an estimated 500 million carriers. The frequent occurrence of occult infection in close contacts of carriers,

parenteral vertical transmission and high incidence of insidious chronic liver disease or cancer of the liver among the carriers signify a major public health problem. Hepatitis B virus causes transient and chronic infections of the liver, is a member of the family Hepadnaviridae. Transient infections with Hepatitis B virus may produce serious illness with approximately 0.5% of infections resulting in fulminant hepatitis and chronic infections also may have serious consequences: nearly 25% result in untreatable liver cancer (Baumert *et al*., 1998; Wai and Fontana, 2004; Marcellin *et al*., 2005). It causes 62% of all reported liver cancers. It is the most common cause of death worldwide leading to a million deaths annually (Francis, 1999). Hepatitis B Virus (HBV) causes acute hepatitis of varying severity (Heermann *et al*., 1999). The virus persists in 2-10% of adult patients (Bowyer and Sim, 2000) leading to liver cirrhosis and hepatocellular carcinoma (Aki *et al*., 1999; Locarnini *et al*., 1998). There are many reasons for chronic HBV infections but one is that the virus causes chronic, noncytocidal infections of hepatocytes, which are the principal cell type of the liver. After infection hepatocytes with HBV continuously shed virus into the bloodstream, ensuring that 100 % of the hepatocyte population is infected (Seeger and Mason, 2000). HBV has a partially double-stranded DNA. The circular genome of approximately 3200 bp comprising 4 overlapping open reading frames: the *S* gene, which codes for the surface proteins (HBsAg); the *X* gene, which codes for the regulatory gene; the *P* gene, which codes for DNA polymerase; and the *C* (core) gene. The latter is divided into the precore region of 29 amino acid codons and the core region of 181 codons by 2 in-frame initiating ATG codons. This results in the transcription of the pregenomic RNA that is essential for HBV replication, and of the nucleocapsid protein or the precore RNA that translates into HBeAg protein that is released into the bloodstream of infected patients (Tacke *et al*., 2004) (Fig.1). Particularly, these reading frames overlap substantially, with half the viral genome encoding more than one protein product approximately (Uysal *et al*., 2001; Locarnini, 2004; Onganer, 2004).

The hepatitis B virus (HBV) is an enveloped DNA virus with an icosahedral capsid replicating via reverse transcription. The crystal structure of the capsid is known. It has a diameter of 36 nm and is formed by one protein species (C protein). The viral envelope contains three different coterminal proteins (S, M, and L proteins) spanning the membrane several times. These proteins are not only released from infected cells as components of the viral envelope but in 10,000-fold excess as subviral lipoprotein particles with a diameter of 22 nm containing no capsid. Assembly of the capsid occurs in the cytosol and results in packaging of a 3.5 kb RNA molecule together with viral

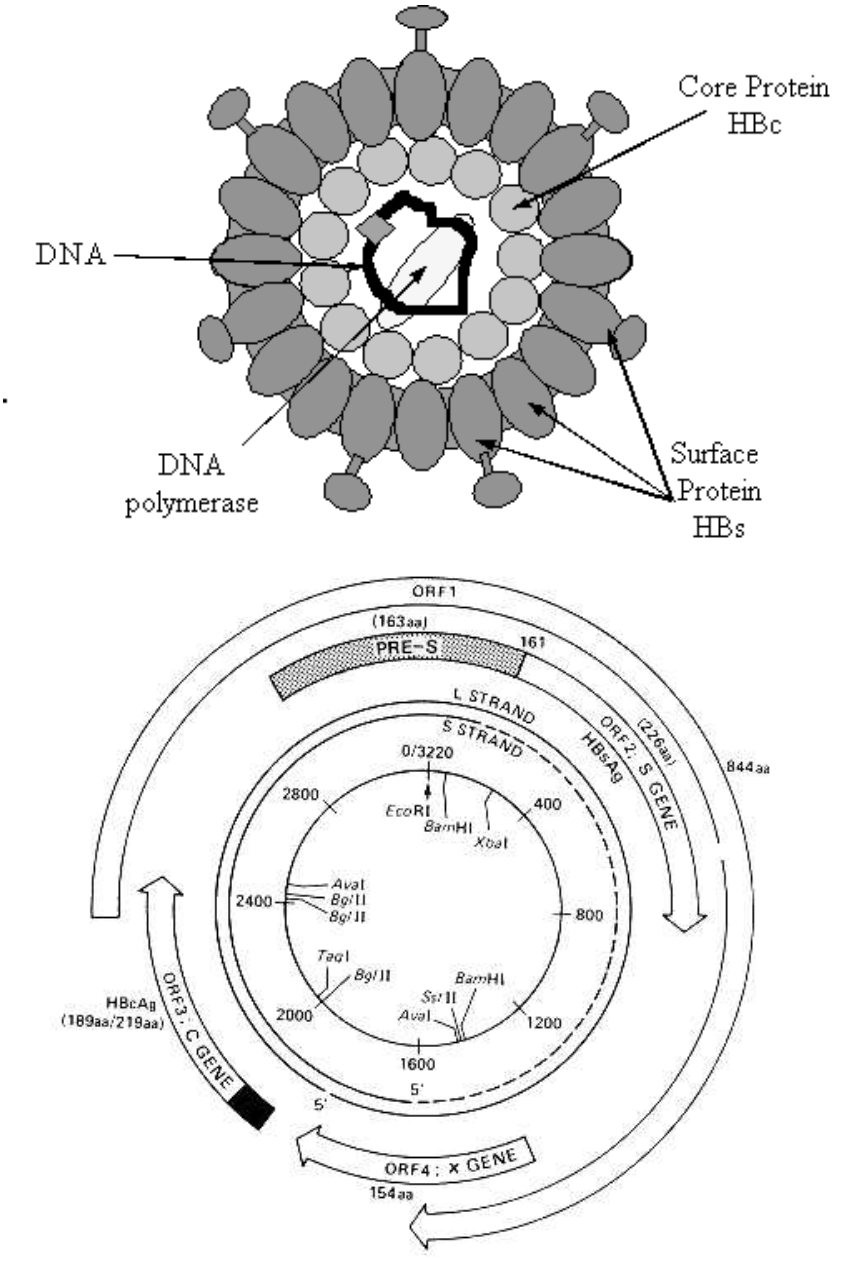

Fig. 1. Structure and genomic organization of hepatitis B virus.

and cellular factors. This newly formed capsid cannot be enveloped. Rather, synthesis of the viral DNA genome in the lumen of the capsid by reverse transcription is required to induce a budding competent state. Envelopment then takes place at an intracellular membrane of the pre-Golgi compartment. The S and the L protein, but not the M protein, is required for this process. The L protein forms two different transmembrane topologies. The isoform exposing the N-terminal part at the cytosolic side of the membrane is essential for budding. In this domain, a 22 amino acid (aa) long linear stretch has been mapped genetically to play a vital role in the morphogenetic process. This domain probably mediates the contact to the capsid. A second matrix domain was mapped to the cytosolic loop of the S protein. A similar genetic approach identified two small areas on the capsid surface, which might interact with the envelope proteins during envelopment (Volker, 2004).

The clinical course of infection with HBV varies, depending on the one hand on the patient's age and immune response and, on the other hand, as increasing evidence is showing, on the virus strain infecting the individual. Overall, less than 1% of acute infections lead to fulminant hepatitis and death. Approximately 0–10% of infected adults become chronic carriers of HBV (Sherlock, 1985; Bläckberg *et al.*, 2000). Perinatal transmission leads upto 90% of chronic carriership. Chronic carriers often lack symptoms but may have histological evidence of hepatocellular damage from mild inflammation to cirrhosis and HCC.

Immunochemical investigations of the viral antigens and molecular characterization of the viral DNA have elucidated the nature of the HBV infection underlying acute, chronic, and oncogenic disorders of the liver in man (Tiollais *et al.*, 1981). Several gene fusions have been constructed in which coding sequences for antigenic regions of the pre-S sequences of HBV, hepatitis B surface antigen, and the envelope protein of human immunodeficiency virus were linked to the 3' end of first 144 residues of hepatitis B core antigen. The sequences were expressed efficiently in *Escherichia coli* to give stable products that assembled to form particles morphologically similar to hepatitis B core antigen itself. The products exhibited the antigenic and immunogenic characteristics of both the hepatitis B core antigen epitopes and the epitopes carried by the additional sequences, thus illustrating the value of such proteins as immunological reagents and potential vaccines (Stahl and Murray, 1989).

HBV genotypes

Currently hepatitis B virus is categorized into eight genotypes A to H. The HBV genotype system was first introduced by Okamoto *et al.* (1988), with four genotypic groups (A to D) distinguished by intra-group difference of <5.6% and inter group differences of >8.0% between the genomes of HBV. Subsequently the genotypes were extended to include genotypes E, F, G and H (Arauz *et al.*, 2002; Norder *et al.*, 1993; Stuyver *et al.*, 2000). Distribution of different genotypes is given in Table I.

TABLE I.- DISTRIBUTION OF DIFFERENT GENOTYPES OF HEPATITIS B VIRUS.

Group	**Area / Regions**
A *(adw, adw2*)	Origin - N. Europe - Sub-Saharan Africa
B (*adw*)	Confined to - Eastern Asia (China)
C (*adr, adr4, adrq, ayr*)	Far East (Japan)
D (*awy, ayw2, ayw3, ayw4*)	Mediterranean - Near, Mid East, South Asia
E (*ayw4*)	W. Sub-Saharan Africa, south to Angola
F (*adw4, adw4q*)	New World - Brazil, C. & S. America
G (*adw4, adw2*)	New World - Brazil, N. & S. America, France
H (*adw4*)	Central America

Moreover, subgroups of HBV genotype have also been reported in genotypes A (A' and A-A') (Bowyer *et al*., 1997; Kramvis *et al*., 2002) and B (Ba and Bj) (Sugauchi *et al*., 2002). According to genotype HBV virulence and pathogenicity differ in each location (Lok *et al.*, 2000; Kidd-Ljunggren *et al.*, 2002**).** Genotyping helps to clarify the routes of infection and virulence of the virus**.** Examination of sequence diversity among different isolates of the virus is particularly important since variants may differ in their patterns of serological reactivity, replication of the virus, activity of the liver disease, prognosis and response to treatment. Patient infected with genotype C have a more aggressive phenotype than those with genotype B (Orito *et al.*, 2001; Kao *et al.*, 2000a, b). However, isolates of the same genotype can cause different clinical manifestations *e.g*. between subgroups Ba and Bj (Sugauchi *et al.*, 2003).

HBV core gene

The HBV core gene is divided into the precore region of 29 amino acid codons and the core region of 181 codons by two in-frame initiating ATG codons. The heterogeneity at the 5' end of the core gene transcript causes

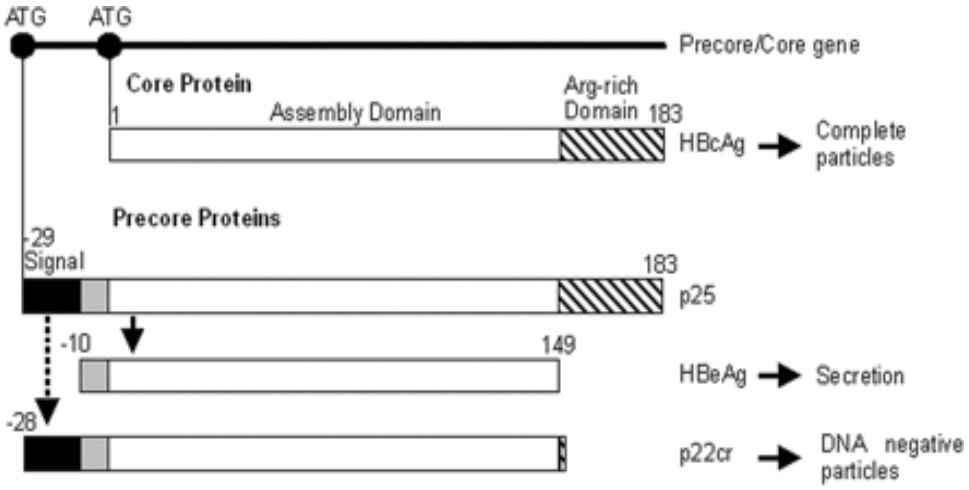

Fig. 2. Schematics of precore/core genes and their products.

initiation of translation from either the precore or core ATG codon to display two related proteins. The major core gene transcript (pregenomic RNA) has the 5' end downstream of the precore ATG codon and thus can express core (nucleocapsid) protein only, while a subset of transcript (precore mRNA) has its 5' end located upstream of the precore region to display a longer protein form, the precursor to hepatitis B e antigen (HBeAg). Efficient translational initiation from precore ATG codon prevents core protein expression from this subset of mRNA species. The 1896 precore (PC) mutation is the most frequent cause of HBeAg-negative chronic HBV infection. Detection of the 1896 PC mutation has application in studies monitoring antiviral therapy and the natural history of the disease (Therese *et al.*, 2005). Maturation of HBeAg requires two proteolytic cleavage issues en route the secretory pathway. The N-terminal 19 residues of this 210-amino-acid (*i.e.*, 29 plus 181 amino acids) protein target the nascent protein to the endoplasmic reticulum, where it is cleaved off. The C-terminal arginine rich sequence of 34 residues is removed subsequently by a furin-like protease during passage through the Golgi apparatus. So, the mature HBeAg protein differs from core protein by 10 extra residues at the N terminus and lacks C-terminal DNA-binding sequence. Formation of intramolecular disulfide bond between two cysteine residues (precore residue 26 and core residue 61) creates the unique secondary structure of HBeAg distinct from core protein (Parekh *et al.*, 2003; Nassal *et al.*, 1993; Wasenauer *et al.*, 1993). Although gene C possesses two in-frame initiation ATG codons, it is accountable for at least four different polypeptides, p25, p22, p21 and p17 (Scaglioni *et al.*, 1997) (Fig. 3).

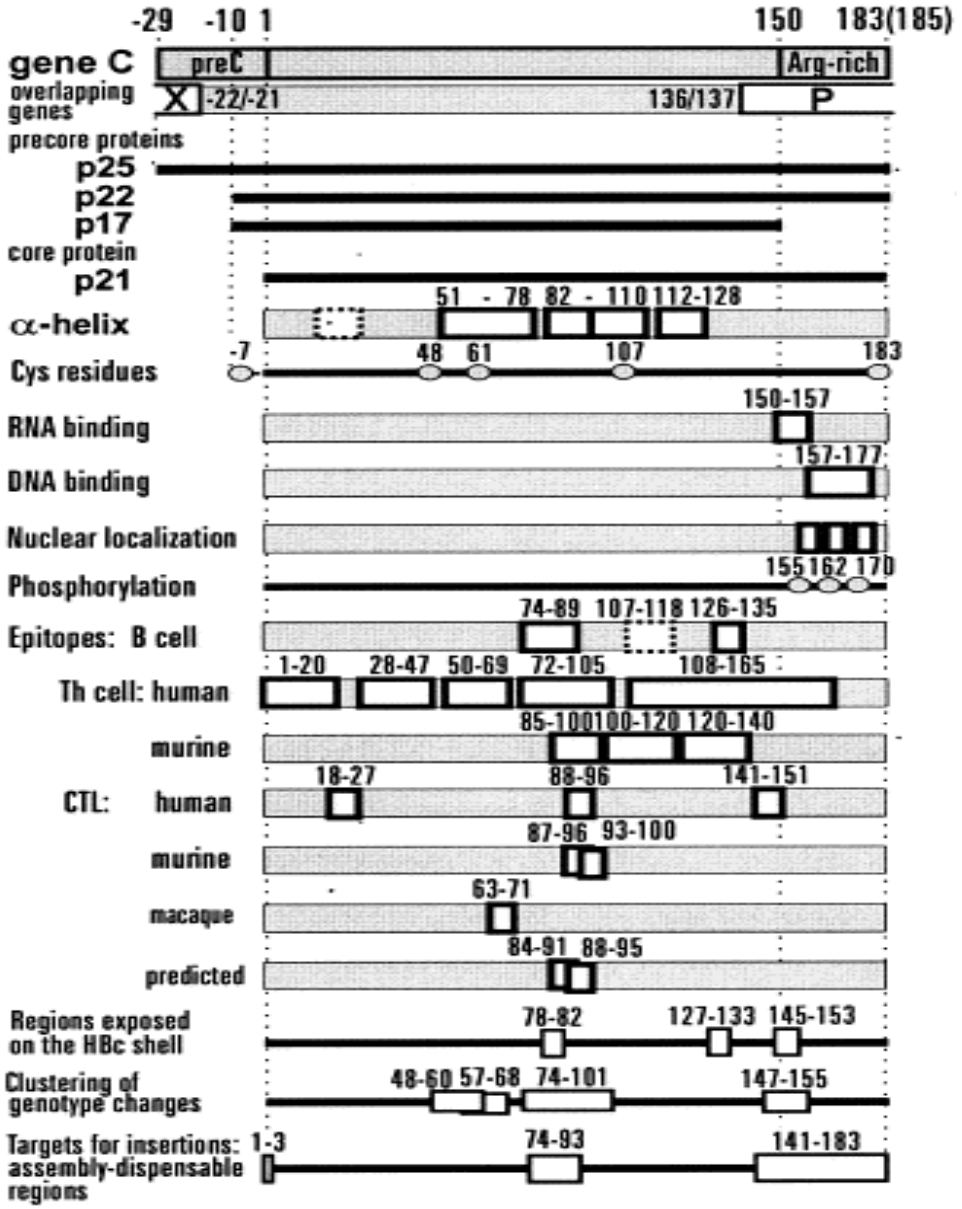

Fig. 3. Portrait of HBV gene C. Structural and functional peculiarities of the Hepatitis B core molecule.

At its 5' end, the precore/core gene contains two closely spaced ATGs (*black dots*) enclosing the precore region, which encodes a 29-aa precore sequence. Translation of the core mRNA results in the production of the cytoplasmic core protein, which assembles into icosahedral capsids enclosing the RNA pregenome, and then the nucleocapsids are enveloped and released as complete particles. Precore protein p25 is directed to the ER by a 19-aa-long signal sequence (*black boxes*) located at its N terminus. This signal sequence is removed during translocation into the ER, and then the C-terminal 34-aa-long arginine-rich domain (*hatched boxes*) is eliminated. Mature HBeAg is then secreted. P22cr is a novel precore protein identified with HBV DNA-negative particles (Kimura *et al*., 2005).

Gene C represents the only non-overlapping sequence in the HBV genome (Fig. 3), although it remains the mostly evolutionarily conserved. The HBV core sequence demonstrates high similarity (65–67% at the amino acid level) to cores of rodent: woodchuck (WHV), ground squirrel (GSHV), and arctic ground squirrel (ASHV) hepadnaviruses, but only traces of homology with cores of bird: duck (DHBV), and heron (HHBV) hepadnaviruses (Norder *et al*., 1993). Recently, the woolly monkey hepatitis B virus (WMHBV), a probable progenitor of the human viruses, was found (Lenhoff *et al*., 1998). The WMHBV core is practically identical to HBV cores (85–87% similarity at the amino acid level). HBV, WHV, and GSHV, but not DHBV core proteins, are able to cross-oligomerize into mixed particles (Chang *et al*., 1994).

The structure of core

Hepatitis B virus core antigen (HBcAg), encodes the viral capsid protein. Assembly of cores into the icosahedral subviral core particles needs the creation of dimers of the core subunit (Chang *et al*., 1994), stabilized through two disulfide bonds, followed by the assembly of 120 dimers into a shell with a diameter of 36 nm and a triangulation number (*T*) of 4 (Crowther *et al*., 1994). Expression of cores with small truncations at their C termini can induce the formation of smaller shells consisting of 90 dimer subunits and a triangulation number of 3 (Zlotnick *et al*., 1996). The structure of the cores was resolved first at 7.4 Å resolution by cryoelectron microscopy (Bottcher *et al*., 1997; Conway *et al*., 1997) and then at 3.3 Å resolution of X-ray crystallography (Wynne *et al*., 1999). The folding of the protein is characterized by four α-helices and the absence of β-sheets. By biochemical analyses, the structural data revealed two regions required for the dimerization of core monomers and for the subsequent assembly of the dimers into core particles (Konig *et al*., 1998; Zlotnick *et al*.,

1998). Two separate regions of the core polypeptide which provide targets for interaction with envelope components, are exposed on the surface of core particles. One of the two sites forms spikes that extend from the interface of the dimerization sites; the other is located just downstream of the α-helix believed to mediate the multimerization of dimers (Conway *et al.*, 1998).

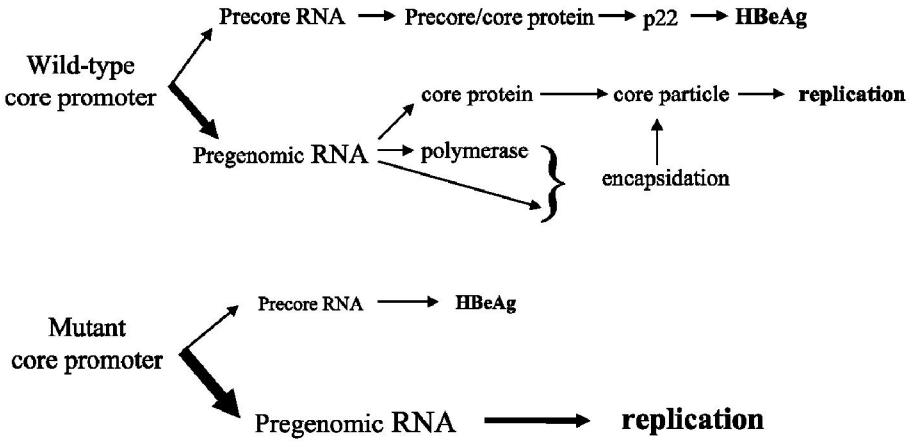

Fig. 4. Regulation of HBeAg expression and genome replication by core promoter mutations. Upper panel: the core promoter directs the transcription of precore RNA and pregenomic RNA. The precore RNA is the messenger for HBeAg. The pregenomic RNA serves as the messenger for both core protein and polymerase. Via the encapsidation signal it can be encapsidated into newly formed core particles together with the polymerase, where it is converted to viral DNA. Therefore, pregenomic RNA is the sole component required for HBV genome replication. Lower panel: Naturally occurring mutations in the core promoter reduce the transcription of precore RNA, thereby diminishing HBeAg expression. The mutations also up-regulate the transcription of pregenomic RNA, thus enhancing genome replication (Tong, *et al.*, 2005).

Out of the four core Ag epitopes, one is on the region around the symmetry axes and three are located on spikes. All three of the latter epitopes involve the immunodominant loop: for the linear epitope 312, as a single copy; for the two conformational epitopes, 3105 and F11A4, with both loops per spike participating. Although the same peptide features in all three epitopes, it interacts with the respective Fabs in quite different ways and combinations. These epitopes are close together and overlapping, the entirely different binding aspects of their cognate Fabs, and their respective binding affinities (unpublished results). All three conformational epitopes bridge interfaces between adjacent subunits: in two

cases (3105 and F11A4), across the dimer interface; with 3120, between adjacent dimers. Such character explains their discontinuous character. Of the three, only 3120 exhibits quasi-equivalent variations in binding affinity, suggesting that there is more play in the interdimer interactions than in the intradimer interactions (Belnap *et al.*, 2003).

Precore/core mutations and their impact

Core promoter mutants

The core protein or nucleocapsid of HBV can be divided into two major domains, the N-terminal assembly domain (up to amino acid position 144) and the functionally important, arginine-rich C-terminal domain of approximately 40 amino acids. The C-terminal domain is required for binding of the pregenomic RNA and subsequent genomic replication and has been shown to contain important B-cell and cytotoxic T-lymphocyte epitopes (Lohr *et al*., 1993; Jung and Pape, 2002). Sequence variation within the core promotor (CP) in natural isolates is limited because of its pivotal role in viral replication. Two highly conserved regions have been identified: nts 1770–1808 and nts 1813–1849. This is to be expected because these regions have been shown to be crucial in the regulation of transcription, however CP mutations were the only independent factor that was associated with HCC (Yuen *et al*., 2003; Sumi *et al*., 2003; Ding *et al*., 2002; Orito *et al*., 2001). Mutations within these regions usually occur as mixed populations with the wild-type sequence. This must have a compensatory effect, overcoming the potentially lethal effect of the mutations and allowing viral replication to proceed. The pre-C mRNA initiation sites map within the first region (Chen *et al*., 1995), as does TA4 (AT-rich sequences) that controls pgRNA (pregenomic RNA) synthesis. Mutations within TA4 severely reduce the synthesis of pgRNA, leaving synthesis of the pre-C mRNAs unaffected (Yu and Mertz, 1996). The second conserved region contains the most essential element of the CP, namely, the initiation site of pgRNA transcription (Chen *et al*., 1995) as well as coding for the C-terminal of the X protein. The overlap of this region with the 5' end of the pre-C ORF containing direct repeat 1(DR1) is a further reason for the low sequence divergence. Because mutations within this region and, in particular, basic core promotor (BCP) may dysregulate viral gene expression and contribute to disease progression, they have been the focus of a number of studies (Kramvis and Kew, 1999). The p25 precore protein is started at the first ATG codon and becomes targeted by a signal peptide to a cell secretory pathway where N-terminal processing will create a p22 species (Fig. 1). The latter undergoes further modification at the C-terminal region, after position

149, to generate p17, or e-protein, which is secreted from the cell as HBe antigen (Pumpens and Grens, 1999). Tong *et al.* (2005) found the gradual loss of HBeAg expression and enhancement of viral replication capacity over the course of chronic HBV infection (Table II).

TABLE II.- CUMULATIVE EFFECT OF CORE PROMOTER MUTATIONS ON GENOME REPLICATION AND HBeAG EXPRESSION.

Mutations	**Genome replication**	**HBeAg expression (%)**
Wild-type	1	100
A1762T/G1764A	2	80
A1762T/G1764A + T1753C	4	70
A1762T/G1764A + C1766T	8	25
A1762T/G1764A + T1753C + C1766T	8	20

Point mutations

Core promoter 1762/1764 and 1753/1762/1764 mutations have been demonstrated *in vitro* to reduce HBeAg expression of the same level. Interestingly, we found that a minor percentage (7.6%) of HBeAg-negative chronic hepatitis B (CHB) patients were infected by HBV of triple core promoter (C1753T, A1762T and G1764A) mutations in Taiwan. Not surprisingly, this HBV mutant was predominant in genotype C. One recent report regarding HBV infection in French patients suggested that 1753/1762/1764/ 1766 mutations conferred lower HBeAg expression than dual BCP (A1762T and G1764A) mutations (Parekh *et al*., 2003). Triple core promoter (C1753T, A1762T and G1764A) mutations were significantly predominant in genotype C. On the contrary, nucleotide 1809–1817 mutations tend to link to genotype B.

Liver specific core promoter is composed of the core upstream regulatory sequence and the basic core promoter. The naturally occurring mutations are clustered around the basic core promoter and heterogenous in nature, except that the A1762T and G1764A mutations are usually present. Several groups extensively studied the A1762T/G1764A double mutation (Table II) by site-directed mutagenesis and transfection experiments in human hepatoma cells. Controversies do exist, possibly due to the weak effect of the mutation. In general, the double mutation has been found to down-regulate precore mRNA production, resulting in reduced HBeAg expression (Buckwold *et al*., 1996; Tang *et al*., 2001; Yu and Mertz, 2003). The liver-specific core promoter is responsible

for the transcription of two subsets of 3.5-kb mRNA species: the precore mRNA (the messenger for HBeAg) and the shorter pregenomic RNA (the messenger for core protein and potymerase; also the template for reverse transcription). The mutations also moderately up-regulate viral genome replication, possibly through the increased transcription of pregenomic RNA (Tong, *et al.,* 2005).

Deletions

At least 20 deletion patterns in the BCP, overlapping the *X* gene, have been detected (Fig. 2). The position of the deletions varies and their size is generally restricted to 8, 20 or 21 bp. The deletions encompass either TA1 or TA2, or both, and overlap transcription factor binding sites (Kramvis and Kew, 1999; Kao *et al.*, 2003).

These deletions may therefore affect the function of the BCP. *In vitro* studies have demonstrated that deletions in the BCP cause reduced production of pre-C mRNA and enhance the levels of pgRNA in the culture medium (Günther *et al*., 1996a; Moriyama, 1997). At another level, all deletions within the CP would predictably influence the expression and function of the X protein. Most deletions result in a frameshift and truncation of the X protein at its C-terminal end (Kidd-Ljunggren *et al*., 1997). The loss of *trans*-activating activity may be an additional reason for the decreased viral replication seen in ASCs and in patients with no serological markers (Kramvis and Kew, 1999).

Most of the HBV genomes in sera of patients with severe liver disease contained deletions or insertions, or both, in the CP, whereas no difference was found in sera from immunosuppressed patients with mild or no liver disease (Günther *et al*., 1996b).

Frame-shift-mutations and in-frame deletions that truncated the core-protein could be observed in immunocompromized patients (Schories *et al*., 2000) and in chronic carriers (Alexopoulou *et al*., 1997). Two frame-shift-mutations shortened the corresponding core-proteins to 29 aa or to 65 aa, respectively (Schories *et al*., 2000), and an in-frame deletion caused a loss of the codons 94 to 101 (Alexopoulou *et al*., 1997). In-frame deletions usually spanned various regions between the codons 80 to 130. Shortened core-proteins may be non functional. However functional coreproteins seem to be required for the nucleocapsid assembly. This would make the core-deletion mutants dependent on the wildtype virus. The observation that these variants only were found together

with the wildtype HBV (Gunther *et al*., 1996a; Marinos *et al*., 1996; Yuan *et al*., 1998) and transfection assays could confirm this opinion (Schories *et al*., 2000). Core-deletion-mutants mostly were observed in patients with long course infections. It was supposed that their selection might not be favored by immunological features but by an enhanced expression of the polymerase. The over-expression of the polymerase is supposed to be inhibited by the two start-codons J (NT 2163 to NT 2165) and C2 (NT 2177 to NT 2179) (Table II), which are situated within the C-ORF upstream of the normal polymerase-AUG (NT 2307 to NT 2309) (Fouillot *et al*., 1994).

Duplication

Duplication of sequences between nts 1641–1666 has been described and this region covers the α box of enhancer II (ENII) (and CURS 'core upstream regulatory sequence') and is vital for CP function. This duplication increases the level of pre-C mRNA, HBeAg, core protein protein, POL and intra- and extracellular-particle-associated HBV DNA, and decreases the ratio of pre-S/S to pre-C/C mRNAs and the HBsAg and pre-S protein levels (Günther *et al*., 1996b).

The economy with which HBV utilizes its genome is very evident in the region covered by the CP. The DNA spanning the CP region (nts 1591–1849) has a number of important functions (Table II). First, it contains ENII, which has dual functions *in vivo*: it stimulates the S and X promoters from a downstream position and the CP from an upstream position. Second, the BCP within this region controls the transcription of both pre-C mRNA and pgRNA. Furthermore, the 3' end of the CP overlaps with the 5' end of the pre-C/C ORF that codes for HBeAg and contains DR1. Third, this region overlaps the X ORF that encodes the X protein, the C-terminus of which has been shown to have trans-activating functions. Therefore, any mutations within the DNA sequence of the CP could influence viral replication, HBeAg production and the amino acid sequence of the X protein. Sequence conservation within this region is therefore crucial in maintaining active viral replication, and variation may contribute to the persistence of HBV within the host, leading to chronic infection and, ultimately, hepatocarcinogenesis (Kramvis and Kew, 1999).

Insertions

Insertions within the BCP have been described in those undergoing orthotopic liver transplantation (Laskus *et al*., 1994). This insertion occurred shortly before, or during, FH and created a binding site for Hepatocyte nuclear

factor 1(HNF1). Thus, the emergence or presence of a novel HNF1, or putative HNF3, site may be related to fulminant exacerbating hepatitis in immunosuppressed patients. This concurs with the presence of such strains in renal transplant patients with severe liver disease but not in those with mild disease (Günther *et al*., 1996b).

TABLE III.- MAJOR MUTATIONS IN HEPATITIS B CORE GENE.

Mutations	**Types**	**References**
A1752G	Point Mutation	**(**Yen-Hsuan *et al*., 2004)
C1773T	Point Mutation	**(**Yen-Hsuan *et al*., 2004)
A1775G	Point Mutation	**(**Yen-Hsuan *et al*., 2004)
G1799C	Point Mutation	**(**Yen-Hsuan *et al*., 2004)
TT1802–3CG	Point Mutation	**(**Yen-Hsuan *et al*., 2004)
A1846T	Point Mutation	**(**Yen-Hsuan *et al*., 2004)
T1850A	Point Mutation	**(**Yen-Hsuan *et al*., 2004)
A1762T	Point Mutation	(Kramvis and Kew, 1999)
G1764A	Point Mutation	(Kramvis and Kew, 1999)
A1762T/G1764A	Point Mutation	(Parekh *et al*., 2003)
A1753T/G1762A/C1764T	Point Mutation	(Parekh *et al*., 2003)
T 1753C/A 1762T/G 1764A/C 1766T	Point Mutation	(Tong *et al.*, 2005)
$1810^{T}1811^{T}$	Point Mutation	(Sato *et al*., 1995)
1653^{T}. 1653^{T}	Point Mutation	(Yuh and Ting, 1991; López-Cabrera *et al*., 1991)
$1766^{T}1768^{A}$	Point Mutation	(Günther *et al*., 1996b)
1809^{T} or 1812^{T}	Point Mutation	(Kozak, 1986)
1809–1817	Point Mutation	(Parekh *et al*., 2003)
G1896A	Point Mutation	(Yuen *et al*., 2004b)
NT 2163 to NT 2165	Deletions	(Fouillot *et al*., 1994)
NT 2177 to NT 2179	Deletions	(Fouillot *et al*., 1994)
NT 2307 to NT 2309	Deletions	(Fouillot *et al*., 1994)
Between nts 1641–1666	Duplication	(Günther *et al*., 1996b)
Between nts 1591–1849	Duplication	(Kramvis and Kew, 1999)
T1740	Point Mutation	(Sugauchi *et al*., 2002)
A1838	Point Mutation	(Sugauchi *et al*., 2002)
G2020	Point Mutation	(Sugauchi *et al*., 2002)
C2167	Point Mutation	(Sugauchi *et al*., 2002)
G1838	Point Mutation	(Sugauchi *et al*., 2002)
A2020	Point Mutation	(Sugauchi *et al*., 2002)

Consequently, mutations that reduce core promoter activity or alter pregenomic RNA *in vitro* should be cautiously interpreted, as it is unclear whether they are clinically meaningful. In order to obtain clinically meaningful data samples from well-characterized clinical phenotypes need to be used for *in*

vitro functional experiments. One such study (Parekh *et al*., 2003) used high and low viremia samples in a cross sectional study of genotype A HBeAg positive patients. They found an association between core promoter mutants and low viremia in serum of their patients, but discordance between experimental *in vitro* results from cell culture and *in vivo* viral load. Patients with low viremia developed high replicator strains, and, conversely, patients with high viremia developed low replicator strains when transfected into hepatoma cell lines. The explanation for this discrepancy between cell models and *in vivo* clinical samples is unclear, but could be due to differences in transcription factor expression in hepatoma cell lines or higher removal of viral strains in patients with increased immune response, such as those following HBeAg seroconversion. In vitro methods generally utilize viral replication in hepatoma cell lines as a surrogate to reflect viral load in patients. However, until a validated cell model is found that correlates closely with clinical viral load, evaluation of replication efficiency in cell models cannot be inferred with confidence as reflecting altered *in vivo* viral load. Alternatively, cellular production of virus may be unchanged or even enhanced, but the immune removal of virus could be more efficient after seroconversion, thus resulting in a net viral load that is low (Cheng *et al*., 2006).

The sequence located around the precore initiation codon (nt1808–1817) is conserved among all genotypes of HBV (Ahn *et al*., 2003). The occurrence of the 1896 mutation is restricted by the secondary structure of the encapsidation signal (ε), (Lok *et al*., 1994; Tong *et al*., 1992; Laskus *et al*., 1994) which is transcribed from the same region of the HBV genome coding for HbeAg (Kramvis and Kew, 2005). In genotype D a G to A mutation at nt 1896 stabilizes the hairpin structure (signal encapsidation) by creating a U-A pair, which might be advantageous for viral replication (Junker–Niepmann *et al*., 1990; Niitsuma *et al*., 1995). This mutation changes the precore-codon 28 (Trp) into a stop codon and therefore prevents the expression of HBeAg. It was found in more than 95% of the precore-mutations (Santantonio *et al*., 1991; Chu *et al*., 1996; Ulrich *et al*., 1990; Yuan *et al*., 1995). Strains of the genotype A, however, in which the stop-codon 28 mutation destabilizes the hairpin structure need an additional C to U mutation at nt 1858 to stabilize the encapsidation signal (Rodriguez-Frias *et al*., 1995). The precore-Cys-23 was found to be highly conserved in Taiwanese hepatomas (Hosono *et al*., 1995), while in Italian hepatomas this cys could be changed (Clementi *et al*., 1993; Manzin *et al*., 1992). HBV genotype A has been recently divided into African/Asian (Aa) and European (Ae) subgroups. Genotype Aa is different from all the other genotypes in possessing unique nucleotide changes including G1809T, C1812T, G1862T, and G1888A (Sugauchi *et al*., 2004).

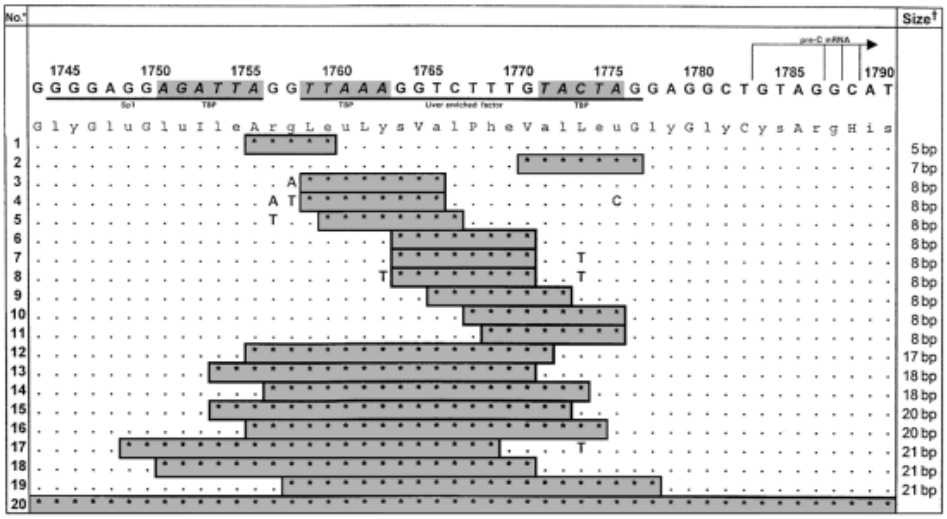

Fig. 5. Deletions in the basic core promoter (BCP) of hepatitis B virus (HBV). The nucleotide sequence and deduced amino acid sequence of part of the BCP (1742–1849) of HBV DNA, aligned to sequence EMBL X 70185, are shown. Transcription factor binding sites are underlined and AT-rich regions are shaded in grey.

The clinical relevance of those HBV variants with mutations located in the core promoter or precore region and evaluation of the impact of the core promoter and precore HBV mutants on viral loads and clinical manifestations among HBeAg-negative CHB patients deserves further study.

Clinical significance of HBV pre-core/core mutants

There is evidence that HBV mutants can influence not only the course of infection but also the clinical manifestation of the disease. Most of our knowledge of the clinical significance of HBV mutants is based on patients with mutation in the pre-core gene. By far, the most common mutation in the pre-core gene is a single base change of *G* to *A* at nt 1896. This point mutation has provided the stabilizing effect needed for the emergence of a new mutant (Carman *et al*., 1989). The single-base change at codon 28 is known to prevent transcription of the pre-core gene, resulting in the failure to encode the infected cell to secrete HBeAg. These patients, though lacking HBeAg, still show persistent HBV-DNA positivity and active viral replication (Carman *et al*., 1991). Recently, four HBsAg carrier surgeons were found to have transmitted the virus to their patients despite their anti-HBe status.

A wide geographical variation seems to emerge for the pre-core mutants. As high as 25% of chronic HBV infections in patients in Delhi, India, were found to be caused by mutants. Similarly, high prevalence rates have been reported in HBV-endemic areas in the Mediterranean basin, including Italy and Greece, and Far Eastern countries, such as China and Japan (Guptan, *et al.,* 1996; Brunetto, *et al.,* 1989; Zhang *et al.,* 1996 and Erhardt *et al.,*2000)**.** In contrast, the mutant is less dominant in non-endemic areas, such as Europe and North America (Liang, *et al.,* 1994; Zaneth, *et al.,* 1988). A high association of fulminant hepatitis has been reported with this mutant in Japan. In general, patients infected with this mutant are more likely to progress to cirrhosis and hepatitic insufficiency, compared to those infected by the wild virus. In a study of chronic hepatitis patients in France, a significant increase in cirrhosis among pre-core mutant infections. This was attributed to patients having early childhood infections. There are reports that these pre-core mutants could induce resistance to interferon therapy (Erhardt *et al.,* 2000), increase incidence of fibrosing cholestatic hepatitis in liver transplant patients (Fang *et el.,* 1993), increase susceptibility to hepatitis D co-infection (Raimondo *et al.,* 1991) and cause fulminant hepatic failure (Kosaka *et al.,* 1991).

Efficacy of HBcAg as a vaccine

HBcAg and HBeAg are highly cross-reactive at the T-cell level (Milich *et al.,* 1987, 1988). It therefore may be speculated that HBeAg presents immunogenic epitopes to the T-cells thus protecting the HBcAg expressing hepatocytes against the immune system. After the selection of an HBeAg-negative mutant the epitopes of the HBcAg might come under the pressure of the immune system (Carman *et al.,* 1993). Variants within the core epitopes in Spanish patients were found to be significantly correlated with precore-mutations that prevented the expression of the HBeAg (Rodriguez-Frias *et al.,* 1995). However, in Japanese patients core mutations could not be correlated with the appearance of precore-stop-mutations (Wakita *et al.,* 1991)**.** This might be due by different host factors. During the asymptomatic period of infection the virus replication is reduced by CD8 + cytotoxic Tcells (CTL) whose target is the HBcAg. Mutations inside the CTL-epitopes of the C-gene therefore might create immune escape mutants leading to chronic viral persistence and severe liver disease (Kreutz, 2002) HBcAg has been proposed as a vaccine platform largely because of its ability to self-assemble even after insertions of heterologous sequence at the NH_2 terminus and COOH terminus and internally in the monomer subunit and because of the enhanced immunogenicity of HBcAg. Because

HBcAg is derived from a human pathogen there are a number of practical limitations to its use as a vaccine carrier in humans. For example, preexisting anti-HBc antibody in individuals previously infected with HBV may affect immune clearance of the modified HBcAg through the formation of immune complexes. Secondly, anti-HBc antibodies induced during vaccination with modified HBcAg may compromise the utility of the anti-HBc assay used for diagnostic purposes. Most importantly, the approximately 400 million global chronic carriers of the HBV demonstrate very poor T-cell responses to all HBV structural proteins, including HBcAg, due to immune tolerance and, therefore, an HBcAg-based vaccine is not likely to be very effective in these individuals. Immune tolerance to the HBcAg is especially relevant in countries where HBV infection is endemic, where chronic carrier rates can be as high as 20% of the population. Because the rodent core proteins are not derived from a human pathogen, their use as vaccine platforms may solve a number of the "preexisting immunity" problems associated with the use of HBcAg. For example, because the rodent core proteins are not significantly cross-reactive with HBcAg at the antibody level preexisting anti-HBc antibodies will not immune complex with modified rodent core particles and the anti-core antibodies induced by vaccination with modified rodent core particles should not compromise the anti-HBc diagnostic assay. Furthermore, because the HBcAg and rodent core proteins are minimally cross-reactive at the Th cell level the use of the rodent core proteins may offer a means of circumventing the Th cell tolerance to HBcAg present in chronic carriers of HBV. Although we have compared Th cell recognition between HBcAg and the rodent core proteins in mice, the predominant Th cell recognition sites on HBcAg recognized by humans have been mapped to aa 1 to 20, aa 50 to 69, and aa 117 to 130 (Bertoletti *et al*., 1991). The first two of these HBcAg Th cell sites are not highly conserved on the rodent core proteins and predict low levels of Th cell cross-reactivity between HBcAg and the rodent core proteins in humans, as demonstrated herein for mice. (Billaud *et al*., 2005). Particulate HBcAg elicits immune responses and provides T-cell 'help' for antibody responses to HBsAg. It has been used as a carrier to enhance the immunogenicity of heterologous epitopes (Schodel *et al*., 1994). HBcAg is a virus-like particle easy to work with because of its high-level expression and efficient particle formation in many expression systems, including bacteria. Appropriate target sites within the HBcAg sequence, in which epitope sequences of foreign antigens can be inserted, are therefore of interest. The N- and C-termini of HBcAg, as well as its major immunodominant region or tip of the spikes (e1 epitope aa 76-81) have been used to insert foreign epitopes derived from the VP2 protein of human rhinovirus type 2 (Brown *et al*., 1991; Clarke *et*

al., 1987) the simian immunodeficiency virus envelope (Yon *et al*., 1992) or the V3 loop of HIV-I gp120 (Von *et al*., 1993; Borisova *et al*., 1996; Brunn *et al*., 1997). Insertion of 39 amino acids of the 'a' domain of HBsAg (positions 111-149) was the first successful attempt to mimic a conformational epitope on the surface of chimeric HBcAg particles (Borisova *et al*., 1993) but there is a paucity of data concerning the use of e2 epitope. The amino acid residues 74-83 forming a 7 nm-long spike protruding from the surface of core particles may play a key role in the architecture of particulate, native HBcAg that determines its antigenicity and possibly its immunological properties (Borisova *et al*., 1996). It was, therefore, unexpected that insertion of the 'a' fragment into the e2 site without linkers resulted in the destruction of the native HBcAg structure. In contrast, insertion of the '*a*' determinant of HBsAg (111-149) into the e2 epitope using linkers maintained the antigenicity of HBcAg, although the immunogenicity of the 'a' determinant of HBsAg was lower than that of the native HBsAg particle (Shahrokhi *et al*., 2006).

An effective HIV vaccine will likely need to induce broad and potent CTL responses. Epitope-based vaccines offer significant potential for inducing multi-specific CTL, but often require conjugation to T helper epitopes or carrier moieties to induce significant responses. We tested hybrid DNA vaccines encoding one or more HIV or SIV CTL epitopes fused to a hepatitis B core antigen (*HBcAg*) carrier gene as a means to improve the immunogenicity of epitope-based DNA vaccines. Immunization of mice with a HBcAg-HIV epitope DNA vaccine induced $CD8^+$ T cell responses that significantly exceeded levels induced with DNA encoding either the whole HIV antigen or the epitope alone. In rhesus macaques, a multi-epitope hybrid HBcAg-SIV DNA vaccine induced CTL responses to 13 different epitopes, including 3 epitopes that were previously not detected in SIV-infected macaques. These data demonstrate that immunization with hybrid HBcAg-epitope DNA vaccinesis an effective strategy to increase the magnitude and breadth of HIV-specific CTL responses (Fuller *et al*., 2007).

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TOXICITY AND UPTAKE OF HEAVY METALS IN PROTOZOA – A REVIEW

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CONTENTS

Abstract.- Heavy metal pollution represents an important environmental problem due to the toxic effects of metals, and their accumulation throughout the food chain leads to serious ecological and health problems. At high concentrations, both essential and non-essential metals can damage cell membrane, alter enzyme specificity, disrupt cellular function and damage the structure of DNA. Several toxic effects are associated with exposure to toxic metal ions, including increased incidence of certain cancers, toxic towards living cells, tissue and organisms serious damage to such major organs as lung, liver and kidney, pulmonary fibrosis and chronic bronchitis, skin ulcers, lung cancer and mutagenic effect on bacteria and impairment of primary immune responses. Cadmium is one of the major metal pollutants and is carcinogenic, embryotoxic, teratogenic and mutagenic and may cause hyperglycemia, reduced immunopotency and anaemia, due to its interference with iron metabolism. Copper ions inhibit macromolecules synthesis and other enzymatic reactions, affect bacterial growth, and dramatically decrease cell numbers. Mercury, another metal pollutant, causes various health problems such as pneumonitis, abnormal cramps, bloody diarrhea and suppression of urine, cancer and hypersecretion of sweat glands are caused by mercurial and mercuric forms of mercury. Zinc has shown inhibitory effects on the growth and enzymatic activities in living organisms. Nickel compounds are found to be nephrotoxic, hepatotoxic, immunotoxic and teratogenic. A wide variety of microorganisms such as bacteria, yeast, algae, protozoa and fungi are found in waters receiving industrial effluents. Many of the microorganisms show adaptation to the toxic materials constantly released in their environment. They have developed strategies to resist, tolerate, metabolize, and to detoxify these toxic substances. Among such microorganisms, protozoans showed fairly high capability to uptake metals from the environment and their use is beneficial because bioremediation has advantages over other techniques as it is cheap, non-destructive and contamination remains localized.

Key words: Metal toxicity, metal detoxification, protozoa, bioremediation.

INTRODUCTION

Heavy metal pollution has become one of the serious environmental problems today. Heavy metals in wastewater come from industries and municipal sewage, and they are one of the main causes of water and soil pollution. Accumulation of these metals in wastewater depends on many local factors such as type of industries in the region, people's way of life and awareness of the impacts done to the environment by careless disposal of wastes (Chipasa, 2003; Shakoori *et al*., 2004; Chisti, 2004). Atmospheric dust deposition can affect biogenic calcification in oceanic regions characterized by trace metal limitation (Schulz *et al*., 2004). Heavy metal contamination and the problems that it poses to the biota have been well documented (Raskin and Ensley, 2000; Meagher, 2000). Accumulation of toxic metals, *e.g*., Hg, Cu, Cd, Cr, and Zn, in humans has several consequences such as growth and developmental abnormalities, carcinogenesis, neuromuscular control defects, mental retardation, renal malfunction and wide range of other illnesses (Thiele, 1995).

Removal of metal pollutants from the contaminated environment has been a challenge for long time. It is important to establish an efficient and low cost method for removal of toxic metal ions (Honjoh *et al*., 1997). Because traditional cleanup processes of heavy metal contaminated soils and waters are expensive and practical only in small areas (Moffat, 1995), researchers have looked for new cost effective technologies that include the use of microorganisms, biomass, and live plants (Ebbs and Kochian, 1997; Miller, 1996; Gardea-Torresdey *et al*., 1996).

Microorganisms may be used to remediate wastewaters or soil contaminated with heavy metals. The metal processing capacity of microorganisms can be used to concentrate, remove and recover metals from aqueous strams and enhance the efficiency of wastewater treatment processes (Amoroso *et al*., 1998). Most of the indigenous microorganisms have developed resistance to the toxic chemicals present in polluted soils and waters. Many of them have achieved the capability of metabolizing toxic metal ions (Haq and Shakoori, 2000). Microorganisms with the ability to grow in the presence of heavy metals and with a significant metal uptake have a potential use in bioremediation of polluted waters (Rehman and Shakoori, 2001; Shakoori *et al*., 2004).

Microbial metal removal has received much attention in the last years due to the potential use of microorganisms for cleaning metal-polluted water (Ledin,

2000). Microorganisms are highly effective in sequestering heavy metals include bacteria, fungi, algae and actinomycetes (Wong and So, 1993). These have been used to remove metals from polluted industrial and domestic effluents on a large scale. Tolerance and removal of toxic metal ions have been studied in bacteria (Volesky and Holan, 1995; Ledin, 2000; Cervantes *et al.,* 2001; Shakoori and Muneer, 2002; Yilmaz, 2003; Deveci *et al*., 2004; Silver and Phung, 2005), cyanbacteria (Inthorn *et al.,* 1996; Wang *et al*., 2005), algae (Matsunaga *et al*., 1999; Rehman and Shakoori, 2001; Davis *et al*., 2003; Feng and Aldrich, 2004; Torricelli *et al*., 2004), fungi (Holan and Volesky, 1995; Yan and Viraraghavan, 2003; Pas *et al.,* 2004). Microbiological detoxification of polluted water is economical, safe, and sustainable (Eccles, 1995).

Microorganisms have acquired a variety and array of mechanisms to remove or detoxify toxic metal ions (Silver and Phung, 2005). They remove toxic metal ions via adsorption to cell surfaces (Mullen *et al.,* 1989), complexation by exopolysaccharides (Scott and Palmer, 1988), binding with bacterial cell envelopes (Flatau *et al.,* 1987), intracellular accumulation (Laddaga and Silver, 1985), biosynthesis of metallothioneins and other proteins (Aiking *et al*., 1985) and transformation to volatile compounds (Robinson and Tuovinen, 1984). Metal resistance is defined as the ability of an organism to survive metal toxicity by means of a mechanism produced in direct response to metal species concerned (Zafar *et al*., 2007).

Protozoans have been found to be present in and metabolizing industrial effluents contaminated by toxic metal ions such as $Cu^{2+,}$ Hg^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+} and Cd^{2+} and other toxic compounds (Schlenk and Moore, 1994; Madoni *et al*., 1996; Haq *et al*., 1998). The long-term survival of protozoa in media containing relatively high concentrations of heavy metal ions shows that these organisms have strategies to tolerate, resist or detoxicate organic substances and heavy metals (Haq *et al*., 2000). Shakoori *et al*. (2004) reported that *Vorticella microstoma* showed remarkable ability to pick up heavy metal ions from the culture medium. The concentration of Zn^{2+} and Cr^{6+} was reduced 99% and 48% after 192 hours, respectively. These microorganisms actively contribute to the amelioration of the effluent quality, since the majority of them feed upon dispersed bacteria (Madoni, 2000).

Nilsson (1981) reported that *Tetrahymena* cells that are forming granules are resistant to higher concentrations of Cu. In fact many metals (Cd, Zn and others) are contained in the granules; these may be regarded as structures involved in the detoxification or metal ion regulation and are normally present in

the stationary phase of growth (Nilsson and Coleman, 1977; Dunlop and Chapman, 1981). Granules may be initially present but we have not looked for their presence at zero time; so cannot confirm whether this detoxification mechanism is operating. In any case the high tolerance found in *Tetrahymena* for Cd is due not only to granule formation but to the formation of a metallothionein (Piccinni *et al*., 1987). The hypotrichous ciliates, which showed tolerance toward toxic metals, are characterized by a stiff and thick cell membrane in the dorsal region and this may be, together with genetic organization, one of the reasons for their resistance to the toxic action of heavy metals (Madoni *et al*., 1992).

Cadmium

Cadmium is a heavy metal contaminant in the environment. It is extensively used in the industry for a number of applications, including electroplating, protection against corrosion and stabilizing plastic (Lebrun *et al*., 1994). It is also obtained as a by-product of zinc production (Nies, 1999). Cadmium (Cd^{2+}) has been utilized eight times more during the last 40 years by mankind than in its entire history; the Cd^{2+} input into biosphere is estimated to be about 30,000 tons/year (Nriagu and Pacyna, 1988).

Cd^{2+} contamination in surface water comes mainly from phosphatic fertilizers used in agricultural operations, which is reflected in municipal water supplies drawing water mainly from river sources. The major route of exposure of Cd^{2+} to humans is via the consumption of vegetables homegrown on Cd contaminated soil. It is well known that soil pH is one of the main soil properties controlling bioavailability of Cd in plants (Vig *et al*., 2003; Millis *et al*., 2004).

Cadmium toxicity

Cadmium is one of the most dangerous heavy metals both to human health and aquatic ecosystems. Cd is carcinogenic, embryotoxic, teratogenic and mutagenic and may cause hyperglycemia, reduced immunopotency and anaemia, due to its interference with iron metabolism (Sanders, 1986). The toxicity of Cd has also been well documented in selective types of almost all major phyla of eukaryotes (Rainbow, 1995; Unger and Roesijadi, 1996; Coeurdassier *et al*., 2004).

Madoni *et al*. (1992) described the sensitivities of the seven ciliate species to cadmium. The 24-h LC 50 values of Cd ranged from 180 µg/l (*P. caudatum*) to 2,650 µg/l (*E. patella*). The toxic effect of cadmium on protozoan colonization

in an experimental microecosystem was studied by Cairns *et al*. (1986) who reported that in a 28-day test the chronic effect level for colonization reduction in tests with cadmium is reached at 0.4 µg/l. Addition of Cd affected the growth rate of *Uronema marinum* even at the lowest dose used (2 µg/ml). The phenomenon of conjugation which appears between days 5 and 7 in controls was delayed starting at day 8 in cells exposed to 2 µg Cd /ml. At higher doses, conjugation was abolished, at least over a grown period of 15 days (Coppellotti, 1994). The 24-h LC_{50} value of cadmium for *Spirostomum teres* was 1.95 mg/l (Twagilimana *et al.*, 1998).

Heavy metals change the structure of the activated-sludge micro fauna by modifying both cell density and species richness. The order of five metals toxicity on protozoan community in activated sludge has been reported: Cd > Cu > Pb > Zn > Cr (Madoni *et al*., 1996). Cd^{2+} is reported to have acute effects on mitochondria, rough endoplasmic reticulum, and golgi apparatus in protozoan at lower concentrations, while higher concentration of Cd^{2+} may cause lysis of cell membrane (Al-Rashid and Sleigh, 1994). Shakoori *et al*. (2004) found that the ciliary movements decreased in the presence of $CdCl_2$ and the cell number was reduced from 1085 to 690 cells/ml. The order of resistance regarding molar concentrations of metal ions of *V. microstoma* against five metals was Zn^{2+}>, Cu^{2+}>, Cr^{6+}>, Pb^{2+}>, Cd^{2+}. Dayeh *et al*. (2005) reported that cadmium was most toxic to *Tetrahymena thermophila* and killed the ciliate at approximately 10 fold lower concentration than needed to kill fish cells.

Cadmium uptake and detoxification

Growth of *Tetrahymena* was not restricted in the presence of Cd unlike in some phytoflagellata, like *Euglena* or *Ochromonas*, where the metal induces an inhibition of growth (Albergoni *et al*., 1980; Piccinni and Coppellotti, 1982). *Tetrahymena pyriformis* can be cultured without damage in the presence of 10-20 µg Cd/ml (Piccinni, 1995; Haq *et al*., 2000). Nilsson (1981) reported that *Tetrahymena* cells that are forming granules are resistant to higher concentration of metal. Infact many metals (Cd, Zn and others) are contained in the granules; these may be regarded as structures involved in the detoxification or metal ion regulation and are normally present in the stationary phase of growth (Dunlop and Chapman, 1981).

The high tolerance of protozoan for Cd is due not only to granule formation but to the formation of a metallothionein (MT) (Santovito *et al.*, 2000; Boldrin *et al*., 2002; Martin-Gonzalez *et al*., 2006). Cd induces a Cd-MT

tetramer in *Tetrahymena* similar in amino acid composition to invertebrate and mammalian vertebrate MTs and very few aromatic and histidine aminoacid residues (Piccinni *et al*., 1987). In *Oxytricha* Cd induces two binding compounds: one is similar to metallothioneins (MTs) containing 25% cysteine, no aromatic amino acids and a typical UV spectrum; the other is unlike the usual metallothionein or chelatin (Irato *et al*., 1995).

The Cd^{2+} induced primary structure of metallothioneins in *Tetrahymena pyriformis* and *Tetrahymena pigmentosa* have been determined. The *Tetrahymena* Cd^{2+} metallothionein chain contains 31 cysteine residues, 14 of which are arranged in seven Xaa-Cys-Cys-Xaa motifs that are characteristic of the metal- binding regions in mammalian metallothioneins. The remaining cysteine residues appear in an atypical context: four groups of Cys-Cys-Cys and two groups of Cys-Xaa-Xaa-Cys and a further solitary cysteine (position 48), which may be in a Cys-Xaa-Cys motif. It should be noted that the *Tetrahymena* metallothioneins contain the G-C-K-C-T-G-C-K-C motif in the region 47-55 (Piccinni *et al*., 1994).

Piccinni *et al*. (1999) identified the Cd-MT genes in *Tetrahymena pyriformis, Tetrahymena pigmentosa* and *Tetrahymena thermophila, which* are identical and encode a transcript of 487 bp, with an intronless coding region of 324 nucleotides. Shang *et al*. (2002) have recently sequenced a second Cd-metallothionein gene (MTT1) from *Tetrahymena thermophila*, which encodes a 162 aa protein, the longest with respect to other MT isoforms. MTT1 gene was more sensitive to cadmium induction compared to copper, and in a certain threshold scale ($\leq$35.2 µmol/l), raising the cadmium concentrations increased the MTT1 expression level (Ting *et al.,* 2005).

The *S. mytilus* growing in medium containing cadmium (10 µg/mL) could reduce 75% of cadmium from the medium after 48 hours, 84% after 72 hours and 91% after 96 hours (Rehman *et al.,* 2008.

Copper

Copper, a cofactor of many cellular enzymes, is an essential element. The biological functions are intimately related to its properties as a transition metal (Shriver *et al*., 1994). Copper is one of the most prevalent biological transition metals, second only to iron. Its concentration in biological habitats, such as seawater, is low, however, so that its accumulation in living cells requires active transport. Free Cu^{2+} is toxic, even in relatively low concentrations, and hence transport within organisms occurs in only complexes, usually in proteins.

Copper toxicity

Copper is rarely found in natural water but is found in man-polluted environments (Fostner and Wittman, 1997; Udom *et al.*, 2004; Andrews and Sutherland, 2004). Any copper present normally originates from industrial effluents, seepage, water from refuse dumps, pesticides or corrosive water that has come into contact with fitting and pipes containing copper. Copper ions inhibit macromolecules synthesis and other enzymatic reactions (Company *et al.*, 2004).

Sudo and Aiba (1973) found that a concentration of between 0.25 and 0.27 ppm of copper halves the specific growth rate of *Vorticella microstoma* and *Opercularia* sp. from activated sludge. Toxicology studies on *Tetrahymena pyriformis* have provided valuable information on the effects of copper on ciliate protozoa such as complete inhibition of growth at 1 mM Cu (Yamaguchi *et al.*, 1973), reduction of copper toxicity by accumulation of Cu in small refractile intracellular granules (Nilsson, 1981) and inhibition of respiration at 0.1 mM Cu (Wakatsuki *et al.*, 1986). Although many studies of Cu toxicity in ciliates have been carried out in monoaxenic cultures, few toxicology studies have been reported on ciliate communities. Ruthven and Cairns (1973) used the percentage of surviving species to assess the effect of copper on protozoan communities, which can be a rough method when work has done in communities with a low number of species.

Copper may also have an effect on the trophic relations of protozoan communities, which is studied by Doucet and Maly (1990) reporting disruption of the predator-prey relationship between *Dinidium* and *Paramecium*. The toxic effects of copper without addition of oxygen are expressed as an acceleration of mortality due to the lack of oxygen in some peritrich species as *Epistylis plicatilis* and *Vorticella convallaria* at 5 and 10 ppm (Gracia *et al.*, 1994).

The LC_{50} values of copper were 1.75 and 3.51µg/l for *Drepanomonas revolute* and *Spirostomum teres*, respectively. *Blepharisma americanum* showed a higher sensitivity to this metal, with a LC_{50} of 1.45 µg/l. The order of toxicity of metals to the ciliate species tested was generally: Cu> Hg> Cd> Pb> Cr> Zn (Madoni *et al.*, 1994). Joshi and Misra (1986) found that the concentration of 5 mg/l was lethal (100% mortality) for *Paramecium aurelia* after 90 minutes exposure to copper oxychloride. Le Du *et al.* (1990) used *Colpidium campylum* to study the toxicity of a copper-cadmium-nickel-zinc mixture by river waters; they found that all the metals were toxic to *C. campylum*, with the exception of

zinc, but that copper could be considered the main source of toxicity in the effluent.

Twagilimana *et al.* (1998) reported the 24-h LC_{50} value of copper was 0.037 mg/l for *Spirostomum teres.* Shakoori *et al*. (2004) found that the movement of *Vorticella microstoma* completely stopped in the presence of $CuSO_4$. The cell population has been adversely affected by the presence of copper ions in the culture medium. The number of the cells was reduced from 1230 to 836 cells/ml in $CuSO_4$. The order of resistance regarding the reduction in number of the cells was, therefore, Pb^{2+}>, Cr^{6+}>, Zn^{2+}>, Cd^{2+}>, Cu^{2+}.

Copper uptake and detoxification

Copper was generally more toxic to ciliate populations than Cd, Hg or Zn. For five of the seven tested species, the 24-h LC_{50} values ranged from 10 to 21µg/l. *Euplotes affinis* showed low sensitivity of 64 µg/l (Madoni *et al.*, 1992). Simanov (1987) reported a 24-h LC_{50} of 500 µg Cu/l for *C. campylum* using copper nitrate. Madoni *et al*. (1996) reported that no mortality was registered for *Opercularia coarctata* when treated with copper, and *Opercularia minima* showed a 56% of mortality at the highest concentration of this metal (6.12 mg/l). *Vorticella convallaria* showed a 95% survival even in the presence of the highest Cu concentration (6.12 mg/l). *S. mytilus* removed 80% of copper (5 µg/mL) from the medium after 48 hours, 84% after 72 hours and 88% after 96 hours (Rehman *et al*., 2008).

Mercury

Mercury is a unique element that has no essential biological function. It is liquid at room temperature and is 13.6 times heavier than water. It normally occurs in small amounts in oceans but the highest deposits of mercury result from industrial discharges (Brunke *et al*., 1993; Zilloux *et al.*, 1993; Gochfeld, 2003). Its unique physical properties have been exploited for a variety of uses such as in mercury switches, thermostats, thermometers, and other instruments. Its ability to amalgamate with gold and silver are used in mining these precious metals and as a dental restorative. Its toxic properties have been exploited for medications, preservatives, antiseptics, and pesticides. The most important anthropogenic sources of mercury pollution in aquatic systems are atmospheric deposition, erosion, urban discharges, agricultural materials, mining and combustion and industrial discharges (Wang *et al*., 2004).

Mercury is extensively used for agricultural and industrial purposes due to its stereo specific properties. Municipal supplies have comparatively higher values (0.014 ug/Ll) since significant portion of mercury is associated with suspended solids, which accounts for a major part of the downstream transport of Hg in rivers, In surface waters Hg exists as Hg^{o}, Hg^{+} and Hg^{2+} and in well-aerated waters (Eh>0.5v), Hg^{2+} dominates whereas under reduced condition Hg° exist (Moore, 1991). Mercury is used in chlorine production, electrical apparatus production, chemical industry as a catalyst and protection of seeds from fungi (Sanders, 1986). The major source of mercury is the natural degassing of the earth crust, including land areas, rivers and the ocean and is estimated to be in the order of 25,000 to 150,000 tons per year (Goldwater and Stopford, 1977).

Mercury toxicity

Heavy metals, particularly in industrial effluents, are constantly contaminating our environments, and pose serious threat to human life. The 24-h LC_{50} of mercury for the seven ciliate species ranged from 4.30 µg/l (*Uronema nigricans*) to 190 µg/l (*Euplotes affinis*). High values were observed also for *Aspidisca cicada* (70 µg/l) and *Euplotes patella* (125 µg/l) (Madoni *et al*., 1992). Parker (1979) reported a 24-h LC_{50} of 6 µg/l for *Uronema marinum* and a 100% lethal concentration of 10 µg/l. Dini (1981) testing the toxicity of mercuric chloride on some clones of the marine ciliate *E. crassus*, found 24-h LC_{50} values ranging from 51 to 132 µg Hg/l. Another marine ciliate *E. vannus*, survived in 73.9 µgHg/l supplied as mercuric chloride, but 0.74 mg Hg/l killed all the test specimens (Persoone and Uyttersport, 1975).

The 24-h LC_{50} value of mercury was 0.004 mg/l for *Spirostomum teres* (Twagilimana *et al.*, 1998). Gray and Ventilla (1971) reported that 14.8 µg Hg/l supplied as mercuric chloride was 100% toxic to the marine ciliate *Cristigera* sp. The LC_{50s} values of mercury for *Drepanomonas revoluta* and *Spirostomum teres* were 5.37 and 5.94 µg/ l (Madoni *et al.*, 1994).

Mercury uptake and detoxification

For the freshwater ciliate *Tetrahymena pyriformis*, the LC_{50} was 3.3 mg Hg/l after 96 h exposure to mercuric chloride (Carter and Cameron, 1973). Madoni *et al*. (1994) reported that some higher values were observed for tested ciliates (17.5 to 64 µg/ l), with the exception of *Uronema nigricans* which showed a LC_{50} of 4.3 µg/ l. In comparison with *Daphnia magna*, the two *Euplotes* species were more tolerant to Cd, Hg and Zn. Rehman *et al*. (2008)

reported that *S. mytilus* reduced 60% mercury (10 µg/mL) from the medium after 48 hours, 80% after 72 hours and 90% after 96 hours.

Zinc

Zinc (Zn^{2+}) is an interesting metal nutritionally essential to living organisms in trace amount (µM). The element has multiple intracellular functions as a catalytic or structural constituent of more than 300 enzymes, including alkaline phosphatase, alcohol dehydrogenase, Cu-Zn superoxide dismutase and carbonic anhydrase (Vallee and Falchuk, 1993; Vallee and Maret, 1993). Zinc also plays a critical structural role in many proteins. For example, several motifs found in transcriptional regulatory proteins are stabilized by Zn, including the zinc finger, Zn cluster and RING finger domains (Bohm *et al*., 1997; Schjerling and Holmberg, 1996). Inside cells, Zn is neither oxidized nor reduced; thus the essential roles of zinc in cells is based largely on its behaviour as a divalent cation that has a strong tendency to form stable tetrahedral complexes (Berg and Shi, 1996). Metallic zinc has been used for a variety of applications such as galvanization, the manufacture of brass and other alloys and fabrication of batteries (Barceloux, 1999). The wide involvement of Zn in biological functions has resulted in an expanding interest in understanding the Zn metabolism.

Zinc toxicity

Domestic sources of zinc are corrosion and leaching of plumbing, water-proofing products, anti-pest products, wood preservatives, deodorants and cosmetics, medicines and ointments, paints and pigments, printing inks and colouring agents. The commercial sources are galvanization processes, brass and bronze alloy production, tires, batteries, paints. Plastics, rubber, fungicides, paper, textiles, taxidermy, building materials, special cements, and also cosmetics and pharmaceuticals (Shomar *et al*., 2004). The extensive use of zinc without its recovery has caused contamination of soil and fresh water habitats by this divalent cation (Talbot, 1986; Jung and Thornton, 1997; Vasconcelos and Tavares, 1998). Also due to low melting point of this metal, industrial plants that employ the roasting or heating of zinc compounds for metallic zinc production are potential sources of environmental zinc contamination (Nies, 1992; Barceloux, 1999).

Madoni *et al*. (1996) reported that an 80% mortality and the reduction of species richness to 9 species out of 16 was observed in the presence of 81 mg/l of soluble Zn, and two species were still surviving in the presence of 145 mg/l.

During a study, 0.57 mg/l of zinc caused the disappearance of only one species, and 11% mortality in the whole protozoan community. The LC_{50} values of zinc for *Drepanomonas revoluta* (254 µg/l) and *Spirostomum teres* (672 µg/l) were low in comparison to the other tested ciliates (1,050 to 192,000 µg/l); this emphasizes the high sensitivity showed by these two ciliate species to zinc (Madoni *et al.,* 1994). The 24-h LC_{50} value of zinc for *Spirostomum teres* was 8.93 mg/l (Twagilimana *et al.,* 1998).

Ruthven and Cairns (1973) found that 1.36 mg Zn/l supplied as zinc sulphate was lethal for *Tetrahymena pyriformis*, while other freshwater ciliates were killed by concentrations between 1.2 and 24 mg Zn/l. Parker (1979) reported a 24-h LC_{50} value of 192 mg Zn/l to the marine ciliate *Uronema marinum*. Zn interferes with Ca metabolism is also indicated by suppressed uptake of Ca in *Tetrahymena* exposured to 0.5 and 1mM Zn^{2+} (Jones *et al.*, 1984). *Tetrahymena pyriformis* was used as an alternative method to the standard direct count of viable protozoa under light microscopy to assess copper and zinc cytotoxicity in protozoa (Dias and Lima, 2002; Nicolau *et al*., 2004).

Nilsson (2003) studied that proliferating *Tetrahymena* exposed to high sublethal amounts of Zn^{2+} experiences a major hazard initially. Due to massive entry of Zn, cell motility is affected but once the cells have circumvented the hurdle and restored homeostasis (during the lag period), they can resume proliferation. Hence, apart from the initial hazard, excess amount of Zn has no adverse long time effects on *Tetrahymena*, only a somewhat prolonged generation time, *i.e*. the metabolic cost of the intracellular handling of excess Zn.

Zinc uptake and detoxification

Zinc in excess is toxic. So, both eukaryotes and prokaryotes have developed mechanisms to prevent over-accumulation of zinc, for example, induction of metallothioneins that sequester zinc (Huckle *et al*., 1993; Palmiter, 1998). Another mode of zinc regulation is likely to be at level of transporters that facilitate zinc influx during deficiency and efflux during excess. Despite the obvious importance of zinc homeostasis, little is known about these transporters at the molecular level (Nies, 1995).

Cardinaletti *et al.* (1990) reported that the protozoa community of an activated sludge plant treating wastes containing 0.6-1.2 mg/l of soluble zinc was not affected neither in density and species richness. Zinc was less toxic to the seven ciliate species than the other tested metals. For six species the 24-h LC_{50}

values were similar and ranged from 1.05 to 3.10 mg/l. *Euplotes patella* showed a very low sensitivity to zinc, reaching a 24-h LC_{50} value of 50 mg/l. The order of toxicity of the four metals to the seven ciliate species tested was generally: Cu > Hg > Cd > Zn (Madoni *et al.*, 1992).

Shakoori *et al*. (2004) reported that the presence of Zn did not make any significant effect on the movement of *Vorticella microstoma.* The cell population, which is indicator of mitotic activity, was reduced from 1320 to 880 cells in $ZnSO_4$ solution. The order of resistance regarding molar concentrations of metal ions of *Vorticella microstoma* against five metals was Zn^{2+}> Cu^{2+}> Pb^{2+}> Cr^{6+}> Cd^{2+}. The ciliate showed remarkable ability to pick up heavy metal ions from the culture medium. The concentration of Zn^{2+} was reduced 99.2% after 192 hour when ciliates *(V. microstoma)* were grown in the culture medium containing Zn^{2+} (100 µg/ml). Rehman *et al*. (2008) reported that *S. mytilus* could decrease 90% of zinc (10 µg/mL) from the medium after 48 hours, 94% after 72 hours and 98% after 96 hours.

Nickel

Nickel is a naturally occurring silvery metal that is used in wide variety of consumer and industrial products (Harte *et al.*, 1991). The greatest concentration occurs in the air, soil and water in the vicinity of nickel-producing factories, metal refineries and municipal solid waste incinerators (Harte *et al*., 1991). The nickel burden of the world's fresh waters is about 3.4×10^7 kg, and rivers transport 1.35×10^9 kg $year^{-1}$ of this metal (Nriagu, 1980).

A significant concentration of nickel is present in industrial and municipal discharges (3.8×10^6 kg $year^{-1}$), particularly in steel mill and electroplating wastes. Dissolved nickel levels in unpolluted fresh water usually range from 1 to 3 µg l^{-1}, and input from mixed industrial urban sources may increase to 10-15 µg l^{-1} (Snodgrass, 1980), whereas natural intrusions of nickel-bearing rock have produced total residues of 200 µg l^{-1} in overlying water (Agrawal *et al*., 1978).

Nickel toxicity

Nickel is a problematic heavy metal (Joho *et al*., 1995). Higher concentrations of nickel are toxic (Dalton *et al*., 1985). Ashworth and Alloway (2004) reported during a soil column leaching study that Ni concentration showed potential environmental significance, due to relatively poor retention of Ni by the sludge solid phase.

Nickel has a known effect upon ciliary activity and, in particular, on microtubule sliding in cilia (Zanetti *et al*., 1979). Only a few papers describe the effect of nickel on the feeding rate of the marine hypotrich *Euplotes mutabilis* (Al-Rasheid and Sleigh, 1994) and on the soil ciliate *Colpoda steinii* (Campbell *et al*., 1997). Nickel exerted the smallest toxicity towards the hypotrich *E. patella*, with concentration values of between 6 and 10 mg/l (24-h LC_{50}, 7.70 mg/l). Greatest toxicity was observed for *Spirostomum teres* with minimum and maximum values of 0.13 and 0.25 mg/l (24-h LC_{50}, 0.17 mg/l). Higher sensitivity to nickel was displayed by two species of *Paramecium* (*P. bursaria* and *P. caudatum*). These free-swimming ciliates had a similar response to the heavy metal, with 24-h LC_{50} values of 0.36 and 0.49 mg Ni / l, respectively (Madoni, 2000).

Larsen and Nilsson (1983) found that, in an organic medium, nickel ions inhibited the movement of the ciliate *Tetrahymena* at a concentration of 352 mg/l, which also reduced the rate of endocytosis. Madoni (2000) reported in a study that seven out of the 12 treated species showed sensitivity to nickel in a range of concentrations between 0.83 and 2.0 mg/l, with 24-h LC_{50} values from 1.02 to 1.40 mg/l. *Spirostomum teres* showed the highest sensitivity not only for nickel, but also for copper, mercury, and zinc. This micro-aerophilic ciliate is able to tolerate low dissolved oxygen values in freshwater environments (Bick, 1972).

Nickel exerted a moderate toxicity to freshwater ciliates. By examining data from literature concerning acute toxicity of heavy metals on ciliates, it follows that nickel was considerably less toxic than cadmium, copper, or mercury. The order of toxicity was generally: Cu> Hg> Cd> Ni> Cr> Zn.

Sewage water often contains considerable amount of toxic metals such as cadmium, chromium, copper, lead, nickel, and zinc (Campbell *et al*., 1988). Daily mean soluble nickel concentrations in activated sludge plants were estimated to range from 30 µg/l (Goldstone *et al*., 1990) to 50 µg/l (Abraham *et al*., 1997). Nevertheless, in plants receiving industrial sewage such as from electroplating or steel mill, higher levels of soluble nickel may depress populations of ciliates, affecting the efficiency of the activated-sludge treatment process.

Nickel uptake and detoxification

The hypotrich *Euplotes patella* showed the lowest sensitivity for both

nickel and most of other tested metals. *E. patella* can resist Ni concentration up to 10 mg/l (24-h LC_{50s}) (Madoni, 2000). The 1-hour LC_{50} of $Ni^{2+,}$ Hg^{2+}, Cd^{2+}, Pb^{2+} and Cu^{2+}, for the protozoan *E. mutabilis* have been reported to be 3.9, 1.0, 0.48, 0.37 and 0.29 µg/ml, respectively (Al-Rasheid and Sleigh, 1994). The 24-h LC_{50} of Ni^{2+}has been reported to be 7.70 mg/l for *Stylonychia pustulata*. These organisms are able to survive in the presence of heavy metal concentrations higher than those normally observed in the activated sludge mixed liquor and actively contribute to the amelioration of the effluent quality (Madoni, 2000).

Rehman *et al*. (2008) reported that *S. mytilus* was found to resist Ni^{2+} up to a concentration of 16 µg/mL. The ciliate decreased 49% nickel after 48 hours, 61% after 72 hours and 73% after 96 hours from the medium containing Ni^{2+} at a concentration of 5 µg/mL

Conventional methods for the treatment of industrial effluents are very costly and require either high energy or large quantities of chemicals, which means adding more chemicals to the environment. Therefore, there is a need for more practical cost effective and efficient method for environmental clean up and decontamination of the industrial wastewater of toxic contaminants including heavy metals. One such method, bioremediation is the use of microorganisms for the treatment of contaminated toxic wastes. Extensive work has been done in different laboratories on the isolation of metal resistant microorganisms (bacteria, yeast, algae and protozoa) from industrial wastes and then use them for decontamination of wastewater of different toxic metal ions (Piccinni and Albergoni, 1996 Salvado *et al*., 1997; Coppellotti, 1998; Fernandez-Leborans and Herrero, 1999; Haq *et al*., 2000; Rehman and Shakoori, 2001: Kamaludeen *et al*., 2003; Viti *et al*., 2003: Megharaj *et al*., 2003; Malik, 2004; Shakoori *et al*., 2004; Rehman *et al*., 2006, 2007, 2008).

The presence of metal resistant ciliate in industrial effluents carrying highly toxic metal ions has indicated adaptation of these organisms to environment containing toxic metals and may be employed for metal detoxification operations (Madoni *et al*., 1994, 1996; Piccinni and Albergoni, 1996; Salvado *et al*., 1997; Fernandez-Leborans *et al*., 1998; Coppellotti, 1998; Fernandez-Leborans and Herrero, 1999; Bonnet *et al*., 1999; Haq *et al*., 1998, 2000; Shakoori *et al*., 2004; Rehman *et al*., 2005, 2006, 2007). The presence of metal tolerant protozoa in industrial effluents indicates ability of these isolates to resist such stressful conditions and metal tolerant protozoans can be employed for the removal toxic metal ions from the wastewaters.

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