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PROCEEDINGS OF THE CONGRESS

Editors

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Dr. A.R. Shakoori

Computerized Composed by: Amjad Ali

PROCEEDINGS

OF

PAKISTAN CONGRESS OF ZOOLOGY

Volume 27, 2007

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

TWENTY SEVEN PAKISTAN CONGRESS OF ZOOLOGY

held under auspices of

THE ZOOLOGICAL SOCIETY OF PAKISTAN

at

BAHAUDDIN ZAKARIYA UNIVERSITY, MULTAN

February 27 to March 1, 2007

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ACKNOWLEDGMENTS

Bahauddin Zakariya University, Multan**,** hosted the 27th Pakistan Congress of Zoology (International).

The Zoological Society of Pakistan expresses its deep gratitude to the Vice Chancellor, Bahauddin Zakariya University, Multan and faculty members and students of the Department of Zoology and Institute of Applied and Pure Biology 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 SEVEN PAKISTAN CONGRESS OF ZOOLOGY (INTERNATIONAL)

BAHAUDDIN ZAKARIYA UNIVERSITY, MULTAN

February 27 to March 1, 2007

PROGRAMME

TUESDAY, FEBRUARY 27, 2007

08:30 AM Registration
10:00 AM Inauguration: Recitation from the Holy Quran
10:05 AM Welcome Address by the Vice Chancellor, Bahauddin Zakariya University, Multan
10:15 AM Address by the President, Zoological Society of Pakistan
10:25 AM Distribution of Medals and Awards
10:45 AM Address by the Chief Guest
11:15 AM Vote of Thanks by the Director, Institute of Applied and Pure Biology
11:25 AM Refreshment

JOINT SESSION I: (Plenary Lectures)

Chairperson: Prof. Dr. A.R. Shakoori
Co-chairperson: Dr. Aleem Ahmad Khan

Speakers: 1. **Dr. G. Rassam:**
Executive Secretary, American Fisheries Society, Bethesda, Maryland, USA
Fisheries; A Global Science in the 21st Century

2. **Dr. Peter C. Wynn**
Faculty of Veterinary Sciences, University of Sydney, Camden NSW 2570, Australia.
Comparative genomics and lactation making more money from milk

01:00 PM Lunch and Prayer

HALL – 1

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

SESSION I

	Chairperson:	Dr. Javed Iqbal Mirza
	Co-chairperson:	Dr. Zahida Tasawar
02:00 AM	Paper reading	
04:30 PM	Tea Time	

SESSION II

	Chairperson:	Prof. Dr. Syed Shahid Ali
	Co-chairperson:	Dr. Farah R. Shakoori
05:00 PM	Paper reading	
06:30 PM	Prayer	

SESSION III

	Chairperson:	Prof. Dr. Shamsuddin Shaikh
	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. Amanullah Khan
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 II: PEST AND PEST CONTROL

SESSION III

	Chairperson:	Prof. Dr. Imtiaz Ahmad
	Co-chairperson:	Dr. Abida Butt
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. Muhammad Ali
	Co-chairperson:	Prof. Dr. N.T. Narejo
02:00 AM	Paper reading	
04:30 PM	Tea Time	

SESSION II

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

SESSION III

	Chairperson:	Dr. Abdul Aziz Khan
	Co-chairperson:	Dr. Zafar Iqbal
06:45 AM	Paper reading	
08:00 PM	Dinner	

WEDNESDAY, FEBRUARY 28, 2007.

JOINT SESSION II: (Plenary Lectures)

Chairman: Prof. Dr. A.R. Shakoori
Co-chairman: Prof. Dr. Peter C. Wynn

09:00 AM 1. **Dr. R.D. Bush**
Faculty of Veterinary Sciences, University of Sydney, Camden, New South Wales, Australia.
Disease investigation in Australian sheep flocks.

2. **Dr. David McGill**
ReproGen. The University of Sydney, Australia.
The mathematical modeling of lactation curves and persistence of lactation in dairy sheep.

HALL – 1

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

SESSION IV

	Chairperson:	Dr. Maqsood Ahmad
	Co-chairperson:	Dr. Abdul Rehman
10:00 AM	Paper reading	
11:00 PM	Tea Break	

SESSION V

	Chairperson:	Prof. Dr. M. Aslam Khan
	Co-chairperson:	Dr. Javed Iqbal Qazi
11:30 AM	Paper reading	
01:00 PM	Lunch and Prayer	

SESSION VI

	Chairperson:	Prof. Dr. A.R. Abbasi
	Co-chairperson:	Dr. Shahid Nadeem
02:00 PM	Paper reading	
04:30 PM	Tea Break	

SESSION VII

	Chairperson:	Prof. Dr. Sikandar Hayat
	Co-chairperson:	Dr. Khalil Ahmad
05:00 PM	Paper reading	
06:30 PM	Prayer	

HALL – 2

SECTION II: PEST AND PEST CONTROL

SESSION IV

	Chairperson:	Dr. M. Afzal Kazmi
	Co-chairperson:	Dr. Bilquees Fatima
10:00 AM	Paper reading	
11:00 PM	Tea Break	

SECTION III: ENTOMOLOGY

SESSION I

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

SESSION II

	Chairperson:	Prof. Dr. M.S. Wagan
	Co-chairperson:	Dr. Alamzeb
02:00 PM	Paper reading	
04:30 PM	Tea Break	

SESSION III

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

HALL – 3

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

SESSION IV

	Chairperson:	Dr. Muhammad Ayub
	Co-chairperson:	Dr. Farzana Parveen
10:00 AM	Paper reading	
11:00 AM	Tea Break	

SESSION V

	Chairperson:	Dr. Aleem Ahmad Khan
	Co-chairperson:	Dr. Syed Anwar Ali Shah
11:00 AM	Paper reading	
01:00 PM	Lunch Break and Prayer Break (Zuhar)	

SESSION VI

	Chairperson:	Prof. Dr. Muhammad Akhtar
	Co-chairperson:	Dr. S.M. Leghari
02:00 PM	Paper reading	
04:30 PM	Tea Break and Prayer Break (Asar)	

SECTION IV: PARASITOLOGY

SESSION I

	Chairperson:	Dr. M. Nawaz
	Co-chairperson:	Dr. A. Arijo
05:00 PM	Paper reading	

06:30 PM Prayer Break (Maghrib)
07: PM General Body Meeting
08:00 PM Dinner

THURSDAY, MARCH 3, 2007

JOINT SESSION III: (Plenary Lectures)

Chairman: Prof. Dr. M. Naseem Siddiqi
Co-chairman: Dr. M. Suleman

09:00 AM 1. **Prof. Dr. Nasim Siddiqui**
Artificial Beef: Fish at your door step

2. **Prof. Dr. Peter C. Wynn**
Game meats – Health alternatives
Wildlife and Milk Product

3. **Dr. Aslam Khan, Lahore**
Dengue virus and mosquitoes in Pakistan

4. **Dr. Inamullah Khan**
Nuclear Institute for Food and Agriculture, Peshawar
Glimpses of scientific signs in the Noble Quran.

5. **A. Rauf**
School of Biological Sciences, University of the Punjab, Lahore
Darwin's Evolution Theory and Islam.

11:00 PM Tea Break

HALL – 1

SECTION IV:PARASITOLOGY

SESSION II

Chairperson: Prof. Dr. F.M. Bilqees
Co-chairperson: Dr. Zahida Tasawar

11:30 AM Paper reading
01:30 AM Lunch and Prayer Break

HALL – 2

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

SESSION VI

Chairperson: Prof. Dr. Q.B. Kazmi
Co-chairperson: Dr. Itrat Zehra
11:30 AM Paper reading
01:30 AM Lunch and Prayer Break

02:30 PM Concluding Ceremony
Recitation
Congress Report by President ZSP
Award Ceremony
Concluding Remarks by the Chief Guest
Vote of Thanks
03:30 PM Refreshments

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

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Website: www.zsp.org.pk

CITATIONS

RECIPIENT OF ZOOLOGIST OF THE YEAR AWARD 2007*

Mr. Mohammad Irshad
Senior Entomologist, National Insect Museum, Institute of Plant & Environmental Protection, NARC, Park Road, Islamabad

Mr. Mohammad Irshad has spent 43 years in the service of Zoology especially Entomology. He has vast research, as well as research management experience. He has contributed about 126 publications on different aspects of Entomology. He has contributed papers on taxonomy of mites and insects, biological control of insects and weeds, integrated pest management of different crops, chemical control, resistance to insecticides, loss assessment/ reduction in grain storage. One of his papers was given the best paper award by Pakistan Society of Sugarcane Technologist in 1981.

Mr. Irshad has developed economically viable and environment friendly technology for the control of storage grain pests, which cause huge economic losses. These technologies have been transferred to farmers and commercial grain handlers through training courses, leaflets, brochures, lectures and television programmes. By adopting these techniques farmers are saving millions of Rupees.

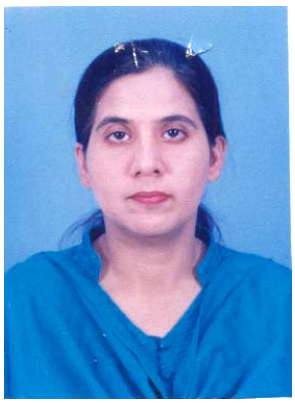
*Other nominees for this award were Prof. Dr. M. Arshad Azmi, Prof. Dr. Muhammad Khan Lohar, Prof. Dr. M. Nazir Bhatti, Dr. Abdul Aleem Chaudhary.

For the first time in Pakistan Mr. Irshad studied resistance in the storage pests and developed resistance management techniques. His work on control of sugarcane pests is outstanding and unique. In the Punjab, he along with this team managed the infestations of Gurdaspur borer during 1978-1979 through non-chemical methods. Losses worth millions of Rupees were saved by the techniques developed by him. In NWFP, his work led to successful management of sugarcane *Pyrilla* and other pests. Thus aerial spraying was stopped and reliance on pesticides was minimized. As a consultant in the Philippines, he developed IPM technology of cocoa pod borer, a devastating pest of Cocoa. These control techniques are now used extensively in the Far Eastern countries.

Mr. Irshad has published eight books, and authored 2 chapters in other books. He supervised theses of M.Sc. students of NWFP Agricultural University, Peshawar and Arid Agriculture University, Rawalpindi.

Now, he is being awarded “Zoologist of the Year Award 2007” by the Zoological Society of Pakistan for his significant contributions in the filed of Zoology.

RECIPIENT OF
PROF. DR. MIRZA AZHAR BEG GOLD MEDAL 2007*



Dr. Zakia Khatoon
Senior Scientific Officer, Food & Marine Resources Research Centre, University of Karachi, Karachi.

Dr. Zakia Khatoon, Senior Scientific Officer has been working in the Food & Marine Resources Research Centre of PCSIR Labs. Complex since 2002. She is a Fishery Biologist, working on Fin Fish culture and on other aspects of fish. Presently she is engaged in a PSDP Project entitled "Production of *Artemia* cysts, biomass and its products". The study is in progress and expected to develop *Artemia* culture and their by-products. Before Dr. Khatoon joined PCSIR, she served as Research Associate in research projects sponsored by Pakistan Science Foundation, Islamabad at Centre of Excellence in Marine Biology, University of the Punjab. She was also engaged as Co-operative Teacher/Research Fellow to teach Fisheries courses at the Centre of Excellence in Marine Biology, University of Karachi. She has published thirteen research papers in national and international journals and had compiled three research project reports.

*Other applicant for this award was Mr. Shahid Hafeez.

RECIPIENT OF
PROF. DR. NASIMA TIRMIZI GOLD MEDAL 2007*

Prof. Dr. Javed Mustaquim
Director, Centre of Excellence Marine Biology, University of Karachi, Karachi.

Professor Javed Mustaquim did his M.Sc. in Zoology from Karachi University in 1972 and Ph.D. in Zoology from London University in 1982. He joined Karachi University in 1974 as Research Officer and became Assistant Professor of Marne Biology in 1984. He was selected as Associate Professor in 1993 and full Professor of Marine Biology in 1997.

Prof. Mustaquim was appointed Director of the Centre of Excellence in Marine Biology at Karachi University in 2005. He has published several research papers and completed many research projects as Principal Investigator, funded by UGC, PARC and PSF. He was involved in Asia-Link Project on Climate Change sponsored by European Union. In 2006, a new species of polychaete worm (*Lumbrinereis mustaquimi*) was named by Dr. Luis of Mexico in recognition of his publications on polychaetes from Pakistan.

*Other applicant for this award was Dr. Razia Sultana.

RECIPIENTS OF
GOLD MEDALS AWARDED BY THE ZOOLOGICAL SOCIETY OF PAKISTAN

1. Muzaffar Ahmad Gold Medal 2007

Fourteenth Muzaffar Ahmad Gold Medal 2007 was received by Miss Saira Nazar for obtaining first position in the M.Sc. Zoology examination of the University of the Punjab. Thirteen medal have already been given.

2. Ahmed Mohiuddin Memorial Gold Medal 2007

Sixth Ahmed Mohiuddin Memorial Gold Medal 2007 was given to Syeda Nida Fatima Jafri, who obtained first position in the M.Sc. Zoology examination of the University of Sindh, Jamshoro.

3. Prof. Imtiaz Ahmad Gold Medal 2007

Seventh Prof. Imtiaz Ahmad Gold Medal 2007 was given to Mr. Ishtiaq Ajnum for obtaining first position in the subject of Entomology for his M.Sc. Zoology examination of the University of Karachi.

4. Mujib Memorial Gold Medal 2007

Fourteenth Mujib Memorial Gold Medal 2007 was given to Nazia for obtaining first position in Parasitology for her M.Sc. Zoology examination of the University of Karachi.

5. Muhammad Afazl Hussain Qadri Memorial Gold Medal 2007

Twelve Muhammad Afazl Hussain Qadri Memorial Gold Medal 2007 was given to Nazia for obtaining first position in M.Sc. Zoology examination of the University of Karachi.

6. Prof. Dr. S.H. Naqvi Gold Medal 2007

This medal was not given this year.

7. Prof. Dr. A.R. Shakoori Gold Medal 2007

Three applications were received, but none of the applicants was recommended by the selection committee for the award.

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HEXAVALENT CHROMIUM RESISTANT BACTERIA ISOLATED FROM INDUSTRIAL EFFLUENTS AND THEIR POTENTIAL USE IN BIOREMEDIATION OF TOXIC WASTE WATERS

QAZI JAVED IQBAL, SADIA ANDLEEB, ABDUL REHMAN AND A.R. SHAKOORI*

School of Biological Sciences (ARS), Department of Microbiology and Molecular Genetics (AR), and Department of Zoology (QJI, SA), University of the Punjab, New Campus, Lahore 54590, Pakistan

Abstract.- The present study is aimed at assessing the ability of seven Cr^{6+} resistant bacterial isolates (CMBL.Cr3-Cr9), from industrial waste of tanneries in Sialkot, Pakistan. The minimum inhibitory concentrations of Cr^{6+} ranged between 6-8 mg/ml. The isolates showed optimum growth at 37°C and pH 6.0-8.2. Large sized plasmids (23kb) were present in all the isolates, which could not be cured with ethidium bromide. For determining the location of the gene for chromium resistance on chromosomes or plasmid, the DH5α cells of *E. coli* were successfully transformed with the plasmids of the bacterial isolates. The transformed cells did not grow on selective medium containing 300 µg/ml of Cr^{6+}, though they grew well on selective medium without Cr^{6+} which showed that resistance gene was not located on the plasmids. The isolates could reduce 70-82% of Cr^{6+} present in the growth medium (100 µg/ml) within 24 hours of incubation, which indicated that these isolates were efficient metal accumulators and therefore could be used for remediation of industrial wastewater.

Key words: Heavy metal resistance, plasmid curing, chromium accumulation, industrial wastewater, bioremediation**,** hexavalent chromium, chromium-resistant bacteria.

INTRODUCTION

Industrial wastes contain variety of toxic chemicals, *viz.* heavy metal ions, toxic aromatic hydrocarbons, pesticides etc. Increasing environmental pollution by heavy metals results from their increasing utilization in industrial processes (Nriagu and Pacyna, 1988). One of the most common polluting metals is chromium, arising from discharged effluents from leather tanning, chromium plating, cleaning and processing, wood preservation, and alloy preparation. The toxicity, mobility and bioavailability of Cr depend fundamentally on its chemical form. Chromium in the environment might be present mainly as Cr^{3+} and Cr^{6+}.

* Corresponding author: Email: arshak@brain.net.pk

Cr^{6+} is highly soluble and about 300 times more toxic than Cr^{3+}. On the other hand, Cr^{3+} precipitates at the average pH of natural waters. Tannery wastewaters contain mainly Cr^{3+} (Rutland, 1991). The nature and behaviour of Cr in wastewater depends on the physicochemical conditions of the effluents originating from various industrial sources (Kotas and Stasicka, 2000).

The biological effects of Cr depend on its oxidation state. Cr^{6+} is highly toxic to most organisms because it is capable of reacting with redox-active enzymes and small molecules to produce Cr^{5+}, Cr^{4+} and Cr^{3+}, as well as oxygen- and sulphur-centered radicals. All these species can damage DNA. Hence, the ability of Cr^{6+} to damage DNA depends on cellular redox systems. One such system depends on glutathione and produces Cr^{5+} and sulphur-centered glutathione-ethyl radical which may attack DNA (Shi and Dalal, 1990).

Several studies indicate that chromium is teratogenic in animals. It has been found that teratogenic effects of Cr^{6+} are more severe than Cr^{3+} (Jacquet and Draye, 1982). Cr compounds have been shown to cross the placenta and to induce abnormalities and lethality in mice (Junaid *et al.*, 1995), cleft palates and lethality in hamsters (Gale, 1986), abnormalities in chick embryos (Asmatullah *et al*., 1998, Asmatullah *et al.*, 1999, Gilani and Alibhai, 1999), and lethality in human embryos (Bona *et al.*, 1992) as well as in adults (Kurosaki *et al.*, 1995). Cr^{6+} has also been found to be toxic to osteogenesis (Puleo and Huh, 1995).

At present, chemical processes are commonly used to remove heavy metals from wastes. Such methods have several disadvantages, for instance, unpredictable metal ion removal, high reagent requirements, and the generation of toxic sludges (Ciba *et al.*, 1999). Recently, microbial bioremediation has emerged as an alternative technique to such traditional chemical treatments (Brierley, 1999).

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 (Parsek *et al*., 1995). Several heavy metal tolerance mechanisms have been reported in different types of cells *viz*., cell wall binding, decreased cell membrane permeability, active extrusion, uptake by vacuoles or organelles, and complexation with chelating agents such as metallothionein and phytochelatin proteins (De-Filippis and Pallaghy, 1994; Reed and Gadd, 1990). 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.

In the present study we are reporting hexavalent chromium resistant bacteria, isolated from waste water of tanneries of Sialkot, Pakistan, which have extraordinary ability to uptake heavy metal from the medium. The uptake ability of the bacteria has also been assessed with a view to highlight their potential to detoxify industrial wastes containing heavy metals.

MATERIALS AND METHODS

Sample collection

The water samples were collected from the industrial effluents of Sialkot and transferred to the laboratory in half filled screw capped oven sterilized bottles at 180ºC for 20 minutes. The pH and temperature (ºC) of the site of sample collection was also noted.

Isolation of chromium resistant bacteria

For isolation of chromium resistant bacteria, 10 ml of the wastewater sample was spread on Luria-Bertani (LB) agar plates (NaCl 1g, tryptone 1g, yeast extract 0.5 g in 100 ml distilled water, pH adjusted at 7.2 to 7.5, 1.5 g agar added and autoclaved) containing 0.5 mg of Cr^{6+}/ml of the medium, and then incubated at 37ºC for 24 hours. Isolated colonies were picked up with sterilized wire loop and streaked on LB agar medium plate containing 1 mg Cr^{6+}/ ml. It was again incubated at 37ºC for 24 hours. This process was repeated with successively higher concentrations of Cr^{6+} increasing 500µg every time, until the isolates stopped growing.

Physical and biochemical characterization

The isolates were Gram stained (Benson, 1994). The morphology of the colony was studied, and the sizes of colonies and cells were estimated using scales and ocular micrometer at 100X. Average of 3 cell measurements was taken.

For biochemical characterization the isolates were tested for catalase activity, motility, oxidase activity, nitrate reduction, and Voges-Proskauer test. Some specific tests were performed for further characterization of isolates such

as endospore formation test, acid formation test, utilization of different sugars, gelatin agar test, growth at 10ºC, 45ºC, 60ºC, growth in medium containing 6.5% and 7.5% NaCl and growth at different pH. The procedures adopted for all the above physical and biochemical tests were taken from Benson (1994), Cheesbrough (Cheesbrough, 1984), Collee and Marr (1996), and those of Bergey's Manual of Determinative Bacteriology.

Determination of optimum growth conditions

For optimum growth of the bacterial isolates, two parameters *i.e.* temperature and pH were considered. For determination of optimum temperature, 5 ml LB broth was added in 4 sets, each of three test tubes, for each isolate, autoclaved and inoculated with 20 µl of freshly prepared log phase culture of isolates. The four sets of tubes were incubated at 25ºC, 30ºC, 37ºC and 42ºC. After an incubation of 10 hours, their absorbance was taken at 600 nm and a graph was plotted with temperature at x-axis and absorbance along y-axis. The temperature at maximum growth was taken as optimum temperature.

For determination of optimum pH, test tubes having 5 ml LB broth were prepared in 9 sets, each containing 3 test tubes, for each isolate and their pH was adjusted at 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0, and then autoclaved. These tubes were inoculated with 20 µl freshly prepared culture of the isolates. After incubation period of 9-10 hours, their absorbance was taken at 600 nm wavelength and then graph plotted between pH (along x-axis) and absorbance (along y-axis). This graph provided information about the optimum pH.

Growth curves of isolates

For each isolate 50 ml LB broth was taken in one set consisting of 3 flasks, autoclaved and then inoculated with 50 µl of the freshly prepared inoculum. These cultures were incubated at their optimum temperature in a shaker at 60-80 rpm. An aliquot of culture was taken out in an oven sterilized tube, at regular intervals of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 24, 36, and 48 hours and their optical density was determined at 600 nm. A graph was plotted between the time of incubation (along x-axis) and optical density (along y-axis). The period of lag phase, log phase and stationary phase of each isolate was determined from the growth curve.

Estimation of Cr^{6+} processing ability of the isolates

Bacterial isolates were grown at their optimum pH and temperature in LB broth containing 100 µg Cr/ml in a conical flask. A control was run having100 µg Cr/ ml but no inoculum was added. Then 5 ml bacterial culture was taken out from each flask after 4, 8, 12, 16, 20, and 24 hours after inoculation, centrifuged at 3000 rpm for 15 minutes to separate the cells. The supernatant was used for chromium estimation by AA1275 atomic absorption spectrophotometer (Varian, USA) at 428.9 nm wavelength. A graph between concentration of Cr^{6+} and time in hours was plotted, which showed percentage reduction in the amount of chromium in the medium.

Determination of location of Cr processing gene

For determination of location of Cr resistance gene, either on the bacterial chromosome or on the plasmid, two pronged approach was adopted. If the Cr resistant isolate becomes sensitive to Cr after plasmid curing, the gene would obviously be plasmid borne. This would be further confirmed if the *E. coli* DH5α transformed with the isolated plasmid of Cr-resistant isolate acquires the resistance to Cr.

For plasmid curing, LB broth (5 ml) was added in 7 sets, each of 8 test tubes, for seven isolates. In each set, different concentrations of ethidium bromide (stock solution of 5 mg/ml was prepared) *viz*. 100,200, 300, 400, 500, and 600 µg/ml were added and inoculated with 50 µl log phase growth of each isolate and then incubated at 37ºC for 24 hours in a shaker. The culture having minimum growth was selected and 50 ml was spread on LB plates and incubated at 37ºC for 24 hours. Using grid pattern replica plating methodology (Ohman, 1988), one agar plate and one selective medium plate (with 100 µg Cr^{6+}/ ml) was prepared for each isolate. The plates were incubated at 37ºC for 24 hours. The boxes on the grid having growth were counted as well as those having no growth were also counted on both plates, selective and LB medium. The presence of colonies on LB plate and absence on selective medium were considered the cells having been cured.

For transformation (Sambrook *et al.*, 1989), the plasmid DNA (50µl), isolated according to Holmes (1984) was added to 0.2 ml of the competent cells of *E. coli* DH5α and incubated on ice for 30 minutes. Tubes were put in water bath at 42ºC for 2 minutes.1.5 ml of pre-warmed LB broth (at 37ºC) was added

and cells were allowed to grow at 37ºC for 4 hours in an incubator. The cells were spread on LB agar plate containing 300 µg Cr^{6+}/ml and incubated overnight. Competent cells were spread on LB plates without chromium, as control. Appearance of colonies on plates is an indication of positive result and confirms the presence of chromium resistance gene on the plasmid, while no growth on the plates shows its presence on the chromosome.

Statistical analysis

Observations were made and all the experiments run in triplicate. At least three separate plates/flasks were usually maintained for one treatment. The averages of control and experimental groups were compared and significant differences evaluated by using Student's 't' test of significance.

RESULTS

Chromium resistant bacteria

For plasmid curing Seven chromium resistant bacterial strains (CMBL Cr3-CMBL Cr9) were isolated from Sialkot industrial area, where the temperature ranged from 22ºC to 25ºC and pH ranged from 6 to 8.2. The MIC of chromium for various bacterial isolates varied from 6.0 to 8.0 mg /ml.

Table I shows physical and biochemical characteristics of various bacterial isolates. On the basis of this characterization CMBL.Cr3 and CMBL.Cr 9 have been identified as *Marinococcus albus,* CMBL.Cr4 and CMBL.Cr5 as *Kurthia* sp., CMBL.Cr6 and CMBL.Cr8 as *Vagococcus fluvialis* and CMBL.Cr7 as belonging to genus *Roseobacter.*

Growth curves

Figures 1 and 2 show the effect of temperature and pH on the growth of bacterial isolates. All the strains showed optimum growth at 37ºC, where as the optimum pH value varied from 6 to 8. Figure 3 shows growth curves of bacterial isolates grown in LB medium. Lag phase of all isolates is 3 hours except CMBL.Cr3, Cr4, Cr6 and Cr9 where lag phase is 6, 9, 9 and 12 hours, respectively. Log phases of isolates were 36 hours for CMBL.Cr3, Cr4, Cr6, Cr7, Cr9, while it was 24 hours for CMBL.Cr5 and Cr8.

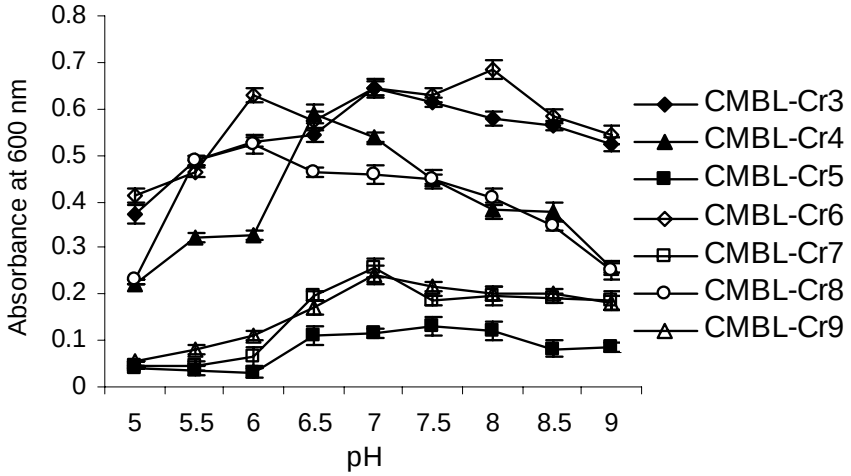


Fig.1. Effect of pH on the growth of chromium resistant bacterial isolates from industrial effluents of Sialkot.

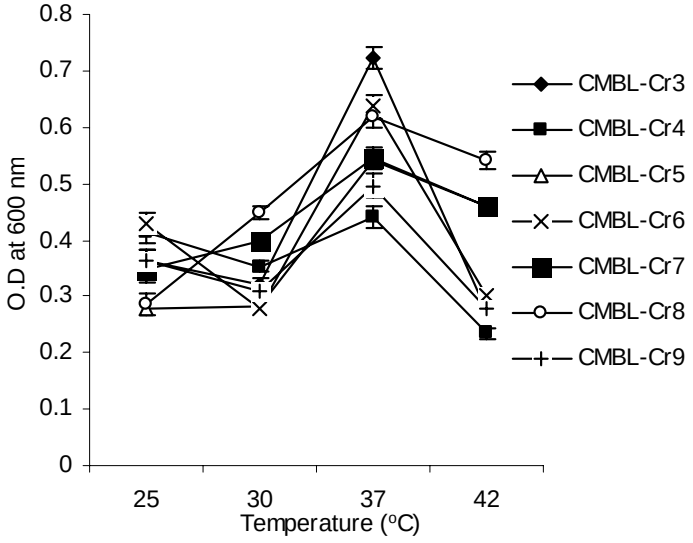


Fig. 2. Effect of temperature on the growth of chromium resistant bacterial isolates from industrial effluents of Sialkot.

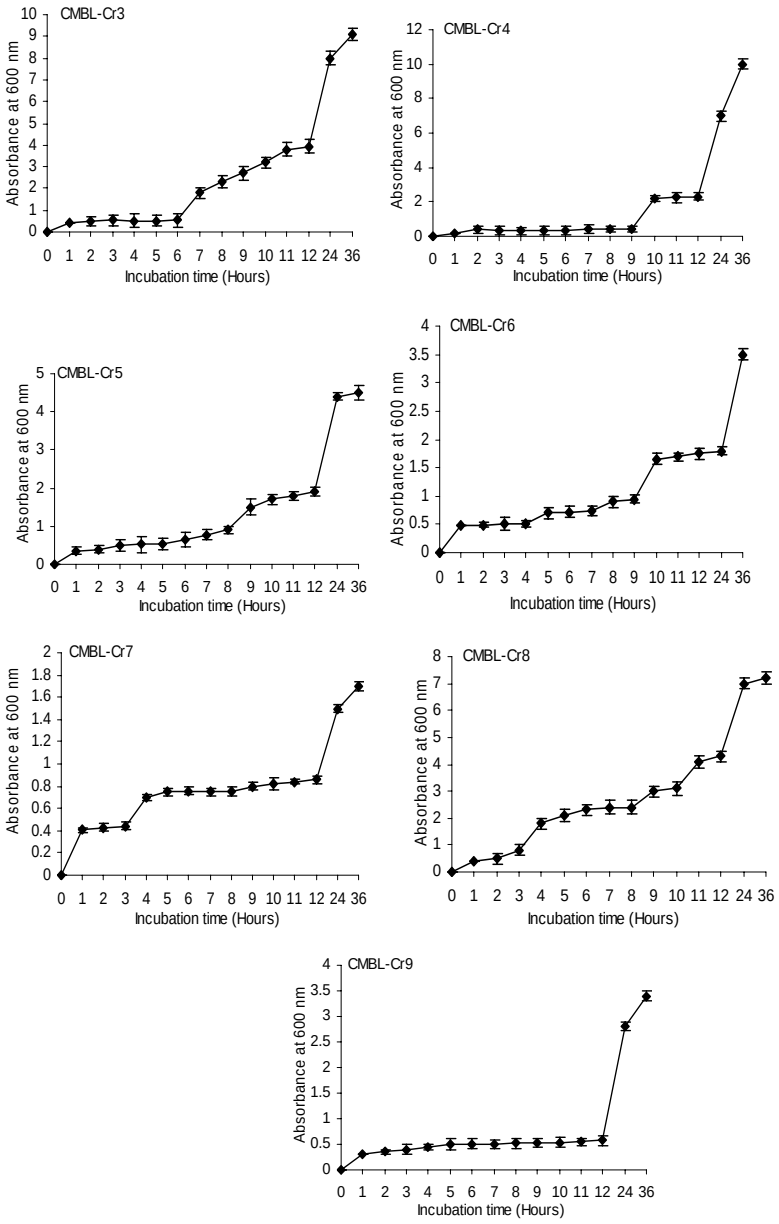


Fig. 3. Growth curves of chromium resistant bacterial isolates in LB medium.

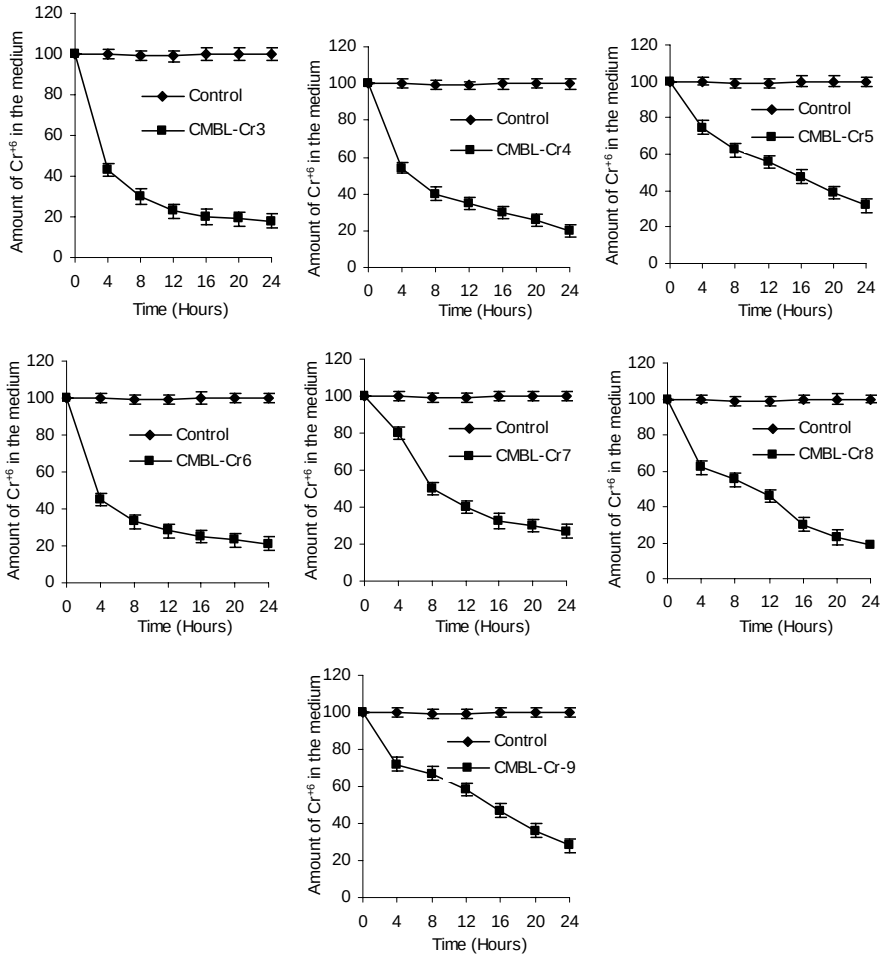
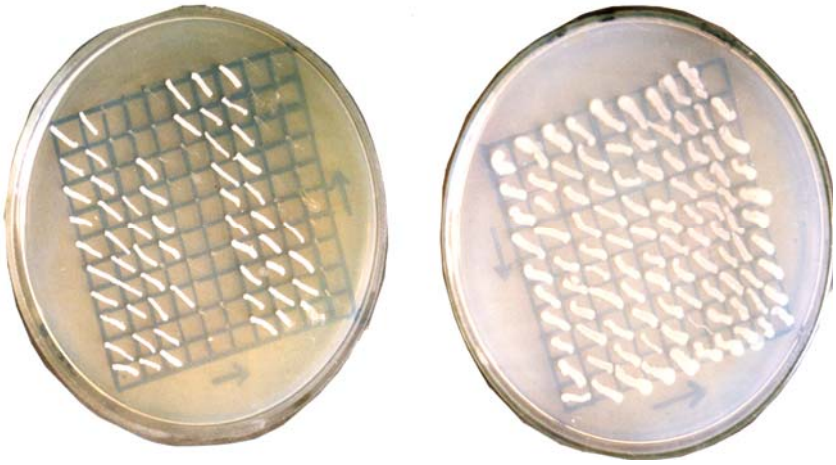


Fig. 4. Metal processing ability of chromium resistant bacterial isolates The bacterial isolates were grown in LB medium containing 100 µg Cr^{6+}/ml at 37ºC for 24 hours. Every 4 hours samples of the medium were taken out and processed for Cr^{6+}/ml estimation. The graph shows percent reduction in the amount of chromium in the medium.

Metal processing ability

The ability of the isolates to accumulate the metal was checked. CMBL.Cr3, Cr4, Cr5, Cr6, Cr7, Cr8 and Cr9 could accumulate 82%, 80%, 75%, 79%, 72.5%,

81.85% and 71.7% Cr^{6+}, respectively (Fig. 4). The isolates were very efficient in metal accumulation.



(A) **(B)**

Fig. 5. Growth of ethidium bromide treated colonies of Cr^{6+}-resistant CMBL-Cr7 on LB agar plate without Cr (A) and with 100µg Cr^{6+}/ml (B). Since almost all the bacterial colonies (99 out of 100) survived and showed robust growth in the presence of Cr, it implies that Cr resistant gene is not present on plasmid, which is removed after ethidium bromide treatment. The Cr resistant gene is apparently on the chromosome.

Location of heavy metal resistance gene

The bacterial isolates were treated with ethidium bromide and the percentage curing observed was 0% (data not being shown). Since plasmids are present in the bacterial isolates, the heavy metal resistance gene for chromium is apparently not plasmids borne in these isolates.

Transformation of *E. coli* DH5α with the plasmids of the isolates further confirmed the absence of chromium resistant gene on the plasmids. *E. coli* DH5α, both competent as well as non-competent cells showed no growth on selective medium containing 300 µg Cr^{6+}/ml, and competent cells also did not show any growth on the selective medium. The competent cells transformed with plasmids of isolates showed no growth even after 36 hours, on the selective medium, while these showed growth on control plates, showing absence of chromium resistant gene on the plasmid.

DISCUSSION

It is well recognized that microorganisms have a high affinity for metals and can accumulate both heavy and toxic metals by a variety of mechanisms. Microorganisms highly effective in sequestering heavy metals include bacteria, fungi, algae and actinomycetes (Pas *et al.*, 2004; Shakoori and Muneer, 2002). These have been used to remove metals from polluted industrial and domestic effluents on a large scale. Microbiological detoxification of polluted water is economical, safe, and sustainable (Eccles, 1995).

Chromium resistance and bioaccumulation has been studied in yeast (Bingol *et al.*, 2004; Dar and Shakoori, 1999; Pas *et al.*, 2004), protozoa (Haq *et al.*, 2000, Madoni *et al.,* 1996, Shakoori *et al.*, 2004), and plants (Acar and Malkoc, 2004, Bai and Abraham, 2003, Bennicelli *et al.*, 2004, Gardea-Torresdey *et al.*, 2004, Maine *et al.*, 2004). Fungal biomasses have proven useful in biosorption studies for the removal of Cr from contaminated sources (Cervantes *et al.,* 2001). Metal resistant algae have been reported in waste waters and metal polluted environment. These algae process and detoxify heavy metal ions usually through bio-sorption, adsorption and bio-accumulation (Chojnacka *et al.*, 2004, Pena-Castro *et al.*, 2004; Rehman and Shakoori, 2001).

Seven chromium resistant bacteria have been isolated from the industrial effluents, which could tolerate 6 to 8 mg /ml of chromium. Such bacteria have been reported from other laboratory too (Idachaba *et al.*, 2004; Michel *et al.,* 2003; Vala *et al*., 2004). Srinath *et al.* (2002) reported that living and dead cells of *Bacillus coagulans* biosorbed 23.8 and 39.9 mg Cr/g dry weight, respectively, whereas, 15.7 and 30.7 mg Cr/g dry weight was biosorbed by living and dead cells of *Bacillus megaterium*, respectively. A variety of chromate-resistant bacterial isolates has been reported, including strains from environmental and clinical setting; in these natural isolates chromate tolerance is usually associated with plasmids (Cervantes and Silver, 1992).

Reduction of Cr^{6+} to less toxic Cr^{3+} has been suggested as an additional chromosome-encoded chromate-resistance mechanism, in addition to the plasmid-encoded tolerance. *E. cloacae* HOI strain, described as chromate-resistant (Wang *et al*., 1989), is the most studied example of Cr^{6+} reducing bacteria (Ohtak and Silver, 1994). *Bacillus* sp. QCI-2, a strain isolated from a Cr-polluted zone, was also selected by its ability to both tolerate chromate and reduce Cr^{6+} to Cr^{3+} (Campos *et al.*, 1995). A Cr^{6+}-reducing activity was found in

both soluble and membrane fractions of *Desulfovibrio vulgaris* (Lovley and Phillips, 1994).

Metal processing ability of the seven bacterial isolates shows that these isolated strains are very efficient metal accumulator/processor. The maximum metal accumulated was by CMBL Cr3 *i.e*. 82%, proving that these strains are very effective in using for chromium polluted environment. Sawada *et al.* (2004) reported a new and an efficient process for the removal of hexavalent chromium from contaminated soils. Chromate anions were removed from the soil to the anodic reservoir by the moving force of electromigration. The adsorbent (immobilized tannin) used was chemically incorporated into cellulose. Cr^{6+} was found to be adsorbed to this adsorbent efficiently. Jin *et al.* (2001) reported a NADH-dependent Cr^{6+} reductase gene from *Pseudomonas aeruginosa* HP104. The enzyme of Cr^{6+} reductase reduces the compounds to the relative stable and non toxic Cr^{3+} state.

With an object to locate gene for chromium resistance, the bacterial isolates were treated with ethidium bromide. The bacterial isolates were not cured. These results indicate that ethidium bromide is not a good curing agent for these isolates or the chromium resistant gene for these isolates is not present on plasmids. For this, transformation of *E. coli* DH5α was done. The transformation was successful as the competent cells transformed with plasmids of isolates showed no growth on the selective medium containing chromium, while it showed growth on control plates. Hence this clearly indicates that gene for resistance against Cr^{6+} is present on chromosomal DNA in all these isolates. Failure of curing, successful transformation and no growth with transformed cells on chromium selective medium concludes presence of gene for chromium resistance on the chromosomal DNA for these isolates. No doubt bacteria have developed diverse resistance mechanisms towards toxic metals. Additional chromosome-encoded chromate-resistance mechanism, in addition to the plasmid-encoded tolerance, is also reported in bacteria (Cervantes and Silver, 1992; Ohtake and Silver, 1994).

Amelioration of environmental pollution by microorganisms is a promising technology. The objectives can best be achieved by ensuring maximum productivity, by providing optimal environmental conditions for their growth. In present study we have reported the isolation of chromium resistant bacteria which have maximal metal accumulating ability. Removal of toxic metal ions from the environment is a very remarkable step towards biological control of

polluted environment and it is safe for human, animals and plants. This high metal accumulating capability of these organisms can be exploited for metal detoxification operations.

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TABLE I.- GENERAL PHYSICAL AND BIOCHEMICAL CHARACTERISTICS OF CHROMIUM RESISTANT BACTERIAL ISOLATES FROM INDUSTRIAL WASTEWATER.

Biochemical test	**CMBL.Cr3**	**CMBL.Cr3**	**CMBL.Cr5**	**CMBL.Cr6**	**CMBL.Cr7**	**CMBL.Cr8**	**CMBL.Cr9**
Color	Page yellow	Orange	Pale yellow	Off white	Orange	Off white	Yellow
Cell size (μm)	0.2	0.22	0.2	0.2	0.2	0.21	0.28
Shape of cell	Cocci	Rods	Rods	Cocci	Cocci	Cocci	Cocci
Gram-staining	+	+	+	+	-	+	+
Motility test	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+
Oxidase test	-	+	+	+	+	+	-
Nitrate reduction test	+	-	-	-	-	-	-
Endospores formation	-	-	-	-	-	-	+
Gelatin hydrolysis	N.D.	-	-	N.D.	N.D.	N.D.	N.D.
Grown in 6.5% NaCl	+	+	+	+	+	+	+
Grown in 7.5% NaCl	+	+	+	+	+	+	+
Grown at 10°C	-	+	+	-	+	+	+
Grown at 45°C	+	+	+	+	+	+	+
Grown at 60°C	-	N.D.	N.D.	N.D.	-	N.D.	N.D.
Growth at pH 9.6	+	+	+	+	+	+	+
Acid formation from:							
Glucose	N.D.	-	+	N.D.	N.D.	N.D.	N.D.

+, positive;-, negative; N.D. not determined.

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ALTERNATIVE SOURCES OF MEAT PROTEIN TO MEET A PROTEIN HUNGRY WORLD

NICOLE SPIEGEL AND PETER WYNN

Faculty of Veterinary Science, University of Sydney, NSW Australia 2570

Abstract.- The increasing world-wide demand for high quality animal protein has directed our attention to resources not previously exploited in any major way. However the uncontrolled reproductive potential of feral animal populations in the wild and of native wildlife in some environments provides a resource that can be utilized to assist in meeting this demand. Caution must be taken, however to ensure that these resources are not over-exploited. Care must also be taken to ensure the highest standards of food hygiene are followed with care being taken to ensure that zoonoses to not pass to the consumers of these products. Limitations on the use of some of these protein resources due to religious beliefs are acknowledged in this article.

Key words: Game meat, bush meat, Australian marsupials, Wallaby Kangaroo meat, emu meat.

INTRODUCTION

The use of wild animals as a source of protein for human ancestors has been identified as long as 5 million years ago. The appearance of *Homo sapiens* around 250,000 years ago ushered in an era in which animals were hunted for sources of both meat for food and skins for clothing to ensure survival of the species. The animals had to survive also under these same rigorous climatic conditions and so had developed homeorhetic mechanisms to enable them to store energy in the form of fat reserves in times when dietary energy resources were plentiful, which would then be available for mobilization when seasonal feed availability was limited. As a consequence of its highly calorific value, fat assumed great importance in providing a source of energy for human diets.

Animals in the wild undergo seasonal changes in carcass composition in order to survive in environments in which the availability of dietary energy sources fluctuates markedly. In many instances, these animals have adopted the strategy of adjusting their metabolic rate in order to conserve body energy reserves for the most essential of physiological functions including the maintenance of brain, heart, lung, visceral organ and reproductive function. Under such adverse conditions, fat depots are severely depleted with the

consequence that meat from harvested carcasses may be extremely low in fat content.

The influence of domestication

Many species were domesticated as nomadic lifestyles gave way to more permanent farming systems. Species chosen for animal production were generally the most productive and were present in large numbers in that environment. Thus the horse, camel, cattle and goat dominated farming systems in the grasslands of central Asia, while the llama and alpaca of the South American Highlands, the yak of Central Asia and the reindeer of booth North America and Europe were prevalent in these environments. In contrast, pigs, cattle, sheep and horses adapted to and were domesticated rapidly in wide regions of the populated world and as such these animals are no longer considered as game animals. Conventional animal breeding practices have resulted in the evolution of animals that are far more efficient at converting dietary nutrients into carcass tissues with conversion rates around 8kg of dry matter (DM) required to produce 1kg of body tissue for ruminant species. In contrast domestic poultry species are now converting feed (DM) to live weight at the rate of 1.5:1.

Game meat resources

Typically, indigenous animal populations are used for domestic meat production to meet dietary needs for animal protein in both developing and developed countries. These animals are mostly harvested from the wild, however a number of species have been domesticated to conventional farming systems under which their nutritional and health status can be monitored closely. In these systems, animals have typically not been subjected to commercial genetic selection programs, although there may be an inadvertent selection process for animals that are more tractable in semi-confined grazing systems. Inevitably, those with great commercial potential will be subjected to these processes and become part of the mainstream of commercial livestock production systems, and as such, may no longer be classified as game meats. An example is the farming of deer to provide a reliable high quality source of venison to meet the increasing world demand, particularly in Europe.

At the other end of the spectrum some species are exploited as a source of animals for leisure hunting activities. New Zealand, for instance offers an array of wild species for commercial exploitation through recreational hunting including the Red deer, Elk, Chamois, Tahr, wild boar and Sika, Traditionally,

the populations of large cats such as tigers and lions have been decimated by this industry in Asia and Africa. They are of course valued more for their skins than for meat. Other examples of species exploited abound, particularly on the veldts of southern Africa. Rhinoceros, elephants, leopards, wildebeest, zebras, baboons, gorillas, gazelles, bush pigs, hippopotamus, and ostriches, all are hunted by affluent trophy seekers.

Bushmeat

Bushmeat is the term used to describe any wild animal hunted for food, or simply, the meat of wild animals. Bushmeat remains the sole source of animal protein for many communities across the globe. Although duikers (small antelope), rats, porcupines and monkeys are most commonly eaten, bushmeat can include any type of terrestrial wild animal from snails to elephants. While some amphibious or semi-aquatic freshwater animals, such as frogs, turtles and crocodiles, are also regarded as bushmeat, fish are not included under this category.

Meat from Zebras, for instance, is a bushmeat. It is much like horse meat, in that the colour of fresh zebra meat is very dark due to high myoglobin levels, and cooking losses are low. There are several species of African zebra in the genus *Equus* and dressed carcass weights range from around 114 to 187kg, with dressing percentages from 53 to 60% (Swatland, 2004). Other examples of bushmeat include alternative animals that can be used as meat producers include: the one-humped camel, water buffalo, kangaroo, harp seal and ostrich. The kangaroo, for instance, produces a lean carcass, yielding 51.7% by weight of the carcass as meat (Tribe and Peel, 1963). The yields of carcass muscle as a proportion of liveweight is given in Table I.

TABLE I.- A COMPARISON OF CARCASS PROTEIN COMPOSITION FROM DIFFERENT SPECIES (MODIFIED FROM TRIBE AND PEEL, 1963).

Species	**Carcass muscle as % of live weight**	**Number of animals**
Pig	31.9 ± 2.0	20
Cattle (*Bos taurus*)	31.7 ± 1.8	14
Cattle (*Bos indicus*)	32.5 ± 2.0	30
Goat	31.3 ± 2.6	13
Sheep	26.9 ± 0.6	13
Wildebeest	41.6 ± 2.6	5
Gazelle	45.6 ± 2.8	5
Kangaroo	51.7 ± 0.5	12

Bushmeats also are often of high nutritional value and can compare well with the quality of more conventional meat types. Mineral profiles for different alternative meat species are given in Table II.

TABLE II.- MINERAL COMPOSITION (MG/100 G EDIBLE PORTION) OF MEAT FROM DIFFERENT SPECIES (MODIFIED FROM SALES, 1995).

Mineral	Camel (n=7)	Water buffalo	Harp seal (n=15)	Ostrich (n=12)	Beef	Chicken (n=24)
Zinc	3.81	-	2.80	-	4.3	1.54
Calcium	5.20	7.5	591	1.72	7	12
Potassium	345	324	288	-	350	229
Magnesium	19.63	-	34.2	-	20	25
Sodium	77.91	111	159	70.55	61	77
Copper	0.080	-	0.10	-	0.14	0.053
Manganese	0.007	-	<0.10	-	0.04	0.019
Iron	1.28	2.7	64.6	2.87	2.1	0.89
Phosphorus	189.1	213	504	-	180	173

The conflict between commercial hunters who have no regard for the principles of conservation and sustainable harvesting and indigenous communities intent on maintaining an equilibrium between their needs and those of the animal species to persist in viable and sustainable numbers, remains an important imperative for legislators world-wide. Regrettably commercial utility often outweighs conservationist sentiments and so species become endangered.

Species harvested from the wild: Australian marsupials

Kangaroos, also known as macropods (meaning ‘great footed’ and belonging to the super family Macropodidae) are harvested for both meat and leather production, forming an industry worth over $200 million and employing between 3,000 and 4,000 people in remote rural towns. The industry plays a significant role to the economy of Australia’s rural communities. Of the 60 species of macropod marsupials in existence, half have decreased in number or distribution while only 14 have either sustained or increased their population since 1960 when records were first kept. Ten species are extinct on the mainland while 4 of these are still found in Tasmania. The larger species of macropods continue to thrive, where 4 of the largest and most abundant in Australia

exploited are among the 5 species utilized for commercial harvesting. The list also includes the Whiptail wallaby: Eastern grey (*M. giganteus*), Western grey (*M. fuliginosus*), Reds (*M. rufus*), and Wallaroos (*M. robustus*), as well as the smaller Whiptail wallabies (*M. parryi*)

These species of kangaroos are distributed widely throughout Australia with the Red, the Eastern Grey and the Western Grey all being found in large numbers in the semi-arid and arid regions of the southern Australia. In contrast the Euro and the Wallaroo are found widely throughout the continent while the Whiptail wallaby is confined to the east coast in mid latitudes.

The first exports of Kangaroo meat as a game meat for human consumption occurred in the 1950s and 1960s to the Federal Republic of Germany (Corrigan, 1988). This was short lived, however, due to poor quality standards and the absence of proper government supervision. The export of kangaroo meat then was resumed in the early 1980s, after intensive investigations of the sustainability of the selective culling of kangaroo populations had been conducted by the government in collaboration with the fledgling industry. This involved the inspection of the process of field shooting and short dressing kangaroos in paddocks as well as the further processing in plants, and the approval of these methods and their incorporation into the Export Game Orders. The key issues were the adoption of high standards of hygiene, the absence of use of dogs for hunting, the adoption of head shooting only, and hygienic delivery of kangaroo carcasses to processing plants to minimize chances of Salmonella contamination. An internationally recognized Code of Practice for game meat production, first developed by Codex in association with FAO/WHO Food Standards, provided a basis for the development of Australia's guidelines for exporting kangaroo meat.

Between 1985 and 1987 110,000 kg of edible kangaroo meat was exported mostly to .the Netherlands, France, West Germany, Papua New Guinea and Japan. In comparison, export of non-edible kangaroo meat for pet consumption, exceeded 600,000 kg with most exported to Japan, Hong Kong, Indonesia and Malaysia.

Kangaroo numbers

The national kangaroo population fluctuates dramatically (Fig. 1) according to the availability of feed, which can vary markedly over the various geographic zones. There has been a marked upswing in total numbers since 2000

to reach a population of 60 million. The annual total harvest rarely exceeds 3 million and does not impact on total numbers, nor on the numbers of each individual species. Given the variation in rainfall and therefore feed resources across any single region, the kangaroo population can travel long distances in search of feed.

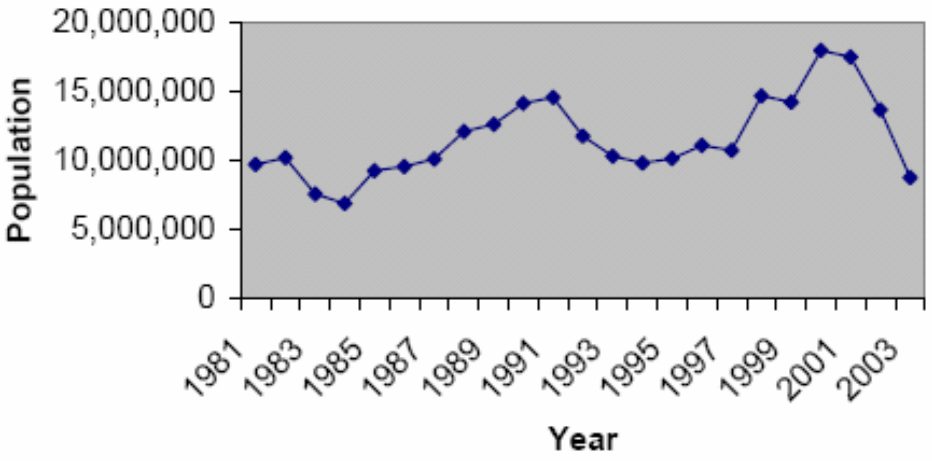

Fig. 1. Fluctuations in the population of red kangaroos (one of the 5 hunted species) in Australia from 1981 to 2003.

Although these policies provoke much controversy in the community, it is generally considered that without adequate control of kangaroo numbers through regulated harvesting, the economic viability of pastoral activities and the environmental sustainability of vast areas of land would be seriously threatened.

The harvesting of kangaroos in Australia is regulated though detailed 'Kangaroo Management Programs', defined uniquely for each state and territory by the relevant National Parks Authority (NPWS, 2003). Annually, each of the kangaroo and/or wallaby species, with the potential to be harvested, is surveyed to determine the estimated total species population via aerial manual survey (Kelly, 2002). Based on the total species population estimate, a maximum allowable quota is set depending on feed availability as well as current and future rainfall predictions: it is usually set between 10-15% of the total population.

The issuing of kangaroo harvest tags is only possible to accredited, licensed kangaroo harvesters and, in certain clearly defined and unusual circumstances, to landholders who can provide proof of excessive numbers of animals on their land. Professional kangaroo harvesters must complete a thorough formal training accreditation covering animal welfare, hygienic harvesting practices and initial carcass inspection. Firearm competency is tested annually, ensuring the continued adherence to the strict guidelines set out in the federal government's 'Code of Practice for the Humane Shooting of Kangaroos'. The level of accuracy required by this code is very high, requiring animals to be only head shot. In brief, kangaroos are harvested at night under spotlight whilst animals are grazing in paddocks. The stunning effect of the light places them in a slightly dazed but fixed upright position, and the instantaneous destruction of the brain by the bullet to the head ensures an effective, clean and minimal stress kill.

TABLE III.- NUMBER OF KANGAROOS KILLED UNDER COMMERCIAL HARVEST QUOTAS IN 1999 (SOURCE: ENVIRONMENT AUSTRALIA 2000).

State	Red	Eastern Grey	Western Grey	Euro/ Wallaroo	Other species[1]	Total	% of quota taken up
NSW[2]	450,020	355,845	122,481	9,296	0	937,642	56
QLD	457,177	570,101	0	119,767	1,279	1,148,324	41
SA[3]	231,327	0	63,672	18,988	0	313,987	42
WA	139,945	0	54,574	4,731	0	199,250	46
Total	1,278,469	925,946	240,727	152,782	1,279	2,599,203	46

[1]Whiptail Wallaby, *M. parryi*; Bennetts Wallaby, *M. rufogriseus*; Tasmanian Pademelon, *Thylogale billardierii*.

Carcass composition, nutritional value and muscle selection

Male and female red kangaroos can reach 90 kg and 40 kg live weight, respectively, but most harvested animals are about half this weight. Male Eastern Grey kangaroos may also reach 90 kg, but are more likely to be around 70 kg, with females reaching half that weight. Dressing percentages are about 65% for males and females, and for red and grey kangaroos, within an empty body weight range from 15 to 40 kg. Carcasses yield about 80% muscle, 14 % bone and 6% waste.

TABLE IV.- NUTRITIONAL VALUE OF KANGAROO MEAT RELATIVE TO OTHER MEATS (FORD AND FOGERTY, 1982).

	Protein (%)	**Fat (%)**	**Kilojoules (per 100g)**	**P/M/S ratio[1]**	**Cholesterol (mg/100g)**	**Iron (mg/100g)**
Kangaroo	24	1-3	500	1.5(1)1	56	2.6
Lean lamb	22	2-7	530	0.1(0.9)1	66	1.8
Lean beef	22	2-5	500	0.1(0.9)1	67	3.5
Lean pork	23	1-3	440	0.2(1.3)1	50	1.0
Lean chicken breast	23	2	470	0.4(1.5)1	50	0.6
Rabbit	22	2-4	520	0.8(0.5)1	70	1.0

[1]P/M/S: polyunsaturated, monounsaturated and saturated fatty acid ratio.

Australia's Kangaroo Industry suffers from an *image problem*, particularly in its own country, where many Australians regard it as an important Australian icon that is seen on the coat of arms and not on the dinner plate. Many still feel that this rich source of animal protein is fit for pet consumption only. This image problem also dates back to the days of European pioneers in Australia, as even though kangaroo provided an important source of food, it did become socially unacceptable as people grew wealthier and eating meat from wild animals suddenly became an indication of poverty.

The social issues raised above, however, have not affected the increasing popularity of kangaroo outside Australia. Currently the industry estimates that it exports kangaroo meat to more than 55 countries, of which the European Union and Russia are the most significant markets, with the USA and Asia becoming increasingly important. It is a lean red meat that claims health benefits when incorporated in the diet, particularly when it comes to cholesterol lowering diets (O'Dea, 1988), and has its own characteristic game meat flavour, texture and colour. A recent press report of high levels of conjugated linoleic acid in this product certainly testifies to its value.

Emu

The emu (*Dromaius novaehollandiae*) originates from the Australian desert and is now ranched in several countries. A typical emu farmed for market will weigh close to 40 kg at 15-18 months, and yield around 12 kg of boneless red meat, 8 kg of fat and 0.6 m^2 of hide. It produces a dark meat that is lean and has a mean muscle pH_u value of 5.5 (Berge *et al.,* 1997). The colour stability of fresh emu meat is generally very poor: Berge *et al*. (1997) noted that the intense

red colour was very sensitive to oxidation, just as the intramuscular lipids are, thus limiting the storage of fresh meat under aerobic conditions to short periods of time only (under 3 days).

The distribution of muscles and primal cuts is similar to that of the ostrich. Lateral limb muscles and their North American names are shown below in Figure 2.

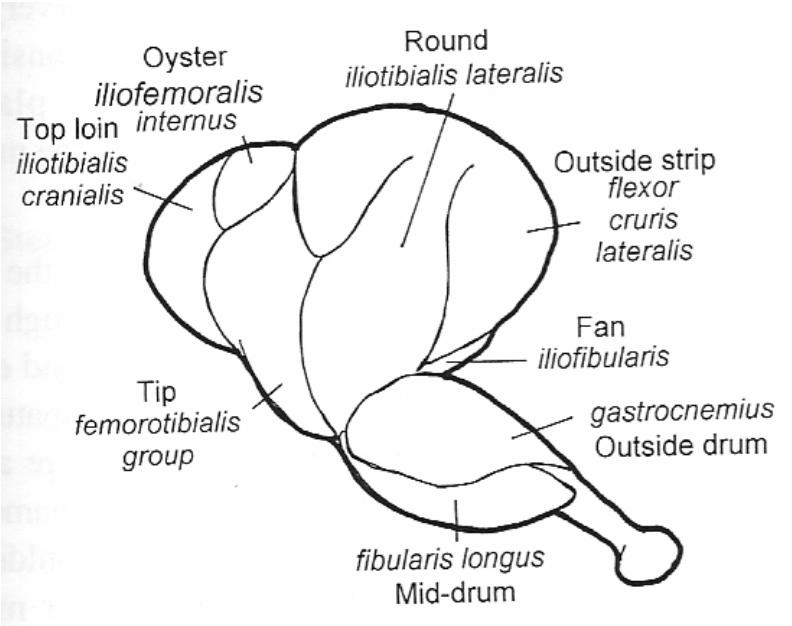

Fig. 2. Leg muscles of the emu.

Emu meat ranks highly as a healthy alternative, as it has low cholesterol levels and a favourable fatty acid profile. Emu meat contains higher levels of linoleic (C18:2 ω6), arachidonic (C20:4 ω6), linolenic (C18:3 ω3) and docosahexaenoic (C22:6 ω3) acids chicken or beef steak (Pegg *et al*. 2006). The ratio of polyunsaturated: saturated fatty acids is more desirable in emu meat (0.72) than chicken (0.57) and beef (0.3) (Wang *et al*., 2000).

Crocodile

Alligators, caimans and crocodiles are all in the Order Crocodylia. The hunting of them for their skins has pushed several species close to extinction, as

illegal hunting remains a problem. However, meat from alligators, caimans and crocodiles is now produced as a by-product of ranching crocodilians for their skins and is commercially available as a gourmet item in the USA, Australia and elsewhere.

The crocodile industry is another small but growing industry. By law crocodiles can only be harvested from commercial farming operations in Australia and in 2002 there were 11 such enterprises holding 68,000 crocodiles. The saltwater variety produce higher quality skins, and thus would probably provide the greater proportion of meat for the industry due to their higher demand. In Australia over 15,000 were harvested in 2001 producing 90 tonnes of meat, most of which was exported.

The major constraint to world trade in crocodile meat is contamination with the parasitic worm Trichinella. Non-encapsulating forms of this parasite have been identified in up to 35% of crocodiles in Zimbabwe, while the practice of feeding infected pig carcasses to crocodiles in Papua New Guinea has resulted in high infection rates in that country (Pozio, 2005). Clearly, this industry needs to develop appropriate feed resources for this burgeoning industry, although at present Australia remains free of this parasite.

Since we know so little about factors influencing meat quality in this species, the focus has been on food safety. Meat cuts are dipped in an antimicrobial solution prior to packing and freezing. Crocodile meat has a firm texture, is light in colour, and has a delicate fishy taste.

In captivity, crocodiles may be given diets of red meat or fish or chicken. Off odours that sometimes occur, which are associated with a grassy or fishy taste, are believed to originate from Hexanal and heptanal (Swatland, 2004). Hexanal has been used as as a biomarker for lipid peroxidation, a process that produces a large number of compounds giving rise to off-flavours and off-odours in foods. Volatile heptanal is known for its characteristic sweet odour that is given off during oxidative processes.

Crocodile can be purchased as body fillets, tail fillets, whole tails or as a whole body. Further processed products are available such as dried crocodile meat also known as jerky.

Other species

Many other feral populations exist in the world that can be exploited for meat production including buffalo, camels, goats horses, pigs and ostriches to name but a few. Regrettably even primate species are hunted for bush meat often with disastrous consequences.

Disease risks

One of the major concerns for consumers of game meats and in particular bush meats are the bacteria, viruses, parasites and unconventional agents that reside within the product and therefore may transmitted to them. Some of these are listed here.

Animal health and disease risks relating to the consumption of kangaroo meat

When considering the consumption of meat from wild, field shot kangaroos, health concerns by the general public still to this day are directed towards 'worms'. Kangaroos exhibit diverse populations of intestinal worms that exist in the gastrointestinal tract namely because the kangaroo is a wild free ranging species that is therefore not drenched like sheep and cattle and so there is no intervention and worm control. For instance, of the strongyloid nematodes found in macropods, there are 40 genera and 171 species described (Hume, 1999), with many species still undescribed. With such a large array of Helminths in macropods, and still scope for more research, it hard to determine the real purpose of such a vast biomass, of nematodes in particular, in macropods, ***i.e.*** parasitic versus symbiotic. Köhler (1985) has reported helminths to commonly ferment carbohydrates to short-chain fatty acids and this is an area of macropodid digestion that should attract further investigation.

Apart from this, other health concerns are related more specifically towards: hydatids, salmonella and *Dirofilaria roemeri*, the latter colloquially referred to as joint worm.

Hydatid disease (*Echinococcus*): there are two causal agents. The major one is *Echinococcus granulosus*, where the mode of infection to humans is through contaminated food, water, hands; careless smoking; lamb marking; handling dogs, dogs' premises, sheep, among others. Animals known to transmit the infection include dogs, dingoes to infect humans, and to re-infect dogs, sheep,

cattle, kangaroos and pigs. The disease is often found in humans often resulting in death, the need for surgery to remove cysts, and tapeworms leading to ill-health (Hungerford, 1990).

Dirofilaria roemeri is probably the most common parasite of macropods. Others include Strongyles, Oxyurids, Cestodes, Trematodes, and Helminth parasites (refer above). *Dirofilaria roemeri* is a large filarid that occurs in fibrous capsules in the the knee joint in Kangaroos. In heavily infected kangaroos this parasite is not just found isolated within the knee joint but can be found on the surface of the muscles of the upper hind limb, particularly on and around the rump muscles and deeper portion of the topside. The intermediate host of *Dirofilaria roemeri* is one of several species of Tabanid flies, the most important being *Dasybasis hebes*. Transmission usually occurs from October to May, with a maximum in April and there is a pre-patent period of about 9 months (Dunsmore, 1978).

So what is the potential risk when consuming kangaroo? Carcasses are inspected at the plant, where the heart, liver, lungs, spleen and kidneys must remain intact with the carcass for inspection by a qualified AQIS meat inspector. However, health inspection of animals in the field by the shooter may assist with the identification of infected carcasses, although there is scope for this problem to be ignored. Thus in order for the industry to maintain credibility, it is important that all products for human consumption undergoes a rigorous AQIS approved inspection procedure. The potential health concerns that kangaroo meat poses to humans was investigated by A. E. Andrew in 1988, but further work has been lacking.

In 1980 Commonwealth legislation was enacted, enabling kangaroo meat to be prepared and exported for human consumption. This occurred only after a thorough investigation of the procedures used in the kangaroo harvesting industry. Andrew (1988) reported that kangaroos suffer from few diseases normally associated with domestic animals and that they present little or no danger to human health. In field studies conducted in 1980 on Red, Eastern Grey and Western Grey kangaroos, a complete absence of the major diseases or conditions associated with domestic animals was noted. Mention was made of the nematode *Dirofilaria roemeri*, which does not affect humans and is mostly confined to the stifle joint. The tapeworm *Progamotaenia festiva* is a common inhabitant of the bile ducts in the liver, however, this parasite is of no public health significance (Andrew, 1988). In terms of hydatid disease, Andrew (1988)

reported that there is no danger of humans contracting hydatid disease through the consumption of meat affected with the disease: the danger lies in fact with the consumption of eggs from the tapeworm through direct or indirect contact with infected dogs.

Birds

The major risks are infection from Psittacosis/Ornithosis (Chlamydia), *Salmonella*/*Campylobacter,* and *Mycobacterium.*

Terrestrial mammals

A number of potential threats exist with terrestrial species. These include: *Salmonella/Campylobacter*: the mode of infection to humans is through contact with faeces such as rat droppings; however, foods of animal origin are the major causes of campylobacteriosis in humans, where various species such as chickens and pigs harbour Campylobacter species.

Campylobacter has been reported to be the leading cause of food-borne bacterial infection, being responsible for an estimated 2.4 million cases (Thakur and Gebreyes, 2005) *Campylobacter jejuni* in humans has generally been considered to be the most important species of Campylobacter causing infection, however, research carried out in Spain and the United Kingdom (Saenz *et al.,* 2000) has suggested that another species, *Campylobacter coli* is just as important as a human pathogen. These studies have highlighted the importance of *C. coli* based on its resistance to various classes of antimicrobials and its prevalence in the indigenous population of Australians as an agent for food borne disease, such as Ringworm, Sarcoptic mange, Lyssa/Hendra and Menangle virus.

Toxoplasmosis is the causal agent in the cat and animals transmitting the infection to humans include cats, sheep, rodents, dogs, cattle pigs, goats and horses. This disease is of great importance in early pregnant susceptible women and it can cause blindness and abortion.

Trichinellosis

Controlling zoonoses

Control of exposure to zoonoses in the population of developed countries

is well advanced, however at the other end of the spectrum, the exposure of native tribes to such horrific viruses as the ebola virus, through the consumption of primate bush meat (eg gorillas and monkeys) stands as a real potential threat. World health regulatory organisations have much ground to cover in some regions to minimise these threats.

CONCLUSION

The continued demand for meats that have a unique and often strong gamey flavour and which are low in fat content will ensure a marketplace for game meats in the future. In addition, the demand for alternative sources of meat is strengthened by any health concerns or incidents that occur relating to the consumption of the more traditional farmed species. For example, the occurrence of BSE in cattle helped increase the consumption and popularity of horse and ostrich meat in Italy.

The challenge remains to maintain the supply of high quality product that will enjoy the confidence of the consumer and at the same time ensure the sustainability of species involved and the environment within which they reside.

In contrast, where game or bush meats provide the sole source of human dietary protein, significant challenges await regulatory authorities since quality control over such product is either non-existent or at best rudimentary. As these procedures are implemented game meats will become more important in the world food equation. The mere fact that kangaroo and emu harvesting ranges and farms are spread as widely as China, Europe and North America bears testimony to this fact. However the restriction to consumption on the basis of religion will limit the use of these resources in Islamic countries among others.

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COMPARATIVE GENOMICS AND LACTATION: MAKING MORE MONEY FROM MILK

PETER C. WYNN, KEVIN R. NICHOLAS, LISA G. RILEY AND PAUL A. SHEEHY

Co-operative Research Centre for Innovative Dairy Products and Reprogen, Faculty of Veterinary Science, University of Sydney, Camden NSW 2570 Australia (PCW, LR, PAS) and Department of Zoology, University of Melbourne, Victoria 3010 Australia (KRN)

Abstract.- Milk and dairy products remain an important component of dietary protein and energy requirements world-wide. With the development of increasingly sophisticated techniques to select superior animals for milk production and greater availability of high quality feeds, milk production from commercial dairy herds has advanced rapidly. In Israel for example most Holstein Friesian cows yield as much as 10,000kg of milk per lactation, while in other countries such as Australia the comparable milk output is closer to 6,000kg. The development of technology to sequence the human genome has opened the way for deciphering the genetic code for other mammalian species including the chimpanzee, mouse, dog and opossum while an additional 24 mammals are being sequenced to low-coverage (2x) funded by the National Institutes of Health in the USA at present. The goal is to create genome assemblies and align resulting sequence to the human genome to permit comparative genomic analysis. The genomic sequence of the marsupial the Tammar wallaby has been the subject of recent investigations along with native Australian monotremes such as the platypus and echidna. These species have developed unique lactational strategies involving the ability to alter milk composition over the course of their lactation cycle. These changes are required to support the array of developmental processes associated with the maturation of the newborn from a highly altricial state through to become an independent rapidly growing young fast developing to attain sexual maturity. The Tammar wallaby, fur seal, platypus, certain lines of mice and echidna each have unique lactational characteristics, some of which would be highly desirable if their regulatory genes were altered within the bovine genome to attain a similar outcome. Our extensive studies of the bovine genome have allowed us to identify genes providing markers for milk production traits over the past 20 years. However advances in productivity have reached a hiatus. As we learn more about the lactation cycles of these other unique species we can apply the power of contemporary molecular genetic techniques and functional genomics to identify genes of importance in controlling the commercially important targets of bovine lactation. Thus genes not previously connected with the regulation of lactation are being uncovered by studying differences in gene expression patterns across the lactation cycles of these species using a comparative genomic approach. The mapping of these genes to QTL's for different production traits combined with the use of genome-wide SNP arrays is

now enabling our geneticists to develop even more powerful selection indices for milk traits in the Holstein Friesian cow.

Key words: Lactation, milk composition, cows, genomics.

INTRODUCTION

Ever since animals were first domesticated by man, milk has been harvested as a ready source of dietary protein and energy in the form of milk fat and lactose. In effect the lactating animal is the ideal source of animal food for domestic consumption since it is produced in small quantities that are readily harvestable over a period of 300 days without any requirement for complex and expensive equipment. In contrast other forms of dietary animal protein such as meat involves the slaughter of animals and therefore an oversupply of product on that particular day. Provision therefore has to be made to store this product under refrigeration or have it preserved in some way to avoid spoilage. Of course the preservation process often decreases the nutritional value of the modified meat.

This utility of milk and its derivatives has resulted in a consistent growth in the demand as living standards improve globally. This has included increased consumption of flavoured milks, yoghurts, fermented milks and in certain countries cheeses. Countries previously thought to be potential exporters of milk have now had to revise their goals as demand outstrips the increase in production. In effect many of these countries will become net importers of these products despite the improvements in dairy technology introduced into their respective countries.

Commercial goals for the dairy industry

Changes in consumer priorities over the past 20 years have altered the direction of genetic selection programs servicing the dairy industry. Increasingly the focus on milk constituents has been shifted from butter fat towards protein as consumers have recognized the close association between the intake of high levels of saturated fats and the incidence of coronary heart disease. Both the casein and whey components of milk protein are important for manufacturing into dairy products providing the balance of amino acids required to support good health for an increasingly discerning world community.

However milk protein is a parameter not easily manipulated through either genetic of nutritional means. This most likely relates to the fact that these

proteins provide a rich source of biologically active molecules that direct the development of the newborn calf. This includes the control of growth processes and most importantly the programming of the central nervous system as basic biological rhythms including feeding behaviour are initiated. Thus in a sense the pattern of secretion of these milk proteins extend the time over which the dam can regulate the development of her young. Thus any major quantitative changes in protein, while being desirable in terms of providing amino acids for growth, may lead to abnormal development including changes in cognitive processes in the brain.

The success achieved in boosting milk output through genetic selection programs has come at a cost of decreased reproductive performance of the high producing cow. Conception rates as low as 25% to first service are not uncommon even in the best fed cows: thus the gains in milk output are counter-balanced by the need to mate more cows at any one time point to meet the production levels required of herds servicing a year-round whole milk market. Clearly metabolic cues are initiated through the satiety centre in the arcuate nucleus in the hypothalamus and related centres through the kiss neuron and its product kisspeptin which in turn controls the hypothalamic GnRH pulse generator and ultimately ovarian function (Fig. 1). The perception of a high level of nutrient partitioning to the mammary gland for milk biosynthesis necessitates the shut-down of ovarian function until the cow perceives that it is able to supply sufficient nutrients to support a growing foetus in addition to servicing the nutrient demands of a newborn calf. Of course the problem is exacerbated if the cow is underfed at peak lactation.

Reproductive inefficiency plagues dairy industries world-wide, although the causes of this problem vary across countries. Infertility in buffalo in Pakistan and Bangladesh most often relates to poor nutritional status of animals resulting from the lack of available feed.

Associated with poor reproductive rates in commercial dairy herds is the prevalence of bacterial infections in either the mammary gland or the uterine tract. Boosting the efficiency of the immune system is one way of achieving lower infection rates in dairy herds. Achieving this goal through quantitative selection is difficult since no single blood or milk parameter provides an overall assessment of cellular and humoral immunity. The multiple inputs to the regulation of the immune system involves a complexity of gene expression across many immune and non-immune tissues including the provision of energy substrate. Activation of the immune system is energetically very expensive for

the animal. Ideally studies on the regulation of the immune system are suited to the use of genomic tools capable of measuring gene expression across the transcriptome of contributing tissues. Of course the most important gains in this area can be achieved through the adoption of high standards of cleanliness in the dairy and in lounging areas to minimize exposure to high bacterial loads.

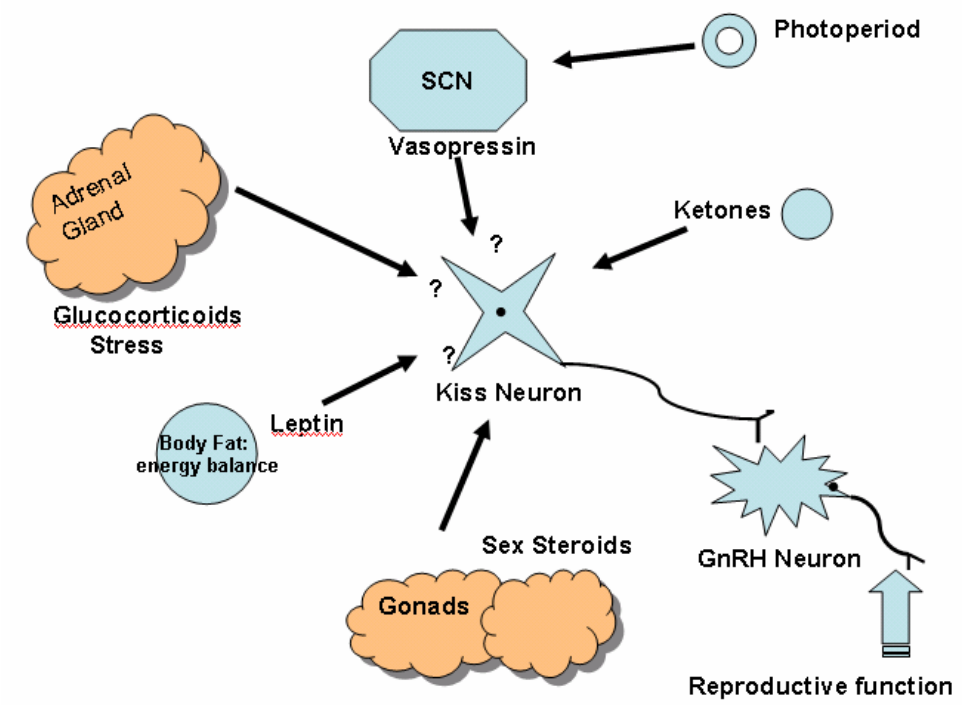

Fig. 1. The relationship between metabolic cues from specific glands/organs to regulate the central Kiss neuron and GnRH secretion

The persistency of lactation is also an important target for genetic manipulation. In spite of the long-term selection for milk output over the past 40 years lactational persistency is highly variable within the Australian Holstein Friesian herd. Thus cows producing at high levels at peak lactation with in the first 60 days post calving are not always the same cows still producing at high levels at 305 days. Again this is a complex trait involving a number of processes including the biosynthetic capacity of the mammary epithelial cell population, the size of the gland and the efficiency of nutrient partitioning to the mammary

arterial vasculature. This complex trait is ideally suited to investigation using transcriptome analysis of mammary tissue collected longitudinally across the lactation cycle. These experiments are difficult since the collection of sequential tissue samples will influence the functionality of the gland over the lactation cycle.

The spectrum of lactational strategies in nature

The evolution of the different physiological strategies to provide sufficient nutrients for the newborn and growing offspring has resulted from the adaptation of genotypes to specific ecological niches in the environment. At one extreme the baleen whale is capable of sustaining a 6 month lactation with milk produced at the rate of 200kg per day containing up to 50% fat with 9-15% protein and 1.2-2.1% ash while at the same time fasting during its migration from the tropics to polar regions (Oftedal, 1997).

At the other end many species are unable to store energy to any degree and therefore meet the nutritional demands of lactation through increasing feed intake: rodent species typify this approach. These changes are also accompanied by dramatic changes in the morphology of the gastrointestinal tract, which may grow by up to 200% at peak lactation (Hammond *et al.*, 1994). However, we have in bred a mouse strain based on Quackenbush lines that has been selected for litter weight and therefore lactational performance for 54 generations capable of supporting litters of up to 18 pups: a comparable control line supports the growth of only 6 pups (Riley *et al.*, 2006). These mice have the capability of storing energy in extensive inguinal fat depots not seen in other mouse strains. Thus as with the dairy cow they undergo the typical negative energy balances observed in high yielding dairy cows as they strive to sustain the nutritional demands of litters of pups that exceed their own body weight. In a sense they have developed an energy storage mechanism reminiscent of the whale but on a scale orders of magnitude smaller.

The fur seal (*Arctocephalus* spp. and *Callorhinus* spp.) has the unique capacity to switch the mammary gland on and off as they cycle between a period of harvesting food off shore and feeding their young on shore. The unique nature of this cycle is that lactation is suppressed to about 20% of capacity while foraging and does not regress to the point of involution observed when lactation ceases. This functional separation of feeding young and maternal foraging provides a unique source of material to elucidate the molecular switches that play

an integral role in the initiation and cessation of lactation (Sharp *et al.*, 2006).

Marsupial lactation is characterized by marked changes in milk composition which are related to the developmental changes that are supported by mammary secretion. Since pouch young are so under-developed or altricial at birth, changes in the composition of biologically active developmental molecules is undoubtedly as important as the provision of nutrients *per se* in supporting the growth of the neonate (Nicholas *et al.*, 1997).

However the changes in milk composition as animals pass from stage 2 to stage 3 at 200 days of lactation are dramatic and therefore of interest to our dairy industry. At this time the gland switches from producing a high carbohydrate, low fat milk to a high fat, low carbohydrate alternative containing a much higher percentage of solids. At this time of switching the expression of specific proteins is initiated which leads to a marked increase in total milk protein output. The functional significance of newly synthesized proteins such as the late lactation protein remains to be elucidated. This not only has implications for the supply of energy for the pouch young, but also will influence milk volume since lactose is the key osmolar moiety in milk.

How do we exploit these extremes of lactation to improve productivity within commercial dairy herds?

The development of technology for sequencing of whole genomes and for the mass screening of gene regulation has revolutionised our approach to genetic profiling, mapping and typing as well as furthering our understanding of underlying physiological mechanisms. Since the publication of a first pass of the human genome, the massive infrastructure developed for this ground breaking project has been used to sequence other genomes of commercial interest.

Our capacity to generate vast banks of genetic code has also resulted in a shift in emphasis to understanding the functions of gene products and their systematic characterization through proteomics. This presents a real challenge for the biologist since each gene potentially yields multiple protein products through post-translational modification. The need for high through-put assays for screening these products for biological activity provides a major challenge, since the patentability of the function of a candidate gene and its novel protein(s) relies on the development of direct bioassays conducted either *in vivo* or *in vitro*: inherently these assays are slow and tedious thereby limiting the capability to

screen for functional activity. Fortunately for the dairy industry the enormous sequencing capacity commissioned for the human genome project has been used to good effect to provide a first pass sequence for the bovine genome. This has lead to the generation of microarrays containing specific nucleotide sequence probes representative of the full complement of transcripts emanating from this bovine genetic code.

Bovine Genome Array developed by Affymetrix can be used to study gene expression of over 23,000 bovine transcripts in a single RNA sample. (www.affymetrix.com/products/arrays/specific/bovine.affx).

These microarrays contain multiple probe pair sets to provide independent measurements for each transcript. This technology provides a quantum leap in accuracy from the initial printed arrays that pioneered this approach to gene discovery.

How does the bovine transcriptome change over the bovine lactation cycle?

As with all physiological experiments the key to success is to minimize the genetic and environmental variation between animals within a trial. This is most likely more important in these microarray expression studies since 20,000 observations are dependent on the integrity of the samples. Poorly contrived experiments can be costly since much of the expense of the study is generated through the bioinformatic analysis of the results. For this reason it is advantageous if animals can serve as their own controls in any experiment. In our studies we have refined surgical techniques for the collection of a limited number of sequential biopsies from the same animal over the lactation cycle. In this experiment biopsies of 4-5 g of tissue were collected using an epidural block on cows for anaesthesia and tissue was stored at -80^{o}C prior to RNA extraction (Sheehy *et al.,* 2004). The key time points for tissue collection were in late pregnancy, peak lactation and involution.

Following the normalization of the data and rejection of false positive analyses through the use of standard bioinformatic procedures a total of 1987 genes or probe sets was found to be differentially expressed between at least one combination of the 3 collection time points (Table I).

Over 70% of the changes in gene expression were accounted for by decreases in gene expression between peak lactation and involution. Many of

these will be associated with the cessation of milk biosynthesis and associated cellular structural remodeling and apoptosis as the gland regresses to a quiescent state. The differences between late pregnancy and peak lactation may have decreased since the late pregnant sample was collected on average 20 days pre-calving. By this time many of the biosynthetic processes have been initiated and so their regulatory genes are already activated.

TABLE I.- CHANGES IN GENE EXPRESSION PATTERNS BETWEEN THE TRANSCRIPTOMES OF MAMMARY BIOPSY TISSUE COLLECTED IN LATE PREGNANCY, PEAK LACTATION AND 5 DAYS AFTER CESSATION OF MILKING (INVOLUTION) FROM MULTIPAROUS HOLSTEIN FRIESIAN COWS PRODUCING BETWEEN 6000-9000L PER LACTATION (N=5 PER SAMPLE)

	Increased expression	**Decreased expression**	**Total**
Pregnancy versus lactation	63	144	207
Lactation versus involution	531	1392	1913
Pregnancy versus involution	30	73	103

A cellular model to elucidate the role of genes in milk biosynthesis

The role of the extracellular matrix in establishing and maintaining the functional integrity of any mammalian cell is well established. When mammary epithelial cells in primary culture are transferred from plastic culture dishes onto Englebreth-Holm-Swarm (EHS) matrix they have the unique ability to communicate with each other to form cellular aggregates. These cells become polarised, form tight intercellular junctions, and secrete milk proteins basolaterally into the culture medium or apically-into a closed lumen within the mammosphere (Blatchford *et al.,* 1995). These lumens are readily visualized through the accumulation of a stain added to the culture well.

We have found that bovine mammary epithelial cell behave as do the mouse cells used in previous studies. Milk protein gene expression is inducible using the lactogenic complex of hormones, insulin, cortisol and prolactin, although the pattern of proteins produced differs from that observed *in vivo*. Thus in a sense this experimental model is indicative of potential bioactivity and any positive results require further evaluation *in vivo*.

One approach that we have taken to investigate the role of genes is to

suppress endogenous gene expression using small interfering RNA (siRNA). This method of sequence-specific, post-transcriptional gene silencing is initiated by short double-stranded RNA fragments 20-25 nucleotides in length that are homologous in sequence to the silenced gene (Soutscheck *et al*., 2004). This method results in variable levels of suppression of mRNA activity: thus negative responses are often difficult to interpret since the level of suppression required to effect a change in milk protein expression is not known. Similarly the role of redundant genes cannot be interpreted since their suppression may simply result in their function being assumed by another gene product. Despite these limitations the approach has yielded some valuable results with some of the genes tested.

The second approach involves the addition of the translated protein product directly to the culture system. The stability of the peptide/protein in culture media must however be investigated since functionally significant concentrations must be maintained in the culture media for up to 4 days to ensure a result is obtained.

Experiments in other species

Similar experiments have been conducted in other species. For example RNA has been collected from mammary tissue from the different stages of the Tammar wallaby lactation cycle with a view to elucidating genes associated with the marked changes in milk composition provided for the pouch young as they grow through to 300 days of age: at this time they are weaned. The RNA has been analysed using a 10,000 expressed sequence tag (EST) array generated by K.R. Nicholas. Preliminary analyses have shown that the marked changes in milk composition are associated with a surprisingly small number of changes in gene expression. Functional analysis of these differentially expressed genes should lead to some novel insights on the regulation of milk composition. Comparative genomic analysis has already shown that genes of interest have yielded bovine homologues which have been expressed as protein products and assessed in lactational bioassays.

The conservation of genetic sequences has provided some unique approaches to the investigation of changes in gene expression associated with the initiation and cessation of lactation in the fur seal. The canine Affymetrix microarray has provided sufficient homology to yield substantial gene expression data using pools of RNA collected from seal mammary tissue. Thus in this

instance the genetic code for species from within the sub-order Canidea has been used as a means of mining information from a species within the sub-order Pinnipedia. Analysis of these data are at an early stage.

Quantitative genetic selection indice

Identification of genes implicated in the various aspects of lactation of interest to dairy production provides the basis for the development of more effective genetic selection platforms. Analysis of gene sequences for single nucleotide polymorphisms (SNP's) using short oligonucleotide arrays will assist in defining markers within chromosomal regions or quantitative trait loci (QTL's) known to be associated with a specific commercial trait. Use of QTL maps in conjunction with microarrays derived from genome expression platforms will increase the power of both technologies to advance animal breeding.

While marker assisted selection is likely to provide the most immediate financial return for an investment in this functional genomics program, knowledge of genes and their functions can be exploited using transgenic, cloning and gene therapy approaches. Often other non-biological barriers to these technologies inhibit advances being made. The manipulation of inbred lines of mice such as the QSi5 provides an ideal target for knock in, knock out and transgenic techniques to specify the function of specific candidate genes in productive tissues. These techniques are by definition slow and expensive.

Proteomic approaches are not limited by genome specificity but are difficult to automate for high through-put analyses. They are important however in identification of post-transcriptional regulation or gene product modifications that have a regulatory role, for example in lactation.

Dairy genomics: what is in it for the small farmer world-wide?

The importance of the key milk components to dairy industries throughout the world varies significantly with cultural background and the need for animal protein and energy in our diets. The keys to success rely on our ability to develop breeds capable of persistent milk output high in protein and yet retaining fertility and a highly functional immune system and then achieving these goals with limited cow feed resources.

Advances in the genomic sciences now provide powerful tools for

identifying key genes, their products and associated markers that boost or limit these factors. The diversity of lactational strategies across the mammalian genome also provides clues for the identification of the genes regulating composition and output within and between lactations and species. Our studies have included investigations of species as diverse as the mouse, sheep, seals and wallabies. These studies may be equally applicable to the buffalo and Sahiwal and Sindhi cattle of Pakistan as they are for Holstein-Friesians in Australia. The challenge remains to harness this knowledge for the benefit of our regional agricultural economies.

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ISOLATION AND OPTIMIZATION OF MILD STEEL CORRODING BACTERIA FROM CORROSION INFLUENCED SOIL SAMPLES*

ARJUMAND SHAH BANO AND JAVED IQBAL QAZI

Environmental Microbiology Laboratory, Department of Zoology, University of the Punjab, Lahore, Pakistan.
E-mail:qazi@scientist.com

Abstract.- Soil samples were collected from surroundings of corroded Sui gas pipelines in sterile glass containers from Sheikhupura, Punjab (I) and Shikarpur, Sindh (II). One gram of a sample was mixed in 10ml of sterilized distilled water and 0.1ml of the prepared sample was spread on Medium I (for bacteria) and Medium II (for actinomycetes). A total of 37 isolates (23 bacteria and 14 actinomycetes) were obtained, pure cultured and preserved. They were optimized for various growth conditions and tested for mild steel corrosion activity. These facultative anaerobes have wide range of growth conditions and metal corrosion levels. The present results are suggestive to speculate that in the field areas wherein the laboratory investigated optimum growth conditions are prevailing to varying degrees, the soil buried mild steel pipelines might had been experiencing the bacterially accelerating corrosion processes. Isolation of bacteria and knowing particular range of physicochemical factor(s) for their optimum growth and other activities may also indicate present or past prevalence of comparable conditions in the field. The present results are helpful in isolating metal corroding bacterial groups in terms of their physicochemical requirements from different field locations.

Key words: Mild steel corroding bacteria, Microbes and corrosion, *Bacillus* sp., *Sulfobacillus* and mild steel corrosion.

INTRODUCTION

In natural and man-made environments corrosion occurs when materials made of pure metals and/or their mixtures (alloys) undergo a chemical change from ground state to an ionized species. Corrosion is an electrochemical process consisting of an anodic reaction involving the ionization (oxidation) of the metal (the corrosion reaction), and a cathodic reaction based on the reduction of a chemical species (Beech and Gaylarde, 1999). In natural environment when an iron surface is exposed to aqueous moisture it then undergoes the following spontaneous reaction.

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

$Fe^0 + 2H_2O \rightarrow Fe^{+2} + 2OH^- + H_2$

The above equation comprises of

(i) $Fe^0 \rightarrow Fe^{+2} + 2e^-$ (at anodic regions)
(ii) $2H_2O + 2e^- \rightarrow 2OH^- + H_2$ (at cathodic regions)

The generation in cathodic reaction was thought to accumulate at the iron surface where it was generated and its building-up to cause passivation (polarization) of the surface, i-e., stopping further corrosion (Ehrlich, 1997). The ferrous iron product of the reaction, Fe(II), is then oxidized to ferric(III), and under neutral conditions, typically forms the amorphous (am) solid $Fe(OH)_3$.

$4Fe^{2+} + O_2 + 2H_2O \rightarrow 4Fe^{+3} + 4OH^-$
$Fe^{+3} + 3OH^- \rightarrow am\ Fe(OH)_{3(S)}$

$Fe(OH)_3$ may be converted to other oxides, including goethite (α-FeOOH) and Hematite (Fe_2O_3). In principle, any set of conditions that promote iron oxidation will accelerate corrosion and any that retard iron oxidation will inhibit corrosion (Lee and Newman, 2003). These reactions are influenced by microbial activities, especially when organisms are in close contact with the metal surface forming a biofilm. The resulting metal deterioration is known as biocorrosion or microbiologically influenced corrosion (MIC) (Beech and Gaylarde, 1999; Beech and Sunner, 2004). A number of microbes represented both by eubacteria and archeobacteria are able to use some metals and metalloids as electron donors or acceptors for operating their energy metabolism (Ehlrich, 1997). In natural environments bacteria attach to metals, colonize the surface and produce biofilms, which are capable of maintaining the surface environment different from surroundings in terms of pH, dissolved oxygen and other inorganic and organic species (Jones and Amy, 2002; Beech and Sunner, 2004). Some authors believe that most cases of MIC happen to occur due to formation of biofilms containing sulfate reducing bacteria (SRB), that grow in consortia with aerobic microorganisms that deplete oxygen from the local environment and provide conditions allowing growth of SRB. Infact, bacteria other than SRB are frequently isolated in association with tubercles formed by metal depositing bacteria (Pope, 1986; Baker *et al*., 2003; Kjellerup *et al*., 2003; Zhang *et al*., 2003). In short, MIC is a result of microbial biofouling process involving formation of biofilms on metal surface (Dubey *et al*., 1995; Lee and Newman, 2003; Pitonzo *et al*., 2004). The effects of biofilms on the corrosion process have

been given more attention for the last several years (Lee *et al*., 1995; Lewandowski and Hamilton, 2002; Beech and Sunner, 2004).

Soil being a complex and extremely variable environment renders it highly difficult to separate purely electrochemical processes from those caused/influenced by microbes involved in underground corrosion (Miller and Tiller, 1970; Matsushima, 2000; Wilmott and Jack, 2000). Microorganisms, particularly bacteria, have been isolated from corroding metals, and knowledge of their physiology and biochemistry has allowed many workers to demonstrate that how they are actually involved in the corrosion processes (Allsopp and Seal, 1987; Zhu *et al*., 2003). The bacteria involved in corrosion are often difficult to work with (Zhu *et al*., 2003). Laboratory based controlled experiments using the defined media and characterized pure cultures often did not yield the expected results because of uncertainties of a number of variables, such as the influence of mixed microbial communities and dynamic physicochemical environment under natural conditions. Obviously set of conditions prevailing in a field location are difficult to simulate while working in the laboratory condition (Allsopp and Seal, 1987). Laboratory growth media cannot accurately reflect the true conditions for pipelines. Further, microbiologists have recognized that many of the vast majority of microbial species cannot be grown in the laboratory media (Maidak *et al*., 2000; Zhu *et al*., 2002). Thus, culture-dependent approaches underestimate the biocomplexity of microbial communities. However, to judge corrosion causing role of microorganisms there is no escape to start with pure culture experiments. Such experiments declare involvement in the process of metal corrosion or otherwise role of a given microbe.

It has, well earlier, been estimated that around 50% failures of underground pipes are due to microbial activities (Booth, 1964; Allsopp and Seal, 1987). Besides the external surface deterioration, 40% of all internal pipeline corrosion in the gas industry has been attributed to microbial corrosion (Grave and Sullivan, 1996; Pound, 1998). This phenomenon is thus of great economic consideration (Vernon, 1957; Brennenstuhl and Doherty, 1990). Replacement costs of biocorroded gas mains in the UK were reported to be £ 250 million per annum (Flemming, 1996). Corrosion problems have cost the nuclear utility billions of dollars in replacement cost alone (Jones-Meehan, 1994). Annual cost of all forms of corrosion to oil and gas industries was estimated $ 13.4 billion, of which microbially influenced corrosion accounted for about $ 2 billion in USA (Graves and Sullivan, 1996; Pound, 1998). Corrosion results in decay of metal structures, loss of metals and more than 33% of world annual total

metal production is lost due to this process in all types of environments. More than 2 million tons of steel and iron is annual requirement of Pakistan, of which 0.2 million tons is lost every year by corrosion (Khan, 1989).

Lack of multidisciplinary approach has probably not allowed investigation of MIC in this country. This paper represents an incipient effort in this regard and reports isolation and characterization of various bacteria involved in mild steel corrosion.

MATERIALS AND METHODS

Isolation of bacteria

Soil samples collected from Sui northern gas pipeline limited, Sheikhupura, Punjab (I) Sui southern gas pipeline company, Shikarpur, Sindh (II) were processed for the isolation of metal corroding bacteria. One gram of a soil sample was mixed in 10ml of sterilized distilled water. Further dilutions of the sample were made by mixing 1ml of a given dilution in 99ml of distilled water. Two selective media were used for the isolation of metal corroding bacteria and actinomycetes. The M-I comprised of nutrient broth (Oxoid) 6.5g, ferrous sulphate heptahydrate 1g, sodium thioglycolate 0.1g, methionine 0.06g, glutamic acid 0.4g, phosphate buffer solution (1M) 50ml, peptone 5g, yeast extract 0.5g and agar 15g/L. And pH of the M-I was adjusted to 6.8-7.2. For the isolation of actinomycetes M-II medium having pH of 6.5-6.6, was prepared by mixing dextrose 2g, casein (dissolved in 10ml 1N NaOH) 0.2g, dipotassium hydrogen sulphate 0.5g, ferric chloride 0.067g, magnesium sulphate heptahydrate 0.2g and mycostatin (Serva) (mixed in cooled sterilized medium at 45°C) 250mg/L. These media were autoclaved at 121°C for 15 minutes and poured in presterilized petriplates. Of the various dilutions 0.1 and 0.5ml were spread on the media plates and incubated at 37°C for 48hrs. Bacterial colonies were streaked further to obtain pure cultures routinely. The pure cultures were subcultured on nutrient agar slants and the growths used for further experiments.

Growth conditions optimization of selected bacterial isolates

Bacterial isolates were optimized for different growth conditions. For this purpose isolates were inoculated in nutrient broths. Overnight incubated cultures were used as inocula (0.1ml) to the same medium for determinations of temperature, pH, aeration requirement, light requirement and inocula sizes growth optima. The inoculated tubes were incubated at 25ºC (RT), 37ºC and

50ºC. For optimization of pH the bacteria were grown in nutrient broths having pH 5±0.2, 7±0.2 and 9±0.2, at their respective optimum growth temperature. The isolates were then incubated on orbital shaker at 100 rpm and without aeration at their corresponding optimum pH and temperatures. The isolates were also grown in dark and light conditions at their corresponding growth optima. Nutrient broth tubes were inoculated with 1%, 5% and 10% inocula and incubated at their corresponding optimum growth conditions. In all the experiments, the isolates were incubated in triplicates for 24 hours and the growth was determined by taking O.D. of the cultures at 600nm on Spectronic 20D, spectrophotometer. In each experiment sterile nutrient broth (un-inoculated) processed otherwise similarly to a given experimental conditions served as controls.

Evaluation of mild steel corrosion ability of the bacterial isolates

The mild steel coupons used in this study comprised of Fe (95.68%), Si (1.916%), Al (0.516%), Mo (0.30%), Co (0.216%), Mn (0.205%), P (0.203%), S (0.20%) and trace elements (remaining constituents). The compositional analyses as well the coupons were obtained from AK Steel Industries, Chah Miran Road, Misri Shah, Lahore.

Pre-harvest preparation of mild steel coupons

Mild steel coupons of 2×2 cm (1mm thick) were degreased with acetone. The degreased coupons were gently polished with 240 grit polishing paper and rinsed with distilled water. After the cleaning they were washed with ethanol followed by rinsing with distilled water and wiped immediately with paper towel and dried in an oven at 80ºC for 10 minutes and then cooled to measure the weight/coupon (g) on an electric balance (Zuo and Wood, 2004**)**. Then each coupon was placed in concavity of a piece of blotting paper folded in quadrant manner and all coupons were sterilized in screw capped glass container at 121ºC for 15 minutes. Transparent culture glass bottles of 100ml capacity containing 20ml of sterilized nutrient broth and mild steel coupons were inoculated with overnight grown cultures of different isolates from sample I and II and incubated for one month in triplicates at 37ºC, for evaluation of mild steel corrosion activity by weight loss method. The uninoculated nutrient broth glass bottles with similarly processed mild steel coupons served as controls. The selected isolates with higher corrosion ability were characterized physiobiochemically following the method described by Benson (1994) and identified according to Konem (1997) and Holt *et al*. (2000).

Post harvest preparation of mild steel coupons

At the end of experiment surface biofilm was removed by immersing the coupons in 25% nitric acid. Dissolution of the corrosion product detached the organic biofilm within five minutes, which removed by repeated careful irrigation with distilled water (Little *et al*., 1997). Next the coupons were processed for cleaning the metal surface by scrubbing vigorously under stream of tap water with rubber stopper. This removed remaining corrosion products. They were then dried and weighed to estimate the weight loss. Weight loss of each coupon was calibrated as percent loss from its initial mass. The weight loss of control coupon was subtracted from the experimental mild steel coupons to calculate their percent corrected weight loss. At the termination of observational period photographs of some of representative control as well as experimental coupons were taken with the help of digital camera.

Statistical analysis

The data were analysed statistically by comparisons between mean values of different parameters employing SPSS 12 programme for ANOVA and paired t-test.

RESULTS

Colonial characteristics of different isolates are shown in Tables I, II. Forty-four isolates were screened from the two samples. Seventeen bacterial isolates including ten of actinomycetes and twenty bacteria including four actinomycetes were recovered on their corresponding selective media from sample I and II, respectively. When these isolates were optimized for different growth conditions seven bacterial isolates of sample I appeared to be mesophilic, alkalophilic, facultative anaerobe and showed best growth in both light and dark conditions (Table III). From the ten actinomycete isolates five, SN-1, SN-2, SN-3 SN-7 and SN-9 showed best growth at room temperature (25°C) while three isolates the SN-5, SN-6, SN-8 appeared mesophilic and the remaining two were found to be thermophilic. Concerning pH all the isolates were found to be neutrophilic, except the SN-6 and SN-7 which were alkalophiles. Incase of oxygen and light requirements different strains preferred different conditions (Table IV). Of the sixteen bacterial isolates from sample II, seven showed optimum growth at room temperature (25ºC), five were found to be mesophilic

TABLE I.- COLONIAL CHARACTERIZATION OF DIFFERENT BACTERIAL ISOLATES OF SAMPLE I AND II ON MEDIA, M-I AND NUTRIENT AGAR.

Sample #	Isolate #	Colonial Characteristics				
		Shape	**Color**	**Margin**	**Elevation**	**Optical density**
I	SNB-1	Irregular (Concentric) [a]	Pink (White)	Lobate (Smooth)	Raised (Flat)	Opaque
	SNB-2	Concentric (Round)	Green (Off-White)	Lobate (Smooth)	Convex (Raised)	Opaque
	SNB-4	Irregular (Round)	Green (White)	Wavy	Raised	Opaque (Transparent)
	SNB-6	L-form (Round)	Yellow (White)	Smooth	Convex (Raised)	Opaque
	SNB-7	Round	White	Wavy	Raised	Opaque
	SNB-8	Irregular (Round)	Green (Light green)	Wavy (Smooth)	Raised	Opaque
	SNB-9	Round	Yellow (Orange)	Smooth	Raised	Translucent (Transparent)
II	SS-1	Round	Yellow	Smooth	Convex (Raised)	Opaque (Transparent)
	SS-2	Round	Pale Yellow	Smooth	Raised (Convex)	Transparent (Opaque)
	SS-3	Round (Concentric)	Light Yellow	Smooth	Raised	Translucent (Opaque)
	SS-4	Irregular Spreading (Round)	Light Pink	Lobate (Wavy)	Hilly	Opaque
	SS-5	Round	White	Smooth	Raised	Opaque Center
	SS-6	Irregular	Pink (White)	Lobate	Hilly	Opaque
	SS-7	Round	White	Smooth	Raised	Opaque
	SS-8	Round	Off white (pale)	Wavy (Smooth)	Convex	Opaque
	SS-9	L-form	White	Wavy	Flat	Translucent
	SS-10	Round (concentric)	Offwhite (white)	Wavy	Convex	Opaque
	SS-11	Oval	Light Yellow (Pale)	Smooth	Raised	Opaque
	SS-12	Offwhite	Irregular (Round)	Wavy (Smooth)	Drop like (Convex)	Opaque
	SS-13	Round	Pink	Smooth	Raised	Opaque
	SS-14	Oval (Round)	Light green	smooth	Raised	Opaque
	SS-15	Round	White	Wavy (Smooth)	Flat	Opaque
	SS-16	Round	White	Wavy (Smooth)	Flat	Opaque

[a] A characteristic appearing different on nutrient agar is given in parenthesis

TABLE II.- COLONIAL CHARACTERIZATION OF DIFFERENT ACTINOMYCETES ISOLATES OF SAMPLE I AND II ON MEDIA, M-II AND NUTRIENT AGAR.

Sample #	Isolate #	Colonial Characteristics				
		Shape	**Color**	**Margin**	**Elevation**	**Optical density**
I	SN-1	L-form (Round) [a]	White Center (Transparent)	Woolly (Smooth)	Raised center (Flat)	Center opaque (Transparent)
	SN-2	Irregular Spreading	White	Wavy	Raised	Opaque
	SN-3	Irregular (Concentric)	White	Wavy (Irregular)	Raised	Opaque
	SN-4	Round	White (Offwhite)	Wavy	Raised	Opaque (Translucent)
	SN-5	Round	White	Smooth	Raised (Flat)	Opaque
	SN-6	Round	Pale Yellow (White)	Irregular (Smooth)	Raised	Opaque
	SN-7	Round (Irregular)	White	Smooth (Wavy)	Raised	Opaque
	SN-8	Irregular (L-form)	White	Wavy (hair like)	Raised (Flat)	Translucent (Transparent)
	SN-9	L-form	White center	Woolly (Wavy)	Raised	Center opaque (Transparent)
	SN -10	White (Transparent)	Filamentous (Round)	Hair like (Filamentous)	Flat	Transparent
II	SSa-2	Round (filamentous)	Transparent (Offwhite)	Filamentous	Flat	Opaque (Transparent)
	SSa-3	L-form (Round)	White	Wavy	Flat (Raised)	Opaque
	SSa-4	Round (L-form)	Light Pink	Smooth	Raised	Opaque
	SSa-5	Round	Offwhite (Yellow)	Smooth	Raised	Opaque

[a] A characteristic appearing different on nutrient agar is given in parenthesis.

and the remaining four thermophilic. The variation in pH of these sixteen isolates felled into two categories; nine were alkalophiles and rests of them were neutrophiles. Eleven of them needed shaking and ten required light for their optimum growth (Table V). The four actinomycetes of this sample had diversed growth conditions' requirements. All levels of optimum growth conditions in terms of temperature and pH were represented by the isolates of this sample (Table VI).

Table III

Table IV

Table V

Table V

Table VI

Table VII

The five bacterial isolates representing the both samples and expressing mild steel corrosion activity from 3.9 to 6.61% in term of metal loss were identified upto species level based on their physiobiochemical characteristics (Table VII). From all isolates of sample I, the two *Bacillus* (SNB-1 and SNB-2) and one *Sulfobacillus* (SNB-4) showed the highest corrosion activity after one month exposure to the mild steel coupons at their corresponding optimum growth conditions. The average corrected weight loss by *Bacillus* isolates was observed as to be 3.9%±1.59 and 4.8%±2.69 for SNB-1 and SNB-2, respectively. While, in the case of *Sulfobacillus* the average corrected lost in weight of mild steel was recorded as 6.01%±3.56, the highest among all the isolates of sample I (Fig. 1). *Bacillus coagulans* (SS-5) and *Bacillus pumilis* (SS-6) bacterial isolates of sample II were found to be the most corrosive to the mild steel and the amount of weight loss by these two isolates were the highest among all the isolates of the both samples. The average corrected loss in mild steel coupons weight appeared to be 6.61%±1.67 and 6.1%±2.17 by the *Bacillus coagulans* (SS-5) and *Bacillus pumilis* (SS-6), respectively (Fig 2). The amount of average corrected weight losses by different isolates of sample I and II are mentioned in Figure 1and Figure 2. The experimental mild steel coupons appeared drastically corroded as compared to the control ones after one month period of exposure (Fig. 3).

DISCUSSION

Three out of seventeen isolates from sample I including actinomycetes caused high corrosion of mild steel in the duration of one month. These mesophilic and alkalophilic bacteria may have a different corrosion impact in fields' environment. The differences are to be worked out in soil immersed coupons instead of laboratory nutrient overfed and growth optimized conditions. Further work on these lines is under progress in this laboratory and is likely to throw light on the roles of these microbes in causing corrosion allied damage to buried mild steel pipeline. One isolate from the sample I that caused the heaviest mild steel weight lost (6.01%) represented *Sulfobacillus sp*. which is not only known to convert sulfate to sulphide but also produces large amount of hydrogen sulfide (H_2S) gas. The microbe is facultative anaerobic sulfate reducer. Sulfate reducing bacteria (SRB) are well known for playing an important role in metal corrosion (Postgate, 1951; Dzierzewicz *et al*., 1992, 1997; Zellner *et al*., 1996) by reducing sulfate and sulfur to hydrogen sulfide (Baena *et al*., 1998). Hydrogen sulfide reacts with iron to form black ferrous sulfide (FeS) which in turn reacts with water to produce an acidic condition. The low pH accelerates the corrosion process by direct mechanism. Biocorrosion is a result of interactions, which are

often synergistics, between metal surface, abiotic corrosion products and metabolite of microbial origin (Beech and Sunner, 2004).

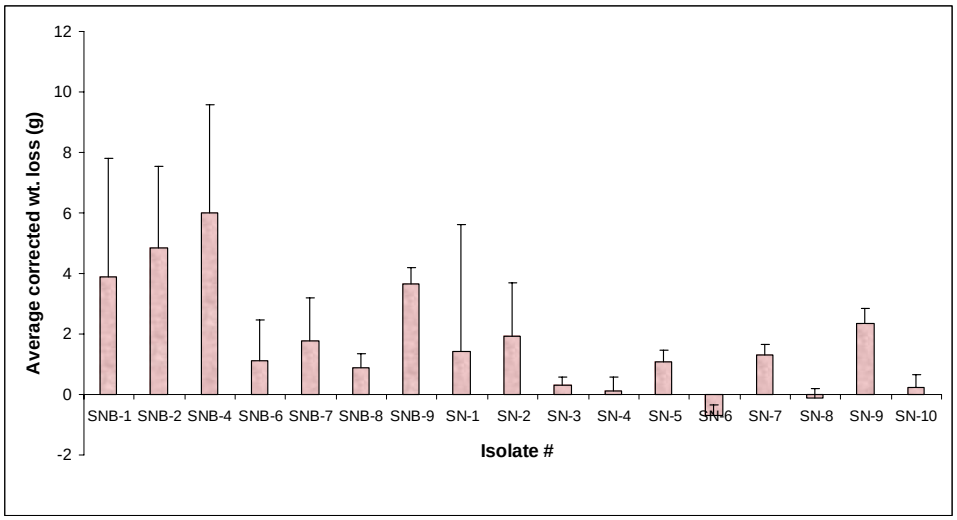

Fig 1. Average corrected weight loss of mild steel coupons by different bacterial isolates of sample I within one month exposure period in nutrient broth.

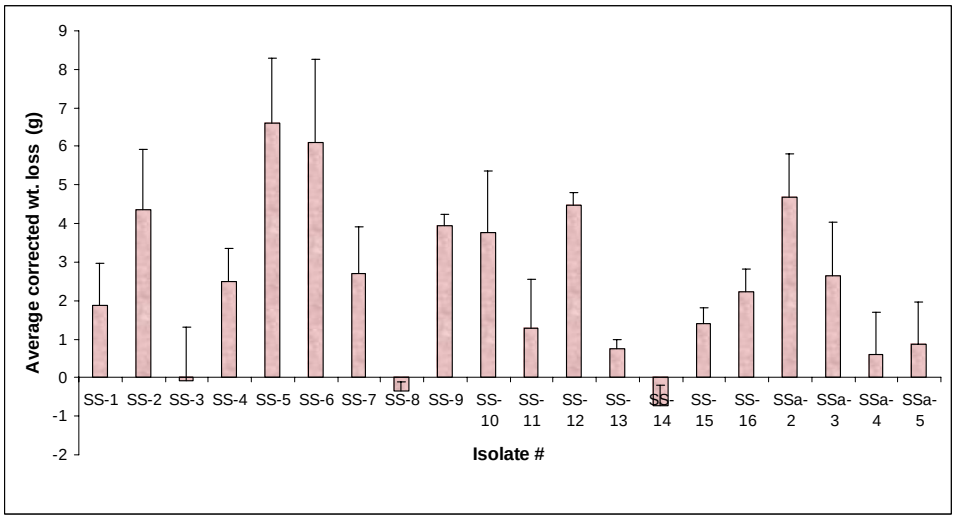

Fig 2. Average corrected weight loss of mild steel coupons by different bacterial isolates of sample II within one month exposure period in nutrient broth.

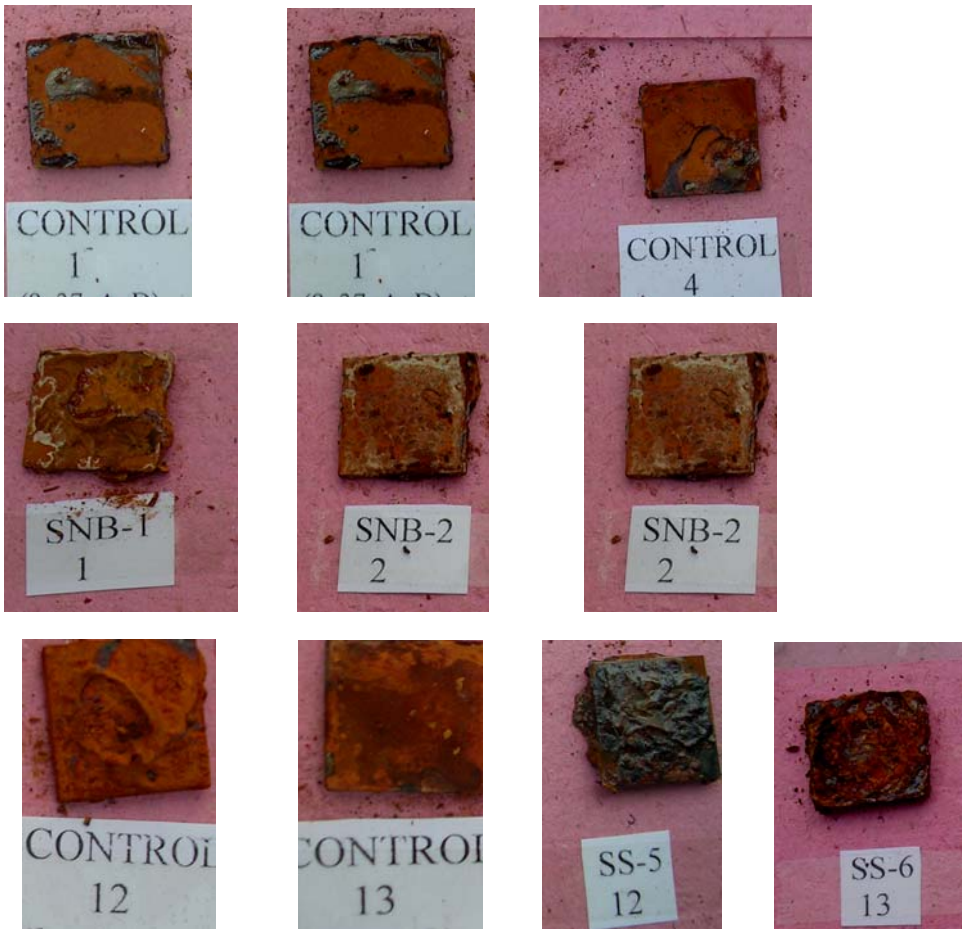

Fig 3. Appearance of the mild steel coupons (2×2cm) exposed to nutrient broth (control) and different cultures for on month period. Excessive corrosion products on the surface of experimental coupon as compared to the respective control ones are evident.

From the sample II, *Bacillus* isolates, *Bacillus coagulans* and *Bacillus pumilis* proved to be extremely deteriorative to mild steel as evidenced by extensive loss in mass of the coupons, with values of 6.61% and 6.1%, respectively. Besides affecting the metal corrosion processes differently, different types of soil physicochemical conditions provide different habitats that direct selective trends for propagation of microorganisms out of indigenous microflora. Thus it is important to study the corrosive bacterial activities with reference to

the nature and depth of soil for buried metal pipes (Matsushima, 2000; Wilmott and Jack, 2000). List of microorganisms involved in MIC in one or the other way is continuously incorporating more species as the research work in this field is progressing. MIC is acknowledged to occur on wide range of metals, however, most reported failure analyses have focused on iron, copper, aluminum and their alloys. In general, damp or wet aerated environments of near neutral pH, characterized by a supply of organic nutrients, would favor the growth of many microbes involved in corrosion (Miller and Tiller, 1970; Kajiyama *et al*., 1996; Ghali, 2000; Li *et al*., 2001).

Here the notion from the above referred literature concerning the corrosion causing /influencing microbes' growth promoting effects of the presence of organic matter in the soil is strengthen by the high corrosion (metal loss) caused by the *Bacillus coagulans* (SS-5) and *Bacillus pumilis* (SS-6) while attacking the mild steel coupons within the nutrient broth. In general, high prevalence of the buried pipelines MIC corrosion in this country within the area characterized by heavy solid organic waste dumping or untreated the sewage leakage/ dumping is a common observation. Such areas/cases are urgently required to be sampled and assessed for the presence and activities of MIC. Bacterial isolates from such environments should be then assessed for their corrosion activities under different sets of physicochemical conditions, especially presence of humus and other acidic and organic matter etc. Such data are highly likely to define the soil characteristics where corrosion and/or MIC should be addressed at top priority level. This would consequently lead reduction in economic burdens posed by the frequency of replacing the damaged (corroded) pipelines in this country.

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TABLE III.- OPTIMIZATION OF DIFFERENT GROWTH CONDITIONS OF THE BACTERIA ISOLATED FROM SAMPLE I GROWN IN NUTRIENT BROTHS.

Isolate #	Experimental condition												
	Temperature			pH			Aeration requirement		Light requirement		Inoculum size		
	RT (25ºC)	37°C	50°C	5	7	9	Aeration	Non-aeration	Light	Dark	1%	5%	10%
SNB-1	8.11 ±0.09	8.26 ±0.02	7.92 ±0.04	7.95 ±0.08	7.45 ±0.78	8.06 ±0.02	8.23 [n] ±0.01	8.07 ±0.03	7.70[d] ±0.3	8.04 ±0.02	6.99[5,10] ±0.01	8.18 ±0.05	8.08 ±0.01
SNB-2	8.04[37] ±0.06	8.14[50] ±0.04	7.95 ±0.07	7.92 ±0.03	4.67 ±2.41	8.08 ±0.06	8.06 [n] ±0.1	8.01 ±0.01	8.38 [d] ±0.3	8.02 ±0.04	7.01[5,10] ±0.07	8.57 ±2.7	8.21 ±0.05
SNB-4	8.11 ±0.06	8.47± 0.08	8.05 ±0.04	7.94± 0.29	7.84 ±0.07	8.36 ±0.01	8.06 [n] ±0.03	8.56 ±0.07	8.54[d] ±0.02	8.45 ±0.02	6.91[5,10] ±0.05	7.74 ±0.01	8.70 ±0.04
SNB-6	6.63 ±0.03	7.49 ±0.50	6.87 ±0.13	6.66[7,9] ±0.05	7.51 ±0.59	8.25[7] ±0.02	6.82 [n] ±0.5	7.98 ±0.2	8.2 [d] ±0.05	7.92 ±0.17	6.75[5,10] ±0.09	8.32 ±0.03	8.35 ±0.04
SNB-7	8.19[37,50] ±0.004	8.39[50]± 0.04	7.23 ±0.27	7.19[9] ±0.09	7.74 ±0.37	8.29 [7] ±0.07	7.93 [n] ±0.07	8.34 ±0.1	8.17[d] ±0.04	8.16 ±0.03	6.63[5,10] ±0.08	8.16 ±0.03	8.28 ±0.09
SNB-8	8.01[37] ±0.02	8.35[50] ±0.07	7.98 ±0.04	7.69 ±0.3	6.96 ±0.8	8.17 ±0.07	8.22 [n] ±0.03	7.95 ±0.01	8.15 [d] ±0.05	8.17 ±0.03	6.74[5,10] ±0.12	8.07 ±0.07	8.10 ±0.04
SNB-9	7.80[37,50] ±0.05	7.98[50]± 0.02	6.92 ±0.01	5.91 ±1.96	7.01[9] ±0.43	7.99 ±0.01	6.80 [n] ±0.02	7.91 ±0.07	7.81 [d] ±0.08	7.80 ±0.04	6.31[5,10] ±0.16	7.90 ±0.01	7.98 ±0.03

Values represent log of C.F.U./ml of 24 hrs incubated cultures and are means of three triplicates ± S.E.M.
The superscript(s) refer to significant difference(s) from the respective group(s) within a respective experiment. (single factor analysis of variance and paired sample t-test). $P \leq 0.05$

TABLE IV.- OPTIMIZATION OF DIFFERENT GROWTH CONDITIONS OF THE ACTINOMYCETES ISOLATED FROM SAMPLE I GROWN IN NUTRIENT BROTHS.

Isolate #	Experimental condition												
	Temperature			pH			Aeration Requirement		Light Requirement		Inoculum size		
	RT (25ºC)	37°C	50°C	5	7	9	Aeration	Non-aeration	Light	Dark	1%	5%	10%
SN-1	8.14 ±0.07	8.04 ±0.06	6.9 ±0.2[25,37]	7.14± 0.40	7.65 ±0.01	7.61 ±4.4	7.87 [n] ±0.06	7.84 ±0.03	7.75 [d] ±0.03	7.86 ±0.11	6[5,10] ±0.17	7.34 ±0.12	7.49 ±4.3
SN-2	8.07 ±0.04	7.86 ±0.07	7.99 ±0.1	7.56 ±0.5	8.12 ±0.03	7.82 ±0.03	8.21 [n] ±0.09	8.19 ±0.1	8.29 [d] ±0.1	8.14 ±0.04	6.10[5,10] ±0.1	7.71 ±0.04	7.78 ±0.1
SN-3	7.86[37,50] ±0.05	7.67 ±0.04	7.43 ±0.01	7.74[7] ±0.03	8.0 ±0.01	7.79 ±0.11	7.87 [n] ±0.1	8.01 ±0.09	7.80 [d] ±0.1	7.49 ±0.18	6.16[10] ±0.1	7.38 ±0.16	7.95 ±0.04
SN-4	7.96[37] ±0.05	7.56[50] ±0.05	7.99 ±0.06	7.6 ±0.06	7.90 ±0.1	7.75 ±0.06	7.62 [n] ±0.14	7.61 ±0.10	7.59 [d] ±0.12	7.64 ±0.04	6.19 ±0.06	7.51 ±0.09	7.54 ±0.17
SN-5	7.49 ±0.28	7.7 ±0.04	7.58 ±0.07	6.92[7,9] ±0.11	7.81 ±0.07	7.75 ±0.04	7.90 [n] ±0.12	7.88 ±0.09	9.18 [d] ±0.75	7.50 ±0.09	6.52 ±0.06	7.78 ±0.11	7.68 ±0.23
SN -6	7.79 ±0.05	8.20 ±0.15	7.99 ±0.06	7.68 ±0.34	8.05 ±0.03	8.09 ±0.03	8.28 [n] ±0.14	7.96 ±0.02	7.53 [d] ±0.76	8.09 ±0.02	7.23 ±0.39	7.86 ±0.43	7.90 ±0.45
SN -7	7.80 ±0.04	7.51 ±0.41	7.06 ±0.4	6.82 ±0.2	7.52 ±0.11	7.60 ±0.18	8.52 [n] ±0.26	8.22 ±0.1	8.04 [d] ±0.03	8.05 ±0.03	6.94 ±0.03	7.73 ±0.4	7.75 ±0.37
SN -8	8.11 ±0.07	8.15 ±0.05	8.09 ±0.06	7.97[7,9] ±0.04	8.21 ±0.03	8.15 ±0.03	8.20 [n] ±0.10	8.15 ±0.06	7.51 [d] ±0.76	7.94 ±0.22	7.31 ±0.29	7.77 ±0.38	7.78 ±0.4
SN -9	7.75[37,50] ±0.04	7.36[50] ±0.04	7.58 ±0.00	7.48 ±0.17	7.89 ±0.22	7.58 ±0.10	7.66 [n] ±0.45	7.79 ±0.4	8.22 [d] ±0.01	8.18 ±0.02	5.83[10] ±0.15	7.09 ±0.05	7.33 ±0.13
SN -10	8.19 ±0.09	8.32 ±0.09	8.43 ±0.08	7.21[7,9] ±0.09	8.38 ±0.07	8.23 ±0.08	7.35 [n] ±0.2	7.75 ±0.4	7.79 [d] ±0.03	7.68 ±0.05	6.98 ±0.12	8.45 ±0.23	8.48 ±0.24

Values represent log of C.F.U./ml of 24 hrs incubated cultures and are means of three triplicates ± S.E.M.
The superscript(s) refer to significant difference(s) from the respective group(s) within a respective experiment. (single factor analysis of variance and paired sample t-test). $P \leq 0.05$

TABLE V.- OPTIMIZATION OF DIFFERENT GROWTH CONDITIONS OF THE BACTERIA ISOLATED FROM SAMPLE I GROWN IN NUTRIENT BROTHS.

Isolate #	Experimental condition												
	Temperature			pH			Aeration Requirement		Light Requirement		Inoculum size		
	RT (25ºC)	37°C	50°C	5	7	9	Aeration	Non-aeration	Light	Dark	1%	5%	10%
SS-1	8.00[37,50] ±0.03	7.87[50] ±0.03	6.98 ±0.05	6.48[7,9] ±0.04	7.66 ±0.09	7.56 ±0.08	7.93 [n] ±0.4	7.88 ±0.08	7.83 [d] ±0.13	8.09 ±0.06	5.68[10] ±0.2	7.66 ±0.09	7.72 ±0.12
SS-2	7.1 ±0.25	7.23 ±0.25	6.96 ±0.07	6.92 ±0.06	7.15 ±0.3	7.6 ±0.06	8.32 [n] ±0.1	8.14 ±0.2	8.23 [d] ±0.0	8.27 ± 0.03	6.45 ±0.2	7.67 ±0.2	8.22[1,5] ±0.09
SS-3	7.66 ±0.09	7.95[50] ±0.09	6.75 ±0.2	6.64[9] ±0.38	7.34 ±0.4	7.91 ±0.03	8.13 [n] ±0.2	8.04 ±0.2	7.86 [d] ±0.03	7.92 ±0.01	6.51[5,10] ±0.06	8.14 ±0.04	8.15 ±0.03
SS-4	7.83[37] ±0.009	7.44 ±0.01	7.71 ±0.06	7.58[7] ±0.07	7.86[9] ±0.08	7.46 ±0.03	8.08 [n] ±0.01	7.98 ±0.07	7.93 [d] ±0.09	7.75 ±0.09	6.76[5,10] ±0.12	8.26 ±0.02	8.39 ±0.06
SS-5	7.89 ±0.05	7.59[50] ±0.05	8.24 ±0.14	7.39 ±0.4	7.49 ±0.3	7.94 ±0.56	7.88 [n] ±0.09	7.77 ±0.01	7.68 [d] ±0.1	7.53 ±0.05	6.20[5,10] ±0.4	7.79 ±0.03	7.82 ±0.50
SS-6	7.81[37] ±0.05	7.03 ±0.05	7.67 ±0.1	7.19 ±0.1	7.09 ±0.4	7.39 ±0.3	7.83 [n] ±0.07	7.24 ±0.15	7.47 [d] ±0.2	7.52 ±0.05	6.72 ±0.11	8.03 ±0.14	8.06 ±0.2
SS-7	7.93[50] ±0.02	7.67 ±0.02	6.79 ±0.5	6.36 ±0.18	6.77 ±0.4	6.86 ±0.4	8.20 [n] ±0.030	7.67 ±0.06	7.63 [d] ±0.07	7.50 ±0.1	6.42[5,10] ±0.07	8.00 ±0.06	8.09 ±0.07
SS-8	6.99 ±0.1	7.07 ±0.1	6.78 ±0.24	8.04 ±0.33	8.36 ±0.08	7.66 ±0.4	7.54 [n] ±0.07	8.52 ±0.04	8.47 [d] ±0.04	8.42 ±0.00	0.83 ±0.07	8.09 ±0.10	8.53[5,10] ±0.11

Continued……

Isolate #	Experimental condition												
	Temperature			pH			Aeration Requirement		Light Requirement		Inoculum size		
	RT (25ºC)	37°C	50°C	5	7	9	Aeration	Non-aeration	Light	Dark	1%	5%	10%
SS-9	7.94 ±0.07	7.72 ±0.07	8.04 ±0.08	7.83 ±0.01	7.52 ±0.36	8.34 ±0.06	8.52 [n] ±0.01	7.81 ±0.11	7.97 [d] ±0.03	8.19 ±0.08	0.80[5,10] ±0.08	7.85 ±0.03	7.85 ±0.03
SS-10	8.33[50] ±0.05	7.88 ±0.05	7.7 ±0.08	2.2 ±2.2	7.88 ±0.22	7.82 ±0.5	8.26 [n] ±0.02	8.05 ±0.1	7.84 [d] ±0.01	7.75 ±0.06	6.85[5,10] ±0.10	7.81 ±1.9	8.18 ±0.2
SS-11	8.23 ±0.06	8.32 ±0.06	6.84[25,50] ±0.2	6.79[7,9] ±0.2	8.27 ±0.02	8.41 ±0.05	7.91 [n] ±0.1	8.51 ±0.03	8.48 [d] ±0.01	8.35 [1] ±0.02	6.91[5,10] ±0.03	8.37 ±0.05	8.44 ±0.01
SS-12	7.86 ±0.08	7.67 ±0.08	7.92 ±0.09	7.49 ±0.2	7.84 ±0.05	7.80 ±0.09	7.36 [n] ±0.06	7.30 ±0.06	7.66 [d] ±0.14	7.39 ±0.11	6.34[5,10] ±0.13	7.69 ±0.05	7.68 ±0.03
SS-13	7.79 ±0.07	7.98[50] ±0.07	7.08 ±0.42	4.26[9] ±2.13	8.00 ±0.01	6.34 ±1.2	8.29 [n] ±0.03	8.46 ±4.9	8.44 [d] ±0.01	8.27 ±0.027	6.88[5,10] ±0.02	8.12[10] ±0.03	6.30 ±2.0
SS-14	7.50[37,50] ±0.27	8.32 ±0.28	8.41 ±0.04	7.98 ±0.14	7.83 ±0.04	8.42[5,7] ±0.04	8.05 [n] ±0.06	8.14 ±0.03	7.85 [d] ±0.01	7.95 ±0.13	6.92 ±0.03	8.32 ±0.01	8.35 ±0.03
SS-15	8.06 ±0.03	7.79 ±0.03	7.68 ±0.43	7.87 ±0.10	8.11 ±0.05	8.05 ±0.1	8.23 [n] ±0.02	8.05 ±0.07	8.10 [d] ±0.03	7.78 ±0.08	6.81[5,10] ±0.04	8.15 ±0.14	8.12 ±0.06
SS-16	8.02 ±0.03	7.81 ±0.02	7.85 ±0.08	7.82 ±0.09	7.89 ±0.5	7.81 ±0.1	8.17 [n] ±0.1	7.91 ±0.03	7.91 [d] ±0.04	7.83 ±0.02	6.23[5,10] ±0.04	7.91[10] ±0.07	8.191 ±0.03

Values represent log of C.F.U./ml of 24 hrs incubated cultures and are means of three triplicates ± S.E.M.
The superscript(s) refer to significant difference(s) from the respective group(s) within a respective experiment. (single factor analysis of variance and paired sample t-test). $P \leq 0.05$

TABLE VI.- OPTIMIZATION OF DIFFERENT GROWTH CONDITIONS OF THE ACTINOMYCETES ISOLATED FROM SAMPLE I GROWN IN NUTRIENT BROTHS.

Isolate #	Experimental condition												
	Temperature			pH			Aeration Requirement		Light Requirement		Inoculum size		
	RT (25ºC)	37°C	50°C	5	7	9	Aeration	Non-aeration	Light	Dark	1%	5%	10%
SSa-2	7.28 ±0.1	7.54[50] ±0.09	8.04 ±0.08	7.65[7,9] ±0.07	8.09 ±0.07	8.05 ±0.01	8.25 [n] ±0.34	7.76 ±0.06	7.85 [d] ±0.07	8.10 ±0.06	6.58[5,10] ±0.09	7.93 ±0.04	8.0 ±0.01
SSa-3	7.40 ±0.17	8.04 ±0.17	7.92 ±0.05	7.52 ±0.08	7.71 ±0.19	7.72 ±0.15	7.94 [n] ±0.2	7.60 ±0.07	7.94 [d] ±0.17	7.93 ±0.09	6.79 ±0.10	8.09 ±0.16	8.11 ±0.02
SSa-4	7.64 ±0.04	7.00 ±0.04	7.44 ±0.08	8.33 ±0.02	8.38 ±0.04	8.39 ±0.03	8.82 [n] ±0.007	8.46 ±0.02	8.29 [d] ±0.06	8.31 ±0.009	4.61[5] ±2.3	7.98 ±0.01	7.82 ±0.2
SSa-5	6.20 ±0.1	7.22 ±0.1	4.63 ±2.32	8.31 ±0.01	8.41 ±0.03	8.42 ±0.06	8.20 [n] ±0.6	7.84 ±0.4	8.14 [d] ±0.08	7.54 ±0.8	6.59 ±0.1	7.39 ±0.28	7.55 ±0.28

Values represent log of C.F.U./ml of 24 hrs incubated cultures and are means of three triplicates ± S.E.M.
The superscript(s) refer to significant difference(s) from the respective group(s) within a respective experiment. (single factor analysis of variance and paired sample t-test). $P \leq 0.05$

TABLE VII.- PHYSIOBIOCHEMICAL CHARACTERIZATION AND IDENTIFICATION OF THE BACTERIAL ISOLATES POSSESSING HIGH (3.9 TO 6.61% METAL LOSS) MILD STEEL CORRODING ACTIVITY.

Sample #	Isolate #	Characteristic																	Identified as
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
I	SNB-1	+ (Diplo bacilli)	+	-	+	+	+	+	--	+	+	--	--	--	--	--	--	--	*Bacillus sp.*
	SNB-2	+ (Diplo-bacilli)	+	+	+	+	+	+	--	+	+	--	--	--	--	--	--	-	*Bacillus sp.*
	SNB-4	+ (Diplo-bacilli)	+	-	+	+	+	+	--	+	+	--	--	--	--	--	+	+	*Sulfobacillus sp.*
II	SS-5	+ (Bacilli With slime)	+	+	+	+	+	-	--	-	+	+	+	--	--	-	--	-	*Bacillus coagulans*
	SS-6	+ (*Bacillus*)	+	+	-	+	+	-	--	-	-	+	--	-	-	--	--	-	*Bacillus pumilis*

1, Gram's staining and cell morphology; 2, Endospore staining; 3, Capsular; 4, Flagellar staining; 5, Motility test; 6, Catalase; 7, Oxidase; 8, Lecithinase; 9, Acid Production; 10, Nitrate; 11, MR.VP; 12, Citrate; 13, Indole; 14, Starch; 15, Gelatin hydrolysis; 16, Sulphate; 17, H_2S production test

-- test was not required

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RUMINANTS FROM HASNOT, THE MIDDLE SIWALIKS OF PAKISTAN

MUHAMMAD AKBAR KHAN AND MUHAMMAD AKHTAR

Department of Zoology, University of the Punjab, Quaid-e-Azam Campus, Lahore

Abstract. - The fossilized ruminant material was collected from 23 localities present at the vicinity of village Hasnot where at least three fossiliferous units occur. Representatives of families Bovidae, Tragulidae, Cervidae and Giraffidae were found from the localities. *Eotragus noyei* was a smallest boselaphine found from the late Miocene of the Siwaliks for the first time in the world. It is also elaborated in this paper how a palaeontologist identifies the animal by studying the dental remains.

Key words: Ruminants, Hasnot, Miocene, the Middle Siwaliks.

INTRODUCTION

The Hasnot fauna age is 7–5 million years ago which is the part of the Middle Siwaliks (Pilbeam *et al*., 1977; Barry *et al*., 1982; Barry, 1987). The Middle Siwaliks has Neogene Tertiary sediments of age11.2–3.5 million years ago (Barry *et al*., 2002). The Middle Siwaliks is divided into two formations; the lower one is the Nagri Formation and the upper is the Dhok Pathan formation (Colbert, 1935; Pilgrim, 1937, 1939). In the Dhok Pathan Formation two villages are very fertile with paleo biodiversity; these are Dhok Pathan and Hasnot. The Hasnot village is situated at about 70 km west of the Jhelum city in the Potwar Plateau of the northern Pakistan on the east bank of the river Bunha (Fig. 1). The village is surrounded by a number of highly fossiliferous localities at an altitude of about 326 meters and has Neogene freshwater sedimentary rocks. The average thickness of the sequence around this area is about 180 m.

MATERIALS AND METHODS

Surface collection was the primary method of collecting remains of ruminants. As a result, identifiable specimens of ruminants were discovered. Some of the specimens were already found in the collections of the Palaeontology Laboratory, University of the Punjab, Lahore, Pakistan, had been

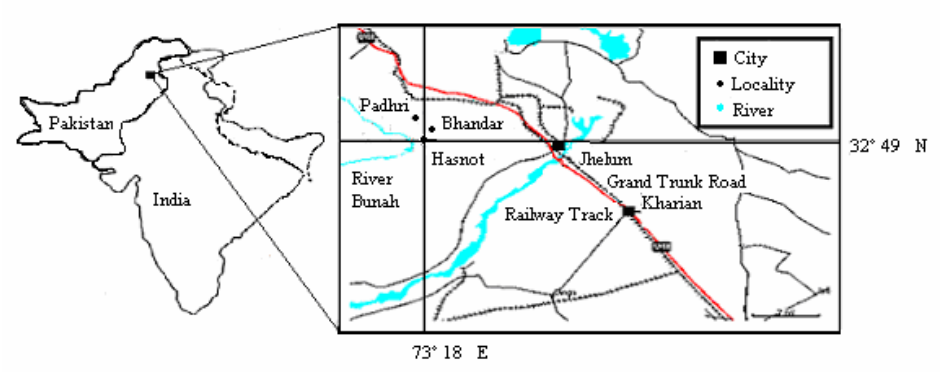

Fig. 1: Location of the study area.

collected previously from localities around Hasnot were also included in this study. Most of the specimens were found partly exposed, thus, excavation methods had to be employed, while a few others were found lying completely exposed on the surface. The embedded material was carefully excavated with the help of chisels and geological hammers. In the laboratory, the material was carefully washed, cleaned, prepared, and broken parts were assembled by using various types of gums (resins) such as Elfy, Elite and Fixin.

Identification parameter

Identification is based on the dental material. Mammals are recognized by their unspecialized teeth. These unspecialized teeth in mammalian groups have variation in their cusp structure. Primates have conical and blunt cusps these are called bunodonts whereas Perissodactyls and Proboscideans have modified cusps, called lophs; these groups are called lophodonts (Kardong, 2002). In artiodactyl cusps are modified into crescentic form and this pattern is called Selenodonty. So ruminants exhibit selenodonty in their tooth structure (Fig. 2).

Tooth morphology

An entostyle can be found in the center of the lingual side of the upper molar and ectostylid is found in the buccal side of the lower molar, completely or partly separate from the rest of the occlusal surface. Tooth length and breadth were measured at occlusal level (Gentry, 1994). Heights were measured on the mesostyle of the upper molar, the metastylid of the lower molar and the protoconid of the lower premolar (Fig. 3).

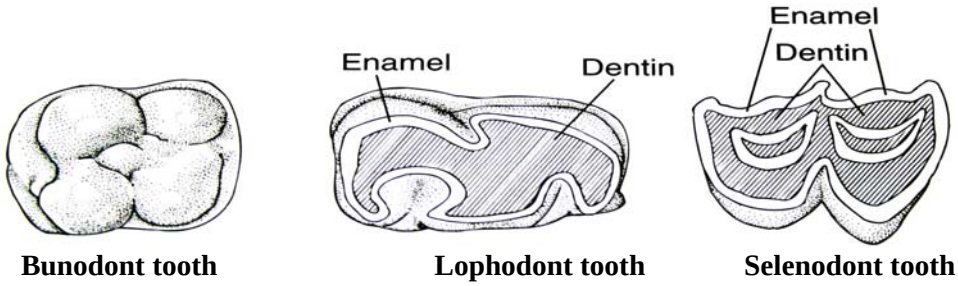

Fig. 2: Modified cusps in mammals.

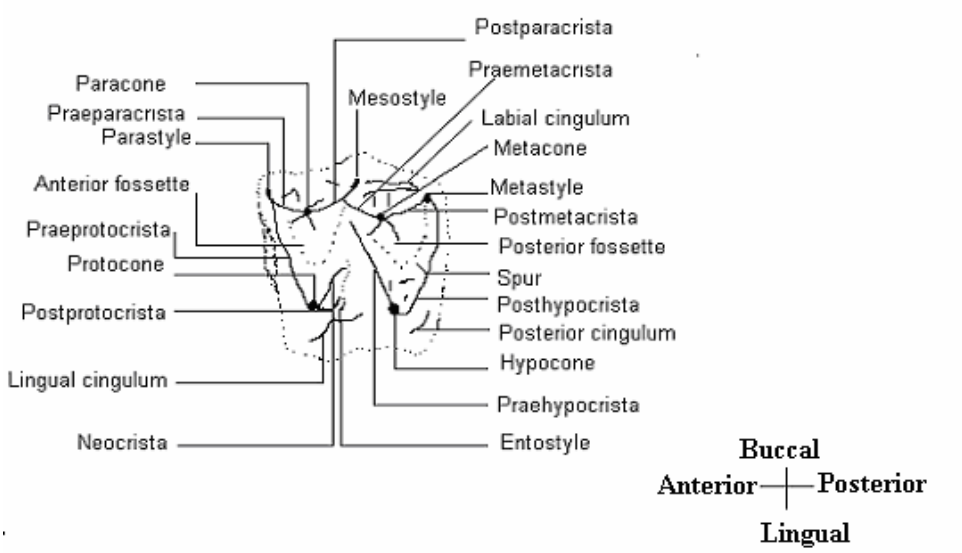

Fig. 3. Terminology used for the molar identification.

Family Bovidae

Tribe Boselaphini

Selenoportax vexillarius, S. lydekkeri, Pachyportax latidens, Eotragus noyei

Diagnosis

Crescentic cusps, moderate to large sized Siwalik bovid; with hypsodont to extremely hypsodont teeth, upper molars quadrate with strong divergent styles, median ribs well developed, entostyle strongly developed and ectostylid

moderately developed, enamel very rugose, crown narrow at the base and broad at the apex.

Tribe Antilopini
Gazella lydekkeri

Diagnosis

Presence of rudimentary entostyles/ectostylids or without entostyles; well developed goat folds, fossettes fairly simple in outline, stylids and ribs moderately developed.

Family Tragulidae
Dorcatherium minus, Dorcatherium majus

Diagnosis

The cheek teeth are prominently hypsodont. The upper molars bear strongly developed styles and basal cingulum. The lower molars are characterized, either by well-developed ectostylids or by vestigial ectostylids and posteriorly directed double fold protoconids.

Family Cervidae
Cervus simplicidense, Cervus triplidense, Cervus sivalensis, Cervus rewati

Diagnosis

Enamel is not much rugose. Styles are not well developed.

Family Giraffidae
Hydaspitherium megacephalum

Diagnosis

The parastyle of *H. megacephalum* is prominent as compared to the mesostyle and the metastyle. The interior median rib is moderately developed, whereas the posterior median rib is weakly developed. *H. megacephalum* generally is smaller compared to the *H. grande* and *H. magnum.*

DISCUSSION

Hasnot is found in a relatively isolated part of the southern Potwar Plateau, 90 km from Dhok Pathan, surrounded by number of fossiliferous localities. 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 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 the 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 found 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).

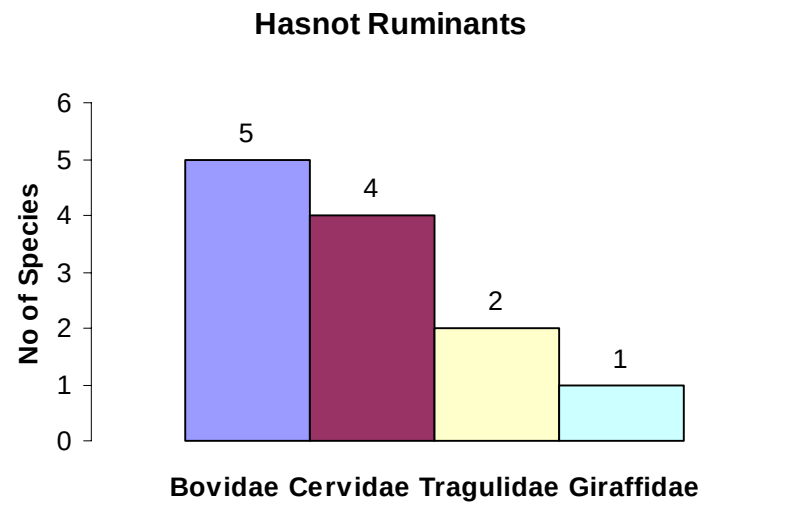

Fig. 4: Hasnot ruminant species.

CONCLUSIONS

Bovids are very common. In the present collection, tragulids were mostly collected from all the fossiliferous sites but cervids were collected mostly from the sites near the Bhandar Bed. Giraffid specimens were collected from the sites close to the river Bunah. Following species of ruminants are found in the present study: *Selenoportax vexillarius, Selenoportax lydekkeri, Pachyportax latidens, Eotragus noyei, Gazella lydekkeri, Dorcatherium majus, Dorcatherium minus, Cervus simplicidense, Cervus triplidense, Cervus rewati, Cervus sivalensis, Hydaspitherium megacephalum* (Fig. 4).

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AVIAN HAEMOPROTIDAE-5: *HAEMOPROTEUS CORACIAE* DE MELLO AND AFENSO, 1935, A PARASITE OF TURKISTAN ROLLER (CORACIDAE) IN BALOCHISTAN, PAKISTAN

YASMIN NAWAZ AND MOHAMMAD NAWAZ

Department of Zoology, University of Balochistan, Quetta, Pakistan
E-mail: nawazzoologist@yahoo.com

Abstract.- Six birds of the genus *Coracias garrulus seminovi* belong to family Coracidae collected from Pishin and Sibi area of Balochistan were examined for haematozoan parasites and one was found to be heavily infested with *Haemoproteus coraeiae*. The incidence was 16.66% and intensity was 2-3/500 RBCs. This is the first locality and host record from Pakistan.

Keywords: *Haemoproteus coraciae*, Coracidae, Balochistan, Pakistan.

INTRODUCTION

The members of the avian family Coracidae are commonly called rollers because of their spectacular aerial acrobatics. It is a small family of 17 insectivorous species that are widely distributed throughout Africa, Asia and Australia. The species under study is called Turkistan Roller collected from Pishin, Quetta and Sibi area. This species is also distributed in Europe, Transcaspian area, Central Asia, Afghanistan and Northwest India. Local nesting migrants among these areas or probably nomadic life, usually inhabit low lands, semi-desert and desert localities with clifs and ravines or bushy vegetations. This bird feeds on large insects and always lives in flock of 5-10 birds.

The Haemoproteus from Corcidae have been recorded in 1913 by Tartakovski (Leningrad) named *Haemoproteus coraciatis.* This species has been designated a nomen nudum because of absence of description. In 1935 de Mello and Afenso described *H. coraciae* and *H. coraciae* Var. *bengalensis* from *C. bengalensis.* Valles (1938) also described *H. coraciae* unaware of the previous work. It was junior homonym of *H. coraciae* de Mello and Afenso 1935 (Bennett *et al.*, 1982). Tendeiro described three species and one variety in 1947 from this family – *H. velaseoi* from *Coraeias abyssinica, H. fontasi* from *C. naevia, H. fantasi* var. cyanogasten from *C. cyanogaster*, and *H. cruzferreirae* from *Eurystomus afar*, primarily on the philosophy of "one-host-one parasite".

The International Reference Centre for Haematozoa has provided the basis for review of the chaotic situation of all previously described *Haemoproteus* spp. also redescription of the haemoproteids of the Coraeidae family (Medona *et al.*, 1986), *H. coraciae* var *bengalensis*, de Mello and Afenso,1935, *H. coriciae* Valles, 1938 nec de Mello and Afonso, *H. velaseoi* Tendeiro, 1947, *H. fontasi* Tendeiro, 1947, and *H. fontasi* var. *cyanogasteri* Tendeiro, 1947 are declared synonyms of *H. coraeiae* and one other species *Haemoprotcus eurystomae* n.sp is also described from *Eurystomus orientalis.* These two species can be separated by different morphological parameters (Bishap and Bennett, 1986).

MATERIALS AND METHODS

The blood smears were air dried, fixed in 100% ethanol or methanol, and stained with Giemsa's stain. Observations were made under 100x lens. The morphometric parameters were obtained by drawing the appropriate cell with camera lucida. Measurements are expressed as Means±SD. The number of specimens measured is indicated by "N" and the nuclear displacement ratio as NDR. All holotypes and paratypes are in the Zoology Department of University of Balochistan, Quetta.

OBSERVATIONS AND RESULTS

Family: Haemoproteidae Doflein, 1961
Genus: *Haemoproteus* Kruse, 1890
Species: *Haemoproteus coraciae* de Mello and Afenso, 1935
Host: *Coracias garrulous semenovi* Loud and Tschusi, (Turkistan Roller)
Locality: Pishin, Sibi, Quetta.

Description

Immature gametocyte (Fig. 1)

Young macrogametocyte lateral to erythrocyte host cell, nucleus in matured cell; surface entire. Young microgametocytes with amoeboid surface, markedly more amoeboid than macrogametocyte.

Macrogametocyte (Figs. 1-3; Table I)

Mature macrogametocyte usually halteridial shaped; margins usually entire but occasionally amoeboid; parasite lateral to host cell nucleus, slightly

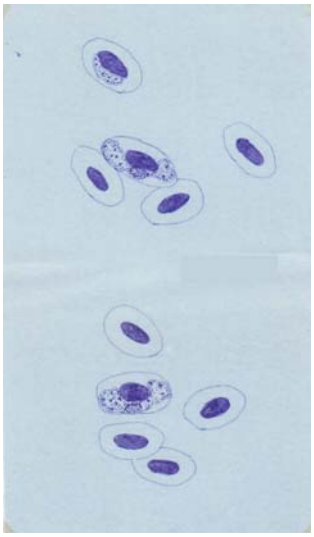

Fig. 1. *Haemoproteus corcaciae*, A, immature microgametocyte and mature microgametocyte; B, Macrogametocyte.

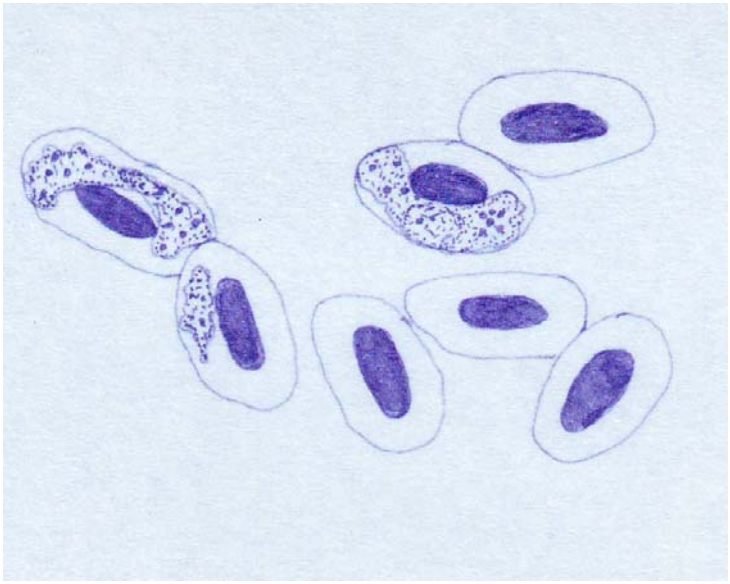

Fig. 2. Immature microgametocyte, mature microgametocyte and macrogametocyte of *Haemoproteus coraciae*.

extend over surface of host cell nucleus; some time dumb-bell shaped; cytoplasm granular; vacuolated staining dark bluish with Giemsa's stain; pigment granules prominent, ovoid, randomly distribution throughout cytoplasm; ranging from 8-10; averaging 8.8 (0.26) in number per parasite; parasite nucleus median, averaging 2.63 (0.10) in length, 1.93 (0.08) in width and 5.09 (0.20) in area, staining deep pink with Giemsa's stain, Parasite measured 14.36 (0.30) in length, 1.96 (0.01) in width and 28.20 (0.74) in area, constituting about 35.58% of host parasite complex; host cell nucleus very little or not replaced laterally. NDR=0.78; host cell hypertrophied 19.53% in length, 8.28% in area and 9.25% atrophied in width; host cell nucleus atrophied 15.23% in length, 12.72% in area and atrophied 1.97% in width.

Microgametocyte (Figs. 1-3, Table I)

Matured gametocyte halteridial shaped; outline amoeboid, not entire; parasite lateral to host cell nucleus; host cell nucleus not replaced; parasite extend over surface of nucleus; cytoplasm light blue in colour with Giemsa's stain; Pigment granules prominent, oval in structure; randomly distributed in cytoplasm; ranging from 8-10; averaging 8.60 (0.20) in number per parasite. Parasite nucleus median, slightly ovoid; averaging 2.83 (0.06) in length, 1.71 (0.01) in width and 4.84 (0.04) in area; staining light pink in colour with Giemsa's stain; Parasite measured 15.59 (0.14) in length, 1.62 (0.03) in width and 25.27 (0.52) in area, constituting 29.38% of host parasite complex; host cell nucleus not displaced laterally; NDR=1.00; host cell hypertrophied 28.37% in length, 17.5% in area and atrophied 8.51% in width; host cell nucleus atrophied 15.23% in length, 12.72% in area and hypertrophied 1.97% in width.

Incidence and intensity of infection

Six birds were examined for haematozoa. Heavy infection with *Haemoproteus coraciae* was recorded from only one bird. Incidence was 16.66% and intensity was 2-3/500 R.B.C

Remarks

Uptill now two morphologically distinguishable species of haemoproteids, *H. eurystomae* and *H.coraciae* have been described (Bishop and Bennett,1986) *H. eurystomae* is a large halteridial parasite that is clearly separable from *H. coraciae* on the basis of larger area, many number of pigment granules,23 compared with 8, larger percent hypertrophy of the area of erythrocyte host cell, and the immature forms are not as amoeboid as those of *H. coraciae.*

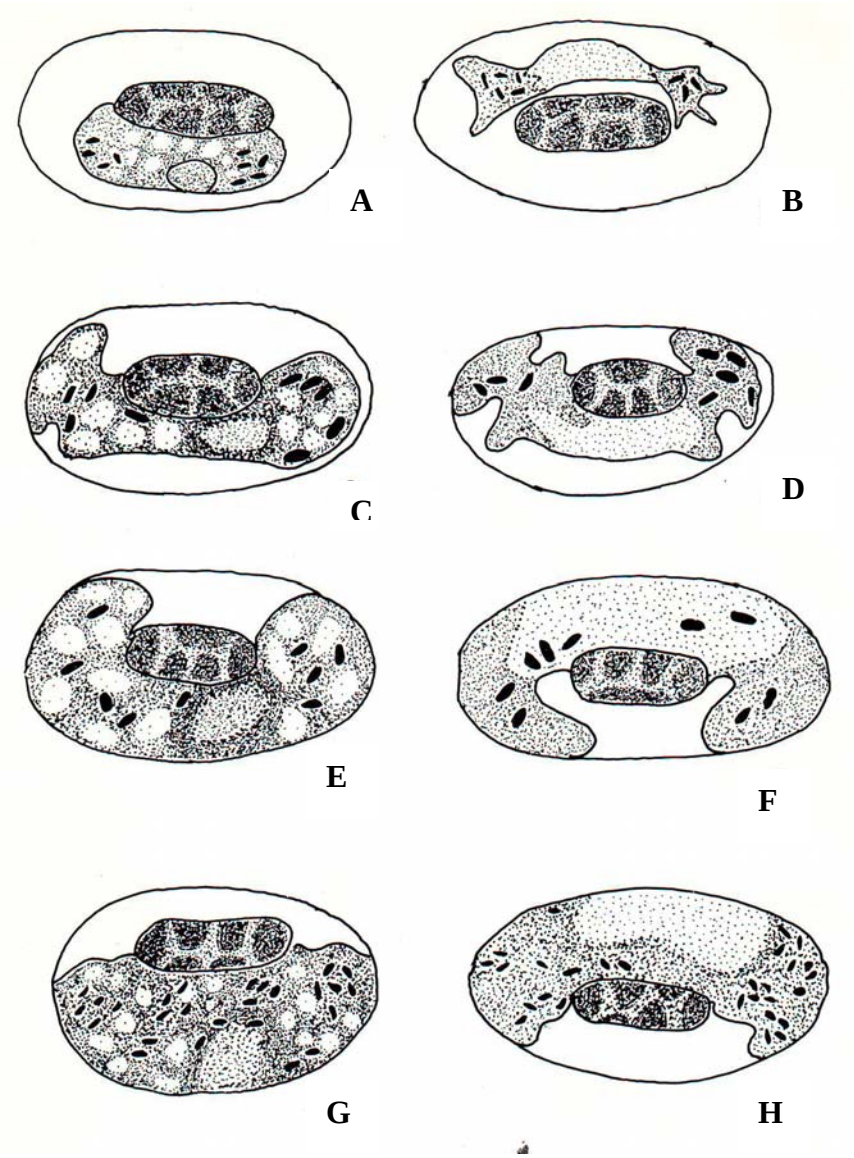

Fig. 3. *Haemoproteus coraciae*. A-C, young macrogametocyte; B, D, young microgametocytes. Note the highly ameboid outlines in comparison with the young macrogametocytes; E, mature macrogametocyte; F, mature microgametocyte; G, H, *Haemoproteus eurystomae*; mature macrogametocyte; H, mature microgametocyte (Bishop and Bennett, 1986).

TABLE I.- MORPHOMETRIC PARAMETERS OF TWO SPECIES OF HAEMOPROTEIDS OF CORACIIDAE. ALL MEASUREMENTS ARE IN µm.

	H. eurystomae **Host: *Eurystomus orientals* (Malaysia) (n=45)**	***H. coraciae*** **Host: *C. bengalensis* India (n=60)**	***H. coraciae*** **Host: *C.g. sememori* (present study) (n=10)**
Unparasitized RBC			
Length	13.8±0.8	12.7±0.9	10.75±0.06
Width	7.7±0.7	7.8±0.7	6.81±0.03
Area	85.8±7.8	79.0±9.3	73.19±0.27
Erythrocyte nucleus			
Length	6.9±0.5	6.3±0.6	6.04±0.02
Width	2.8±0.3	2.7±0.3	2.03±0.02
Area	16.1±2.2	14.1±2.3	12.26±0.07
% area of nucleus of total area	19.00	18.00	16.75
Erythrocyte parasitized by macrogametocyte	n=40	n=75	n=10
Length	14.0±1.5	13.9±0.9	12.85±0.12
Width	8.5±0.9	7.3±0.7	6.18±0.04
Area	93.4±8.4	82.2±9.5	79.25±0.40
% hypertrophy/ atrophy			
Length	+ 1.00	+ 9.00	+ 19.53
Width	+ 10.00	- 6.00	- 9.25
Area	+ 9.00	+ 4.00	+ 8.28
Erythrocyte nucleus			
Length	6.6±0.6	5.7±0.6	5.12±0.05
Width	2.6±0.2	2.7±0.3	2.07±0.01
Area	13.9±1.5	12.6±1.6	10.70±0.10
% area Erythrocyte-Parasite complex	15.00	16.00	13.50
% hypertrophy/ atrophy			
Length	-4.00	-10.00	-15.23
Width	-7.00	No change	+1.97
Area	-14.00	-11.00	-12.72
N.D.R.	0.5±0.2	0.8±0.2	0.78

Continued

	H. eurystomae **Host: *Eurystomus orientals* (Malaysia) (n=45)**	***H. coraciae*** **Host: *C. bengalensis* India (n=60)**	***H. coraciae*** **Host: *C.g. sememori* (present study) (n=10)**
Macrogametocyte			
Length	15.8±1.6	15.0±1.1	14.36±0.30
Width	4.3±0.7	2.6±0.6	1.96±0.01
Area	61.8±7.9	48.5±6.9	28.90±0.74
% erythrocyte parasite complex	66.00	59.00	35.58
Macrogametocyte nucleus			
Length	3.1±0.6	2.3±0.6	2.63±0.10
Width	2.0±0.5	1.4±0.3	1.93±0.08
Area	4.5±1.0	2.3±0.7	5.09±0.20
% area of parasite	7.00	5.00	18.05
No. of pigment granules	22.9±3.7	8.2±1.7	8.8±0.26
Erythrocyte parasitized by microgametocyte	(n=20)	(n=30)	(n=10)
Length	13.9±1.6	14.2±1.2	13.80±0.07
Width	8.2±1.4	7.6±0.9	6.23±0.02
Area	94.2±10.9	86.6±13.5	86.01±0.41
% hypertrophy/ atrophy			
Length	+1.00	+12.00	+28.38
Width	+6.00	-5.00	-8.51
Area	+10.00	+6.00	17.51
Erythrocyte nucleus			
Length	6.7±0.6	5.9±0.6	5.09±0.01
Width	2.7±0.4	2.8±0.3	1.90±0.03
Area	15.0±1.9	13.5±1.8	9.68±0.18
% area erythrocyte parasite complex	16.00	16.00	11.25

Continued

	H. eurystomae **Host: *Eurystomus orientals* (Malaysia) (n=45)**	***H. coraciae*** **Host: *C. bengalensis* India (n=60)**	***H. coraciae*** **Host: *C.g. sememori* (present study) (n=10)**
% hypertrophy/ atrophy			
Length	-3.00	-6.00	-15.72
Width	-4.00	+4.00	+6.40
Area	-7.00	-2.00	-21.04
N.D.R.	0.5±0.2	0.8±0.2	1.00
Microgametocyte			
Length	16.3±1.7	14.6±1.6	15.59±0.14
Width	4.2±0.6	2.4±0.7	1.62±0.03
Area	61.3±6.7	43.2±6.8	25.27±0.52
% area erythrocyte parasite complex	65.00	50.00	29.38
Microgametocyte nucleus			
Length	8.0±2.2	7.1±1.3	2.83±0.06
Width	3.3±0.5	2.1±0.4	1.71±0.01
Area	20.9±5.5	14.0±3.7	4.84±0.04
% area of parasite	34.00	32.00	19.15
No. of pigment granules	23.4±3.8	8.6±1.8	8.60±0.20

Note: Data on *H. coraciae* and *H. eurystomae* are taken from Bishop and Bennett (1986).. Hypertrophy is indicated by "+", and atrophy by "-" sign

The species under study resemble very closely those already described species, *H. coraciae* in morphology, number of pigment granules, in NDR, % atrophy and hypertrophy in length, width and area of the parasitized R.B.C. But differ in Size, Percent covered area, and in host record. On the basis of similarities it has been identified as such with new host record (Table I). The world record of the distributional range of this family, Coraciidae showed that *H. coraciae* was more prevalent than *H. eurystomae* and no mixed infection is on record within this family (Bishop and Bennett, 1986).

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AVIAN HAEMOPROTIDAE-6: *HAEMOPROTEUS HALCYONIS* DE MELLO, 1935, A PARASITE OF KING FISHER (ALCIDINIDAE) FROM BALOCHISTAN, PAKISTAN

MOHAMMAD NAWAZ AND YASMIN NAWAZ

Department of Zoology, Balochistan University, Quetta, Pakistan.
E-mail nawazzoologist@yahoo.com

Abstract.- A bird *Alcedo atthis pallasii* of family Alcidinidae was shot and examined for blood parasites. A blood parasite *Haemoproteus halcyonis* was identified. This is the first locality and host record from Pakistan.

Key words: *Haemoproteus halcyonis*, Alcidinidae, Balochistan, Pakistan.

INTRODUCTION

During the avian survey in Balochistan three genera and three species were observed *i.e. Halcyonis somyrensis* (white breasted king fisher) and *Ceryle rudis* (small pied king fisher) which can be seen around Quetta (Cristison, 1942), and *Alcedo atthis pallasii* Reichanboch, 1851 (the Central Asian king fisher), which can be seen in the area close to Afghanistan border especially in those places where perennial streams are present. One specimen was shot dead in Panjpai during summer and blood smears were prepared. Unfortunately all slides were damaged, only a few pieces of stained slides were preserved and used for the study. This species of king-fisher is also reported from Afghanistan (Pauludan, 1953). This might be migratory in summer in this area for breeding.

In 1971, Levine and Campbell cited 139 species of haemoproteus of which 120 occurred in birds. The majority of these species were described prior to 1940 and many were created on the assumption of "one host-one parasite". Undoubtedly, many of these species are synonyms. In an effort to introduce some order into the current taxonomic chaos of the family Haemoproteidae an attempt was made to review the *Hemoproteus* species in avian families. Bennett and Camphell (1973) on the basis of cross infection and limited experimental evidence available, summarized by indicate strong familial specificity (Bennett *et al*., 1972).

Haemoproteids have few recognizable morphological characters *viz.*, utilization of degree of host cell hypertrophy, degree of host cell nuclear displacement, the size of parasite as measured by its ratio to the host-cell-parasite complex, the degree of parasite encirclement of the host cell nucleus, number of pigment granules and nuclear displacement ratio (NDR). Bennett *et al*. (1972) examined some 200 blood films from 14 species of king fishers infected with *Haemoproteus* species and identified three morphological by distinct species, *viz*., *Haemoproteus enucleator, H. halcyonis* and *H. fusea. Haemoproteus halcyonis* from Alcidinidae is the first report from Pakistan but further research on haemotogoan survey of all king-fishers found in Pakistan is required for the detailed report of *Haemoproteus* species of Alcidinae.

MATERIALS AND METHODS

Blood slide, prepared from the killed king-fisher, were air dried, fixed in 100% ethanol and stained in Giemsa stain. The parasites, micro and macrogametocytes and immature gametocytes were studied using 100x lens. Dimensions of the parasites were measured as described by Bennett and Campbell (1972) and results are presented as Means±SD.

RESULTS

Family: Haemoproteidae Doflein, 1961
Genus : *HAEMOPROTEUS* Kruse, 1890
Species : *Haemoproteus halcyonis* de Melo, 1935
Host : *Alcedo atthis pallasii* Reichanbach, 1851
Locality : Panjpai

Diagnostic features

Heavy infection of *Haemoproteus* sp. with immature and mature gametocytes was observed. Matured gametocytes halteridial; sausage shape, not encircling host cell nucleus. Nucleus median. Double infection very common; pigment granules feebly stained; very few visible towards the poles of the parasite.

Immature parasite

These were observed in the initial stages in the subpolar location,

elongating along the periphery of host cell nucleus, nucleus displaced laterally. Single and double infection present almost in every field of microscope. Parasite, light pinkish in colour; nucleus dark pinkish in colour with Giemsa's stain, nucleus median in position; pigment granules feebly visible.

Gametocytes (Figs. 1, 2; Tables I-II)

Mature gametocytes of large size were seen not extending beyond host cell nucleus, even it does not reach polar margin of erythrocyte; surface entire; halteridial, sausage shaped cytoplasm finely granular, vacuolated, staining light pink with Giemsa's stain. Parasite nucleus light pink and median in position. Due to light stain macro and micro gametocytes can not be distinguished.

TABLE I.- DIMENSIONS OF THE *HAEMOPROTEUS HALCYONIS* RECOVERED FROM *ALCEDO ATTUS PALLASII*. ALL MEASUREMENTS BASED ON 10 PARASITES AND NORMAL AND PARASITIZED ERYTHROCYTES FROM ONLY ONE HOST. ALL MEASUREMENTS ARE IN MICRONS. THE VALUES ARE IN MEAN±SD.

	Parasitized	**Non-parasitized**
Erythrocyte		
Length	14.83±0.14	12.07±0.07
Width	6.29±0.03	6.09±0.07
Area	91.16±1.80	73.58
Parasite		
Length	11.24±0.09	-
Width	3.45±0.03	-
Area	38.55±1.12	-
No. of granules	-	-
NDR	0.36	1.00

The parasite measured, 11.24 (0.09) in length, 3.45 (0.02) in width and 38.55 (1.12) is area. Parasite occupied 42.28% area of erythrocyte. Hypertrophy 22.86% in length, 3.28% in width and 23.89% in area. Nuclear displacement ratio 0.36.

Incidence of infection

Only one bird was examined and found heavily infected with *Haemoproteus halcyonis.*

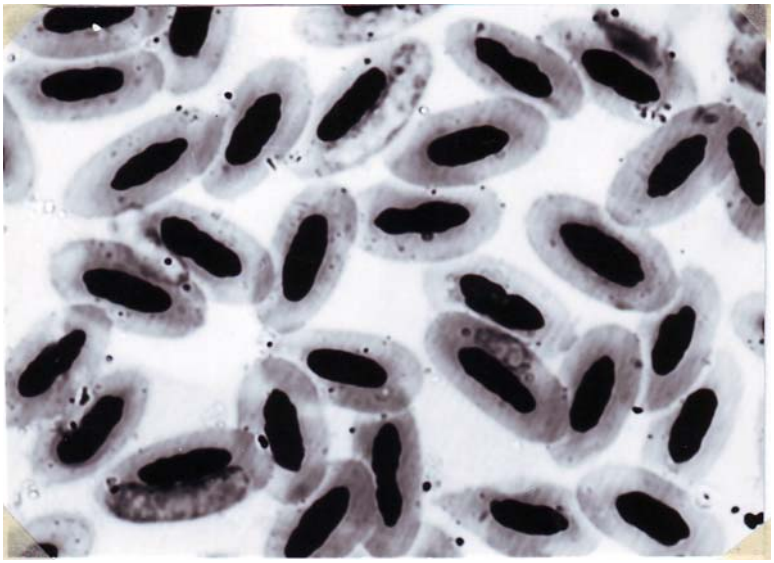

Fig. 1. Gametocyte of *Haemoproteus halcyonis* recorded from king-fisher (*A.a.pallasii*)

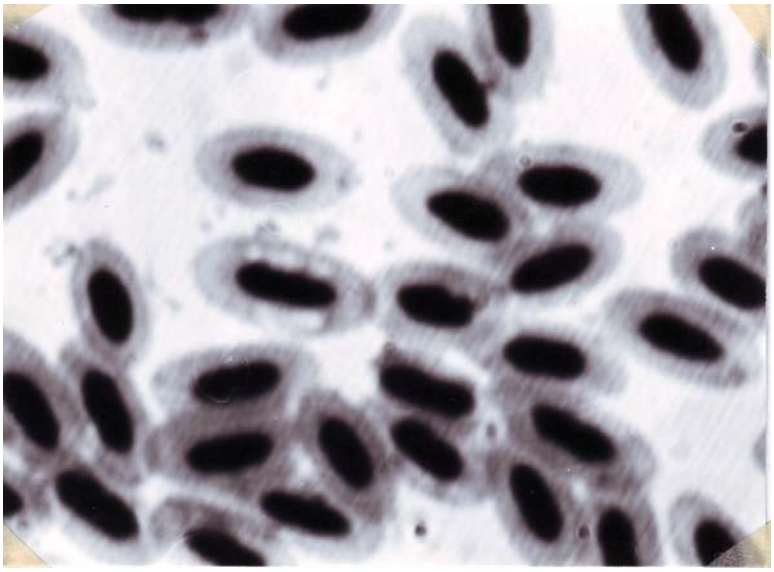

Fig. 2. Gametocytes of *Haemoproteus halcyonis* showing double infection in single erythrocyte.

Table II

Intensity of infection

The infection was very heavy. Immatured and matured gametocytes was observed almost in every field of microscope. Intensity of infection was 3-4/200 RBC.

Remarks

Four species of *Haemoproteus* have been described so far from Alcidinidae (Bennett and Campbell, 1973). *H. enucleator* totally replaces the host cell nucleus. *H. halcyonis* is halteridial oval and cordiform in structure *H. ecae* is fully extended in the host cell and very prominent granules present. *H. fusca* fully encircle host cell nucleus and do not displace it. Other morphometric measurements are given in Table II.

The species under study is sausage shaped, halteridial. After comparing this species with the haemoproteids in Alcidinidae, it is found closely related with *H. halcyonis* in many characters and identified as such. This is the first report of Haemoproteus from Pakistan with new host record.

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TABLE II.- DIMENSIONS OF *H. HALCYONIS*, *H. FUSCA*, *H. ENUCLEATER* AND THE SPECIES UNDER STUDY. ALL DIMENSIONS ARE BASED ON 10 PARASITES AND 10 NORMAL ERYTHROCYTES, THE VALUES ARE IN MEAN±SD.

	H. halcyonis		***H. fusca***		***H. enucleater***		***H. halcynois***	
	Parasitized	**Non-parasitized**	**Parasitized**	**Non-parasitized**	**Parasitized**	**Non-parasitized**	**Parasitized**	**Non-parasitized**
Erythrocyte								
Length	14.5±0.9	14.2±0.9	15.5±1.2	13.7±0.8	16.4±1.4	13.6±0.8	14.83±0.14	12.07±0.07
Width	8.0±0.74	7.8±0.55	7.7±0.8	7.7±0.4	7.4±1.2	7.2±0.06	6.29±0.03	6.09±0.07
Area	83.6±8.8	78.3±9.2	85.5±9.0	74.4±5.1	82.7±12.6	36.5±6.8	91.16±1.80	73.58
Erythrocyte nucleus								
Length	5.90±0.43	6.9±0.46	6.0±0.6	6.8±0.4	-	-	6.73±0.03	5.95
Width	2.45±0.37	2.8±0.26	2.6±0.25	2.8±0.3	-	-	1.78±0.42	1.97
Area	9.4±1.37	11.3±	9.4±0.9	10.9±1.0	-	-	13.17±0.32	11.58
Parasite								
Length	14.4±0.84	-	14.4±0.84	-	14.2±1.4	-	11.24±0.09	-
Width	4.3±0.65	-	4.3±0.65	-	4.8±0.5	-	3.45±0.03	-
Area	59.6±7.1	-	59.6±7.1	-	46.2±6.3	-	38.55±1.12	-
No. of pigments granules	24.4±9.4	33.8±8.0	-	-	14.8±3.0	-	-	-

Note: Data on *H.halcyonis, H.fusca* and *H.enucleater* from Bennett *et al*. (1972) and Bennett and Campbell (1973).

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FIELD PERFORMANCE OF SYSTEMIC FOLIAR AND GRANULAR INSECTICIDES AGAINST RICE STEM BORERS *SCIRPOPHAGA* SPP. IN RICE CROP

MUHAMMAD SARWAR, AKBAR ALI, NAZIR AHMAD, GUL ZAMIN KHAN, MOULA BUX AND MUHAMMAD TOFIQUE

Nuclear Institute of Agriculture, Tandojam- 70060, Sindh, Pakistan.

Abstract.- The use of insecticides for the control of insect pests outbreak is the only practical measure for their management in modern agriculture. During this research trial, granular [Phorate (Thimet 5 G) and Monomehypo 5 G] and foliar [Spinosad (Tracer 240 SC) and Endosulfan (Thiodan 35 EC)] insecticides were included for testing their efficiency against the rice stem borers using Khushboo-95 and Basmati-370 rice varieties. Among these treatments, Spinosad 240 SC was found to be the best toxicant on the basis of declining the abundance of borers' infestation and maximum grain productivity followed by Monomehypo 5 G, Endosulfan 35 EC and Phorate 5 G. This made Spinosad 240 SC as one of the products of choice for the control of rice insect pests.

Key words: Rice stem borers, granular insecticide, foliar insecticide, chemical control.

INTRODUCTION

In Pakistan, rice varieties particularly Basmati are of the international reputation and appreciated for their cooking quality and aroma, and are of great demand at substantial premium in the world market (Chaudhary, 1976). A tremendous irrigation system, encouraging environmental conditions, hard working peasants and fertile soils in Pakistan are very relevant to have bumper paddy crop. The efforts made to increase the rice production has, no doubt, brought satisfactory results but its yield per hectare is very low as compared with other rice growing countries around the globe, which could be attributed to pests attack.

There are a large numbers of insects which damage rice crop right from nursery to the harvest causing considerably high yield losses. About 128 species of insects have been reported attacking the rice crop (Ahmad, 1981). Out of these, rice borers *Scirpophaga* spp. have been playing havoc to rice crop in the past. Rice yellow stem borer *Scirpophaga incertulas* Walker is a very important

pest of rice, which causes yield loss ranging from 5 to 10%. Although the losses are compensated at the vegetative stage, the damage cannot be compensated at the reproductive stage (Kumari and Pasalu, 2003). It is the most important insect pest of rice, attacking all stages of the crop during wet and dry seasons (Padhi and Saha, 2004). An allied species *Scirpophaga innotata* Walker, the white moth borer, is also present in most of the paddy growing areas and damage of this species is almost similar to *S. incertulas* (Atwal, 1986). The insect pests and diseases have caused serious damage to rice crops. The damage caused by rice borers has become more serious year after year and actual losses still reach several millions kilograms (Jiang *et al.,* 2005). In modern agriculture, pesticides are frequently used to increase crop production; especially the control of rice stem borers is not feasible without the help of granular and sprayable formulations of insecticides. To combat the pest resistance and to sustain agricultural productivity, the agrochemical industry has recently introduced certain insecticides with new chemistry bearing novel modes of action. The proposed study was carried out to quest the effects of granular and foliar insecticides with new chemistry against rice stem borers for employing as a component of integrated pest management strategy in rice field.

MATERIALS AND METHODS

The research trials were carried out in the experimental farm at Nuclear Institute of Agriculture, Tandojam. The nurseries of two rice varieties viz., Basmati-370 (P) and Khushboo-95 were sown on 25 May 2004. The seed rate was 5 kg per acre. Both the rice varieties used in this study were obtained from the Plant Breeding Division of the Institute. The nursery was transplanted exactly after 40 days after seedling emergence. All the standard agronomic practices were followed to raise the crop. The efficacy of granular [Phorate (Thimet 5 G) and Monomehypo 5 G] and foliar [Spinosad (Tracer 240 SC) and Endosulfan (Thiodan 35 EC)] pesticides was evaluated against the rice stem borers. These insecticides were acquired from the local market and used at recommended concentrations for the field. Four treatments of insecticides and unsprayed control, were applied against the pests. The experiment was laid out in Randomized Complete Block Design. Each treatment was replicated three times. The plot size was 3 x 1.5 m. Row to row and plant-to-plant distance was kept 25 cm and 25 cm, respectively. A path of 90 cm was maintained among the treatments. All the agronomic practices were applied timely and kept constant for the whole rice field. Urea was applied three times at 15, 30 and 50 days after transplanting. All plots received an initial application of Triple Super Phosphate.

The applications of the insecticides were after 55 and 70 days of nursery transplanting, and the data on pest population were taken before and after seven days of each application. The differences among the treatments for their effects were measured on incidence of larval population of borers and seed yield. Effects of each treatment on appearance of population levels of the pest on rice were explored by recording pest population in 2nd week of September. The data for borers' infestation were taken from 1 m^2 randomly selected area on 16 plants by counting sound and damage hills on the basis of number of dead heart. At the later stage of crop, for borers infestation, data were taken on whitehead basis. Then percent infestation such as dead heart and whiteheads per plant were calculated. The yield of different treatments was recorded at final harvest of crop. The data of experiment repeated three times were subjected to the standard analysis by using statistical techniques. The data were analyzed by using One-way Analysis of Variance and means were compared by LSD test.

RESULTS AND DISCUSSION

The rice stem borers larvae of all generations were found damaging the rice crop. Data for the mean pest population from selective insecticide treatments showed their suppression. The mean pest population per plant in the untreated control was significantly higher than pesticide treatments. Analysis of variance regarding the mean values of pest population recorded in the various treatments (Table I), revealed a significant difference among insecticides. The foliar applied insecticides significantly (P= 0.05) reduced the percent crop damage of the rice by borers compared to the granular and check treatments. However, the most promising in its effect against pest was Spinosad 240 SC, while, Monomehypo 5 G ranked second in protecting crop from the borers followed by Endosulfan 35 EC that had significantly lower percent damage intensity than those in the plots treated with other tested insecticide Phorate 5 G as well as in the control. Based on pooled data, the pest infestation and seed yield levels of variety Basmati-370 was not significantly different from Khushboo-95. But on the basis of data obtained, the results revealed that the effects of insecticides were more evident in Khushboo-95 than on Basmati-370.

On Basmati-370, the % infestation level (2.35 and 2.42% dead hearts) of the Spinosad 240 SC and Monomehypo 5 G treatments had non-significant difference. The % infestation levels (3.07 and 3.31 % deadhearts) in the plots treated with Endosulfan 35 EC and Phorate 5 G had non-significant difference between each other but were significantly different from the control treatment

(5.31%) at P< 0.05. The % infestation levels in the plots treated with Spinosad 240 SC, Monomehypo 5 G, Endosulfan 35 EC and Phorate 5 G had significant differences between each other (2.47, 3.30, 4.11 and 5.33% whiteheads, respectively) at P<0.05. The infestation level in numerical terms was significantly higher (7.24% whiteheads) in non-treated plots from that of in the treated plots. On Khushboo-95, the similar pattern of infestation levels occurred as in case of Basmati-370. The infestation level in numerical terms was higher in non treated plots (4.47% deadhearts; 5.41 % whiteheads) from that of in the Spinosad 240 SC, Monomehypo 5 G, Endosulfan 35 EC and Phorate 5 G treated plots (1.16, 1.72, 1.96 and 2.49 % dead hearts; 1.63, 2.76, 3.58 and 4.10% whiteheads, respectively).

Table I.- Field evaluation of foliar and granular systemic chemicals against rice stem borers.

Variety	Treatment	Insecticide	Borer's infestation (%)		Yield/plot ($4.5\ m^2$) (gm)
			Deadheart	Whitehead	
Basmati-370 (P)	Foliar	Spinosad (Tracer 240 SC)	2.35 def	2.47 f	1483 a
		Endosulfan (Thiodan 35 (EC)	3.07 cd	4.11 c	1233 cde
	Granular	Phorate (Thimet 5 G)	3.31 c	5.33 b	1153 e
		Monomehypo 5 G	2.42 def	3.30 d	1270 cd
	Check	Control	5.31 a	7.24 a	900 f
Khushboo-95	Foliar	Spinosad (Tracer 240 SC)	1.16 g	1.63 g	1490 a
		Endosulfan (Thiodan 35 (EC)	1.96 ef	3.58 d	1333 bc
	Granular	Phorate (Thimet 5 G)	2.49 de	4.10 c	1217 de
		Monomehypo 5 G	1.72 fg	2.76 e	1417 ab
	Check	Control	4.47 b	5.410	945 f

Table I shows yield out put of the insecticide applications, which were compared with control treatment in Basmati-370 and Khushboo-95. In all the treatments the grain yield levels in the plots treated with insecticides had

significant differences between each other, but also significantly different from the control treatment at P< 0.05. In two varieties Spinosad 240 SC was statistically similar in its effects on grain yield/ 4.5 m^2 plot (1483- 1490 gm) this treatment had significant difference from control treatment (900- 945 gm). The tested insecticides (Monomehypo 5 G, 1270- 1417 gm; Endosulfan 35 EC, 1233-1333 gm; Phorate 5 G, 1153-1217 gm per plot) in regard to grain yield were statistically significant on Basmati-370 and Khushboo-95. The grain yield in Spinosad 240 SC, Monomehypo 5 G, Endosulfan 35 EC and Phorate 5 G treatments was 1483, 1270, 1233 and 1153 gm, respectively on Basmati- 370 (P), whilst, 1490, 1417, 1333 and 1217 gm per plot, respectively on Khushboo-95.

The combined effects of the four pesticides resulted in a considerable increase in crop produce. In regard to total percent pest damage, although insecticide treatments showed less damage to that in the control, Spinosad 240 SC ranked first followed by Monomehypo 5 G showing significantly lower percent infestation compared to the control as well as other tested insecticides. These results are in agreement with the findings of Hasan *et al.* (2001), who found Tracer (spinosad) better than the conventional insecticides. Ahmad *et al.* (2003) studied toxicity of spinosad in laboratory and they found no resistance of the pest against this new chemistry insecticide. This made Spinosad as one of the products of choice for the control of lepidopteron insect pests. The efficacy of foliar insecticides was better in reducing the infestation of rice stem borers than granular tested insecticides. Our findings are in accordance with the findings of Ahmed *et al.* (2004) who found foliar treatments most effective than granular insecticides against stem borer insect pests in rice ecosystem. The results suggest that the incidence of rice borers can be safely and successfully managed by adopting granular or foliar sprays in rice field. Of course, these treatments affected field population of borers and the beneficial effects upon treated plants were due to the suppression of borer populations. Field observations revealed that after treatment, the insecticides have favorable effects on plant health. Majority of treated plants showed vigorous blooming responses. Further, it may be due to useful influence of insecticides themselves on physiological and biochemical changes in plant. It is, therefore, concluded that all tested insecticides can be safely used as soil and foliar treatments when intended to control rice pests. Future field investigations to determine the effects of insecticide applications on the populations of beneficial fauna are needed. To control rice pests effectively and reduce yield losses, an organized system of plant protection including chemical control should be adopted.

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