Investigation of pathogenic bacteria of a resident fish, *Lepidocephalichthys guntea* (Hamilton Buchanan), in relation to limnochemistry of a Terai river Lotchka in the Darjeeling foothills of West Bengal, India

Thesis Submitted to the University of North Bengal for the Award of Degree of Philosophy (Ph.D.) in Department of Zoology

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(2014)

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CERTIFICATE

This is to certify that Mr. Rudra Prasad Roy, M.Sc., has prepared the thesis entitled **"Investigation of pathogenic bacteria of a resident fish, *Lepidocephalichthys guntea* (Hamilton Buchanan), in relation to limnochemistry of a Terai river Lotchka in the Darjeeling foothills of West Bengal, India"** for the award of Ph.D. degree in Zoology of the North Bengal, under my guidance. He has carried out the work at the Department of Zoology, University of North Bengal.

(**S. Barat)**

DECLARATION

I declare that the thesis entitled "**Investigation of pathogenic bacteria of a resident fish, *Lepidocephalichthys guntea* (Hamilton Buchanan), in relation to limnochemistry of a Terai river Lotchka in the Darjeeling foothills of West Bengal, India"** has been prepared by me under the guidance of Dr. Sudip Barat, Professor, Department of Zoology, University of North Bengal from the basis of for the award of any Degree or Fellowship previously.

Date: 27.03.2014

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ACKNOWLEDGEMENTS

At the outset with excitement, I wish to acknowledge many people who were always standing by my side from the beginning of my Ph.D. work to its completion. This is my goodness for Prof. Sudip Barat, Head, Department of Zoology, University of North Bengal with his excellent mentorship. He always tried to keep me in touch to reach the destination from starting the journey of this thesis. During the research period of my continuous interaction with him, I have developed a deep respect for him.

It is my pleasure to express heartiest gratitude to the Honourable Vice-Chancellor, University of North Bengal; Registrar, Dean, Head Department of the Zoology and other officials of the University for their academic and administrative facilities extended to me.

I have gained enormously through my interaction with Prof. Debesh C. Deb, Department of Zoology, University of North Bengal. Whenever I faced any problem regarding my research work, paper writing and especially for thesis writing, I used to seek his advice. His door was always open to me. Apart from this I have tried to learn from his honesty, simplicity and how to be a good human being. I received a lot of strength and encouragement from the interaction with Prof. Joydev Pal, Department of Zoology, University of North Bengal. As his great personality attracts me, initially I was hesitant to interact with him but later on I realised he is a man of kind heart and very co-operative to all. One of my inspirations has always been Prof. Ananda Mukhapadhyay, Department of Zoology, University of North Bengal. I always felt very happy whenever I had chance to listen his lecture. I got valuable suggestion and encouragement from time to time from him. I am also very much obliged to Prof. Tapas Kumar Chaudhari, Department of Zoology, University of North Bengal for his expertise suggestion and encouragement from time to time.

This thesis would never have been completed without the crucial guidance and help of Dr. Min Bahadur, Department of Zoology, University of North Bengal. I had bothered him so many times during my Ph.D. work. When I did not get experimental results properly, it was his invaluable suggestions that proved to be the key to achieve the final result. He accommodated me out of his busy schedule, whenever I needed him. My other respected teachers Dr. Soumen Bhattacharya, Mr. Dhiraj Saha and Mr. Tilak Saha, Department of Zoology, University of North Bengal for their encouragement, cooperation and helpful suggestions. I am also thankful to Dr. C. R. Nayak, Computer Centre, University of North Bengal for his efforts in statistical analysis.

The completion of my thesis is also due to the whole hearted support of my senior research colleague Dr. Pritwhiraj Jha. From the initial stage of my Ph.D work, his

undoubtedly support and guidance always gave me strength. It is also glad to express my thanks to other senior colleagues Dr. Tamal Majumder, Dr. Bikash Mitra, Mrs Muhua Rudra and Mrs Soma Das.

I want to express heartiest thanks to my research scholar friends Benoy Kishore Rai, Ritesh Biswa, Gautam Debnath, Pokhraj Guha, Sangita Khewa Subba, Dawa Bhutia, Anjali Km. Prasad and Priyankar Dey. All of those whose heartiest coordinate activities were very graceful. I am also thankful to my others colleagues Manoj Lama, Tanmay Mukhopadhyay, Shubhrajyoti Roy, Kumar Basnett, Ruksa Nur, Sutannuka Chattaraj, Uttara Dey Bhoumik, Mrinal Ray, Tanmoy Dutta, Subhasish Paul, Bappaditiya Ghose, Swati Sing, Sanjib Sarkar, Jayesree Soren and Somit Dutta.

I am thankful to the non teaching staff of Department of Zoology, University of North Bengal.

Tinku, Tarapada, Dip da and Badaru da for their coordination in field work was also urgent part of work, I always is kept their in my heart.

Finally I wish to convey my pronam to my Parents. As I am able to complete my entire thesis work, the reason is they always act as my backbone.

I always remember also so many unknown persons for their helpful activity also required to fulfil to any corner of work.

Place: University of North Bengal

Dated: 27.03.2014 *(Rudra Prasad Roy)*

ABSTRACT

A study was conducted on the limnochemistry parameters at four different sites (S1, S2, S3 and S4) of a Terai river Lotchka of Darjeeling district, West Bengal, and its influence on the opportunistic pathogenic bacterial population with their antibiotic resistant ability of river water and different tissues of resident fresh water loach, *Lepidocephalichthys guntea* (Hamilton Buchanan). Various physico-chemical parameters like Temperature, pH, Free Carbon dioxide, Total Alkalinity, Total hardness, Chlorides, Ammonium-N, Nitrite-N, Nitrate-N and Phosphate-P and different bacterial counts including heterotrophic bacteria, total coliform, *Aeromonas* spp. and *Pseudomonas* spp. were performed along the sampling sites for the river Lotchka during the period from March 2009 to February 2012. The results of limnochemistry parameters did not show much significant difference ($P \leq 0.05$) between the four sites. Temperature of water at the four different sampling sites (S1, S2, S3, and S4) averaged between 26.5±4.6 and 26.9±4.6 °C. The pH of water ranged between 6.7 ± 0.2 and 6.9 ± 0.3 showing slightly acidic condition. The dissolved oxygen content at the four sites of the river water showed similar trend, irrespective of the site of sample collection, with concentration averaging between 3.6±1.2 and 4.1±1.5 mg l^{-1}. The average free carbon dioxide in the water ranged between 15.0±5.8 and 16.7±6.1 mg l^{-1} and was present throughout the period of study as the pH remained acidic. Total alkalinity in the form of bicarbonate alkalinity was only recorded and ranged between 19.8±7.6 and 21.4±7.4 mg l^{-1} indicating carbonate alkalinity was absent in the water body. The present study showed chloride concentration in the water samples averaging between 20.0±4.4 and 23.1±5.3 mg l^{-1}, thereby indicating, that the concentration falls within the permissible limit of 250 mg l^{-1}. Total hardness varied from 62.6±8.6 to 68.5±9.7 mg l^{-1}, thereby showing water to be moderately hard. Concentrations of ammonium-N varied from 0.08±0.13 to 0.10±0.18 mg l^{-1}, Nitrite-N (NO_2-N) between 0.07±0.07 to 0.12±0.09 mg l^{-1} and Nitrate-N (NO_3-N) from 0.17±0.08 to 0.24±0.20 mg l^{-1} thereby showing hardly any nitrogen toxicity. Phosphate-P averaged between 0.23±0.11 and 0.33±0.24 mg l^{-1} in the four different sites. The heterotrophic bacterial counts in the four sites of river ranged from 3.3±1.1 x 10^6 and 4.6±1.4 x 10^6 cfu ml l^{-1} and showed positive correlation ($P \leq 0.01$) with Dissolved oxygen content, Nitrite-N, Nitrate-N and Phosphate-P. The total coliform counts in water recorded ranged from 1.1±1.3 x 10^6 and 1.6±2.6 x 10^6 cfu ml^{-1} and was positively correlated ($P \leq 0.01$) with temperature, dissolved oxygen and nitrite-N. During the study period seasonal variation were observed in all the physic-chemical and bacteriological parameters from all sampling sites. All types of bacterial counts including major water quality parameters namely dissolved oxygen, total alkalinity, nitrite-N, nitrate-N and phosphate-P

showed higher trend in monsoon season followed by summer and winter. However, luxuriant growth of all bacterial types in all the sites of the river was observed throughout the three seasons (summer, monsoon and winter). In the fish, *Lepidocephalichthys guntea* the maximum counts of heterotrophic bacteria 4.2 X 10^6 cfu g^{-1} and total coliform 2.3 x 10^6 cfu g^{-1} more observed to be present in the gut followed by gills and skin. The heterotrophic bacterial count, total coliform count and counts of total *Aeromonas* spp. and *Pseudomonas* spp. were found higher in monsoon followed by summer and winter. An interesting observation was that seasonal trend of bacterial loads were almost found to be parallel in fish tissues and in river water. Results also revealed that bacterial counts in both river water and in the fish were relatively higher. A total of 175 bacteria were isolated from four different sites of river Lotchka and tissues like skin, gills and gut of fish. Isolated bacteria were identified by different biochemical procedures and Polymerase Chain Reaction using genus specific 16S rDNA and tuf gene primers for confirmation of identification. All isolates belonged to the genera of *Aeromonas, Pseudomonas, Salmonella* and *Enterobacteriacea*. Antibiotic susceptibility test of bacterial isolates was also done by Disc Diffusion Method. Among the total isolates of *Aeromonas* spp., *Pseudomonas* spp., *Enterobacteriacea* spp. and *Salmonella* spp. in four different sites of river there were 75 to 100% resistant to Penicillin, Cephalothin and Ampicillin. 25 to 70% isolates were resistant against Erythromycin and Kanamycine. Nevertheless all isolates showed 100% sensitiveness to Streptomycin, Ciprofloxacin, Moxifloxacin, Tetracycline and Gentamycin. Bacterial isolates belong to *Aeromonas* spp., *Pseudomonas* spp., *Enterobacteriaceae* and *Salmonella* spp. of fish tissues namely skin, gills and gut also showed higher range of resistance (57 to 100%) against Ampicillin, Penicillin-G, and Cephalothin belong to the β- lactam class. These isolates showed lower range of resistance (0 to 84%) against erythromycin, kanamycin, streptomycin, ciprofloxacin, gentamicin and tetracycline. The percentage patterns of resistant bacterial isolates were similar in the fish and river water. Among all the isolates, four strains belonged to *Aeromonas* spp. and *Enterobacteriaceae* from the gut of fish, *Lepidocephalichthys guntea* based on their antibiotic resistance ability and was identified up to the species level. For the identification purpose genus specific 16s rDNA and tuf gene based sequencing was performed. The strains (A30S2, ET01, ET02 and ET08) were amplified by 16s rDNA *Aeromonas* specific primers and *Enterobacteriaceae* specific tuf gene based primers. Positive amplicons products were then sequenced. The sequences were compared and aligned with sequence deposited in the NCBI-Gen Bank database. After the comparison with other sequence data base maximum similarities of the strains (A30S2, ET01, ET02 and ET08) were found with *Aeromonas veronii, Enterobacter asburiae, Enterobacter* sp and *Enterobacteriaceae bacterium.* The study, thus

revealed that river Lotchka may be contaminated with antibiotic resistant bacteria due to deteriorating water quality which had vis-a-vis influenced greater harbouring of antibiotic resistant bacteria in the resident fish, *Lepidocephalichthys guntea.*

PREFACE

Water is the most essential part of every civilization. For the fulfilment of this essentiality, river plays a crucial role. This was the main cause of the ancient civilizations to have developed along the banks of rivers. River is the complete part of fresh water ecosystem. Rivers provide fertile soil and water to the area on river banks and any effect on the water quality of rivers is directly reflected on the health and lifestyle of the people depending on it. In developing country like India anthropogenic pressure is the main causing substance to generate river water pollution. In India uncountable small and big rivers are present. All these rivers flow through the inhabitant of villages, cities, agricultural field, industries, medical hub and so on, where numerous factors are responsible for the degrading quality of river water. These water quality degrading factors lead to the river water pollution. and is responsible for ecological disturbance and biodiversity loss also. All these factors rose due to anthropogenic pressures and is one of the major concerns of human and other animal health risk. Bacteria directly take an active part in the formation of all aquatic pollution. Along with their inactivate property, such natural microbial population in turn function as vectors causing rapid and wide spread dissemination of the water borne diseases leading to high rates of morbidity and mortality. All the factors affecting the water quality should be handled with equal attention.

The northern part of West Bengal consisting of the Himalayan range has an ecotourism value. Siliguri the second largest city of West Bengal located in the foothills of Terai region has gradually developed as a profitable businesses centre. As a central hub, many national companies and organizations have set up their offices here. Siliguri is the gateway to the North East of India, Bhutan, Nepal and Bangladesh. Many rivers (small and big) are flowing in and around the Siliguri city. These rivers consist of rich fish faunal diversity. Villages, agricultural fields including tea gardens near the Siliguri city are situated around these rivers also. The rivers of this region are prone to polluting because of receiving run-offs such as agricultural contaminants, domestic, human sewage and pollutant of medical and industrial hub. There is, therefore, a risk for human and aquatic animal health as well as biodiversity loss thus from the ecological point of view special attention or monitoring is essential of these rivers.

BREVIATION

bp= Base pair

^{o}C= Degree Celsius

Temp= Temperature

pH= Power of hydrogen ion concentration

DO= Dissolved oxygen

FCO_2= Free carbon dioxide

TA= Total alkalinity

Cl= Chloride

TH= Total hardness

NH_4-N=Ammonium-nitrogen

NO_2-N= Nitrite-nitrogen

NO_3-N= Nitrate- nitrogen

PO_4-P = Phosphate-phosphorous

HC=Heterotrophic Count

TC=Total Coliform

+ve= Positive

-ve=Negative

Nd= not done

R-Resistance

I- Intermediate

S-Sensitive

AMP-Ampicillin

PEN-Penicillin

CEPHA-Cephalothin

ERY-Erythromycin

KANA-Kanamycin

STREPTO-Streptomycin

CIPRO-Ciprofloxacin

MOXI-Moxifloxacin

TETRA-Tetracycline

GENTA-Gentamycin

Tuf gene- Elongation factor Tu gene

PCR- Polymerase Chain Reaction

SIM- *Sulphide-Indole-Motility*

MEGA- Molecular evolutionary genetic analysis

NCBI-National Centre for Biotechnology Information

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