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Molecular Identification of Toxigenic Cyanobacteria and Effects of Cyanotoxins on Human Renal Cell Lines

by

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Dedication

To my darling twin daughters Vidushi and Virushi

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List of Abbreviations

△	Delta
µg	microgram
µL	microliter
µm	micrometre
µM	micromolar
ACF	aberrant crypt foci
ACR	Albumin to Creatinine ratio
ADME	Absorption, Distribution, Metabolism and Excretion
ANOVA	Analysis of variance
AO/EB	Acridine orange/ethidium bromide
ATCC	American Type Culture Collection
Bax	Bcl-2–associated X protein
BBB	Blood Brain Barrier
Bcl-2	B-cell lymphoma 2
BLAST	Basic local alignment search tool
bp	Base pair
Caspases	Cysteine-aspartic-acid-proteases
cDNA	Complementary DNA
CKD	Chronic kidney disease
CKDu	Chronic kidney disease of uncertain etiology
CO_2	carbon dioxide
Ct	Cycle threshold
CYL	cylindrospermopsin
DAF	Dissolved Air Floatation
DEPC	Diethylpyrocarbonate

DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxy ribo Nucleic Acid
dNTP	Deoxy nucleotide triphosphate (N = A, C, T, G)
DO	Dissolved Oxygen
EC	Electrical Conductivity
EDTA	Ethylene diamine tetra acetic acid
eGFR	estimated Glomerular Filtration Ratio
ELISA	Enzyme Linked Immuno Sorbant Assay
EMEM	Eagles Minimum Essential Medium
FA	fatty acid
FBS	Foetal bovine serum
g	gram
g	Gravitational force
GAPDH	Glyceraldehydes 3-phosphate dehydrogenase
h	hour (Time)
HAB	harmful algal blooms
Hb	Haemoglobin
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
IAP	Inhibitors of Apoptosis
IARC	International agency for research on cancer
IBMBB	Institute of biochemistry, molecular biology and biotechnology
IC_{50}	The half maximal inhibitory concentration
ID	Identification

IP	intra peritoneal
ITS	Internal Transcribed Spacer
KDOQI	Kidney Disease Outcome Quality Initiative
kPa	kilopascal
LC	Liquid Chromatography
M	Molar
MC	microcystin
MC-LR	microcystin LR
$MgCl_2$	Magnesium chloride
min	Minute (time)
mL	milliliter
mM	millimolar
M-MLV	Moloney murine leukemia virus
mRNA	Messenger ribonucleic acid
MS	Mass Spectrometry
MTT	3-(4, 5-dimethylthiazol-2yl) -2, 5-biphenyl tetrazolium bromide
N_2	nitrogen gas
NaCl	Sodium Chloride
$NaHCO_3$	Sodium bicarbonate
NCBI	National Centre for Biotechnology Information
NCP	North Central Procince
ng	nanogram
NGAL	Neutrophil gelatinase associated lipocalin
NHMRC	National Health and Medical Research Council
NKF	National Kidney Foundation

nm	nanometer
NMT	N-methyl transferase
NOD	nodularin
NRPS	non-ribosomal peptide synthetase
NW	North Western
NWSDB	National water supply and drainage board
OATP	organic anion transporting polypeptide
PBS	Phosphate buffered saline
PC	phycocyanin
PC	Principal Component
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PDA	Photo Diode Array
pH	Power of hydrogen
PKS	polyketide synthetase
PP	protein phosphatase
qPCR	quantitative Polymerase chain reaction
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RNasin	Ribonuclease Inhibitor
ROS	Reactive Oxygen Species
RPM	Revolutions per minute
RT-PCR	Reverse transcriptase Polymerase chain reaction
s	second (time)
SDS	Sodium dodecyl sulphate
SIR	significant increased risk

SPE	Solid Phase Extraction
SRB	Sulforhodamine B
TAE	Tris-acetate Ethylene diamine tetra acetic acid
TCA	Trichloroacetic acid
TDI	tolerable daily intake
TFA	Tri Fluoro Acetic acid
TIN	Tubular Injury
Tm	Melting temperature
TN	Total Nitrate
TP	Total Phosphate
TYG	Tryptone, Yeast extract, Glucose
U	Unit
USEPA	United States Environmental Protection Agency
UV	Ultra violet
V	Volt
WHO	world health organization

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Molecular Identification of Toxigenic Cyanobacteria and Effects of Cyanotoxins on Human Renal Cell Lines.

M.A.P.C. Piyathilaka

ABSTRACT

Cyanobacterial toxins became widely recognized as a human health problem arising as a consequence of eutrophication. Present study was planned for the phylogenetic identification of toxic and non-toxic cyanobacteria in Sri Lankan water bodies to understand potential of toxigenicity for future water treatment solutions. Further, present work was planned to evaluate the cytotoxicity and possible apoptotic effects of cyanotoxins (microcystin LR/ MC-LR) and crude cyanobacteria toxin extracts on human embryonic kidney (HEK-293) and human kidney adenocarcinoma (ACHN) cell lines.

Forty water bodies from seven provinces were selected depending on the past history of cyanobacterial and cyanotoxin occurrence for the study to collect water and phytoplankton samples. Physico-chemical and some biological parameters of water were measured at the site itself and in the laboratory according to standard methods. Results reveled that most of the drinking water bodies were having acceptable water qualities. MC-LR and Cylindrospermopsin (CYL) contamination levels were assessed using ELISA method and HPLC and the highest MC-LR level was recorded in Beira Lake (2198.26 ppb).

Preparation of cyanobacterial batch and monocultures were achieved by testing number of culture media and BG11 culture medium was selected as the best. Extraction of

genomic DNA from cultured cyanobacteria and whole cyanobacterial community in reservoirs was achieved by standard lysis buffer method. Presence of Microcystins (MCs) producing genes, *mcy A, mcy B* and *mcy E* in water was screened with PCR. The results of the specific PCR revealed that all the reservoirs having *mcy A, mcy B* and *mcy E* MC producing genes. Detectable level of MCs were not recorded in Borelesgamuwa Lake during the study period either by ELISA or HPLC. Interestingly presence of toxin producing genes in cyanobacteria in Borelesgamuwa Lake showed that there is a potential to produce MCs. Absence of MCs in Labugama, Kalatuwawa, Mahaweli River, Kantale podi wewa, Rathkinda and Minneriya reservoirs were further confirmed by PCR with negative results.

Phylogenetic analysis of some isolated cyanobacteria was achieved by sequencing 16S rRNA gene region. One strain of *Microcystis* spp. was isolated from Beira Lake, Kurunegala Lake, Borelesgamuwa Lake, Giradurukotte reservoir and Nuwara wewa. Other than the strain isolated from Nuwara wewa, all strains were belonged to the same phylogenetic strain.

Toxicity of MC-LR and natural cyanotoxin extracts was assessed using *Artemia* (Brine shrimp) bio assay. Compared to toxicity of MC-LR, toxin extract prepared from Borelesgamuwa Lake had higher toxicity. Further, it was detected that decreasing tendency of IC_{50} values had a tendency to decrease along with the increasing incubation period for all extracts and any degradation of pure MC-LR was not detected during incubation period.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and SRB (Sulphorhodamine B) assays for cell viability revealed a significant decrease in cell

viability in both cell lines after treatment with MC-LR at 50 μM for 24 h ($p<0.001$). Moreover, MC-LR treated ACHN and HEK-293 cells exhibited a marked dose dependent loss of confluence as judged by phase contrast microscopy. Similarly, fluorescence microscopic observations following Acridine Orange-Ethidium Bromide (AO/EB) staining confirmed that both cell types were undergoing apoptosis after treatment with MC-LR for 24 h. Expression of three apoptosis related genes namely, Bax, Survivin and p53 was analyzed by quantitative RT-PCR analysis. Both Bax and p53 genes function as promoters of MC-LR mediated apoptosis in ACHN and HEK-293 cells. Survivin gene acts as a suppressor of apoptosis in lower MC-LR concentration (1 μM) and the gene was up regulated at higher MC-LR concentration (10 μM) tested ($p< 0.001$). A significant increase of caspase 3 ($p<0.0001$) and caspase 9 ($p<0.0001$) activity was detected in both cell lines after exposure to MC-LR for 24 h indicating that MC-LR induces cytotoxicity and a marked apoptosis in both ACHN and HEK-293 kidney cell lines. MC-LR treated ACHN and HEK-293 cells exhibited marked dose dependent cytotoxic and apoptotic effects in the present study contributing to elucidate the nephrotoxic effects of MC-LR on Human kidneys.