ASSESSMENT OF ARSENIC TOLERANT BACTERIA FROM ARSENIC CONTAMINATED GROUNDWATER IN NAWALPARASI DISTRICT OF NEPAL

A
DISSERTATION
SUBMITTED TO THE
CENTRAL DEPARTMENT OF MICROBIOLOGY
TRIBHUVAN UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN MICROBIOLOGY
(ENVIRONMENT AND PUBLIC HEALTH)

BY
SHREE KRISHNA SHRESTHA

CENTRAL DEPARTMENT OF MICROBIOLOGY
TRIBHUVAN UNIVERSITY
KIRTIPUR, KATHMANDU, NEPAL
2008

RECOMMENDATION

This is to certify that **Mr. Shree Krishna Shrestha** has completed this dissertation work entitled **"ASSESSMENT OF ARSENIC TOLERANT BACTERIA FROM ARSENIC CONTAMINATED GROUNDWATER IN NAWALPARASI DISTRICT OF NEPAL"** as a partial fulfillment of M. Sc. Degree in Microbiology under our supervision. To our knowledge, this work has not been submitted for any other degree.

Dr. Anjana Singh	**Dr. Linda S. Smith**	**Mr. Makhan Maharjan**
Head	Country Director	Program Manager
Central Department Microbiology	Filters for Families	Environment and Public Health
Tribhuvan University	Lalitpur	Organization, Kathmandu

Date:

CERTIFICATE OF APPROVAL

On the recommendation of **Dr. Anjana Singh, Dr. Linda S. Smith** and **Mr. Makhan Maharjan**, this dissertation work of **Mr. Shree Krishna Shrestha,** entitled "**ASSESSMENT OF ARSENIC TOLERANT BACTERIA FROM ARSENIC CONTAMINATED GROUNDWATER IN NAWALPARASI DISTRICT OF NEPAL"** has been approved for the examination and is submitted to the Tribhuvan University in the partial fulfillment of the requirement for Master's Degree of Science in Microbiology.

Dr. Anjana Singh, Ph.D.
Head
Central Department of Microbiology
Tribhuvan University, Kirtipur
Kathmandu, Nepal

Date:

BOARD OF EXAMINERS

Recommended by:

…….……

Dr. Anjana Singh
Supervisor

……

Dr. Linda S. Smith
Supervisor

……

Mr. Makhan Maharjan
Supervisor

Approved by:

……

Dr. Anjana Singh
Head of Department

Examined by:

……

Dr. Alenka H. Radojcic, Ph. D.
External Examiner

……

Mr. Binod Lekhak
Internal Examiner

Date …….

ACKNOWLEDGEMENTS

It is my heartiest pleasure to express my sincere gratitude to my supervisor **Dr. Anjana Singh**, Head, Central Department of Microbiology, Tribhuvan University whose expert guidance and encouragement led me to the completion of this thesis work.

I am also indebt to my supervisors **Dr. Linda S. Smith**, Country Director, Filters for Families, Bakundole, Lalitpur and **Mr. Makhan Maharjan**, Program Manager, Environment and Public Health Organization, New Baneshwor for supervision and constant encouragement for the completion and accomplishment of this work.

I am thankful to Dr. Shreekant Adhikari, Dr. Dwij Raj Bhatta, Mr. Binod Lekhak and Ms. Shaila Basnyat for their valuable idea, moral support, and kind co-operation during this study. I am much obliged to Mr. Megha Raj Banjara, Mr. Dev Raj Joshi, Mr. Kiran Babu Tiwari and all the teachers of Central Department of Microbiology for their tremendous support and valuable suggestions during the research work.

I acknowledge profound gratitude to the Parasi Hospital and its staff for providing me laboratory and other facilities. I am immensely grateful to Mr. Somai Kurmi, Mr. Arjun Gupta, Mr. Rajan Poudel, Mr. Sushil Tuladhar and all the families of FFF. I am also gratefully indebted to Ms. Dikshya Pokharel, College of Applied Science.

I am deeply grateful to Mr. Ramesh Khadka, Mr. Rayman Shakya, Mr. Madhukar Thapa, Mr. Nawaraj Karki and other staffs of Central Department of Microbiology for their help. I highly appreciate for kind cooperation and genuine support by my colleagues Narendra Maden, Sanjiv Neupane, Pankaj Baral, Manoj Khadka, Kashi Ghimire and Anil Shrestha.

I sincerely appreciate cooperation received from all my family members especially Ram Krishna Shrestha, Pancha Maya Shrestha, Dr. Ramesh Makaju, Sanu Maya Makaju and my brother Rabi Shrestha.

At last, I would like to dedicate this thesis work to the peoples of Nawalparasi district, especially peoples of Ramgram Municipality; Sunwal, Sarawal, Sukrauli, Swathi, Pratappur and Jahada VDCs.

Date:

_____

Shree Krishna Shrestha

ABSTRACT

Arsenic, considered a human carcinogen, contamination in groundwater drinking water is a worldwide public health issue, including Nepal. To assess arsenic tolerant bacteria from arsenic contaminated drinking water, this research study was carried out from June 2007 to January 2008. Eighty-six water samples were collected from one municipality and six VDCs' wards in Nawalparasi district of Nepal having arsenic concentration above WHO guidelines (10 ppb).

Physico-chemical and microbiological parameters of water quality were determined to be poor. The pH of 11.63% water samples was found below WHO guideline. Sixty two percent (53/86) samples in Nawalparasi district showed arsenic level above Nepal drinking water quality standard (NDWQS) (50 ppb). Filtered water samples showed that 30.16% (19/63) was above NDWQS guideline. Comparison between arsenic concentration of influent and effluent water showed significant difference ($p<0.05$). Within the last 10 years, 66.27% (57) tubewells were constructed whereas 52.63% (30/57) tubewells constructed within 6-10 years. Moderately positive correlation ($r=0.23$, $p=0.83$) was found between arsenic concentration and age of tubewells. At 36-95 feet depth range, 50 (58.14%) tubewells were above NDWQS and 70 (81.39%) tubewells above the WHO guideline. Results showed weekly negative correlation ($r=-0.12$, $p=0.24$) indicating arsenic concentration of groundwater decreases as the depth of tubewell increases.

Skin manifestations were observed in 12 (34%) females and 18 (33%) males, skin manifestations and gender difference was found statistically insignificant ($p>0.05$). Arsenicosis cases were highest (33.3%, 10/30) in 32-41 age groups. Most of the patients (30%) suffered from diffused arsenical melanosis and 20% patients from both diffused arsenical melanosis and keratosis. Mild skin manifestation were observed in most, 66.7% (20/30) patients followed by 7 (23.3%) moderate and 3 (10%) severe. Percentage of arsenicosis cases was higher among respondents using tubewell that were built >10 years (41%) than that were built ≤10 years (32%).

The microbiological analysis of groundwater revealed the presence of total coliforms in 98.84% samples (85) of influent water from tubewell and 71.43% (45/63) effluent water from filter. Among the isolates, *E. coli* (37%) was found to be predominant in influent water which was followed by *P. aeruginosa* (16%), *Citrobacter* spp. (16%) *and K. pneumoniae* (10.5%). Similarly, *E. coli* (39.21%) was found predominant in effluent water which was followed by *P. aeruginosa* (19.61%), *K. oxytoca* (13.72%) and *Citrobacter* spp. (13.72%). Among thirteen arsenic tolerant bacteria isolated from plate count agar incorporated with As (V) >50 ppm, the most predominant isolates were unidentified NP1 (Bluish green) (19.51%) followed by *Pseudomonas* spp. (16.38%), *Bacillus cereus* (11.15%). The mean colony count of samples (<300 cfu/ml) was highest in 10 ppm (216 cfu/ml) and lowest in 200 ppm (70 cfu/ml). The highest MIC value of As (III) was obtained for *E. coli* (mean=15 ppm) followed by NP1 (Bluish green) (mean=10.71ppm). Similarly the highest MIC value of As (V) was obtained for *Pseudomonas* spp. (mean=560 ppm) followed by *E. coli* (mean=360ppm).

Plasmids that were extracted from 8 arsenic tolerant *E. coli* and one arsenic sensitive *E. coli* and run in agarose gel which showed 8 types of plasmid profiles of size ranging from 3.1 to 16.21 kb. The most frequent band was 14.17 kb (in four isolates) followed by 6.16 kb (two isolates) and 8.5 kb (two isolates). Apparently a size difference was observed in overall plasmid profiling. Large size bands were obtained from arsenic tolerant bacteria than arsenic sensitive bacteria (4.36Kb).

Key words: Arsenic, groundwater, arsenic tolerant bacteria, water quality, arsenicosis, plasmid profiles

TABLE OF CONTENTS

	Page No.
Title page	**i**
Recommendation	**ii**
Certificate of approval	**iii**
Board of examiners	**iv**
Acknowledgement	**v**
Abstract	**vii**
Table of contents	**viii**
List of abbreviations	**xiii**
List of tables	**xv**
List of figures	**xvi**
List of photographs	**xvii**
List of appendices	**xviii**
CHAPTER I: INTRODUCTION	**1-4**
CHAPTER II: OBJECTIVES OF THE STUDY	**5**
2.1 General objective	5
2.2 Specific objectives	5
CHAPATER III: LITERATURE REVIEW	**6-30**
3.1 Arsenic, its sources and occurrences in the environment	6
3.2 History of guideline development	8
3.3 Global scenario of arsenic poisoning of groundwater	8
3.4 National scenario of arsenic poisoning of groundwater	9
3.5 Interim Nepal guidelines and policies for arsenic in drinking water	11
3.6 VDCs vulnerability to arsenic	11

3.7 Physico-chemical parameters of water 11
3.7.1 pH 12
3.7.2 Temperature 12
3.7.3 Arsenic 12
3.8 Microbiological parameters of water 13
3.8.1 Coliform bacteria 13
3.8.2 Some intestinal pathogens found in contaminated drinking water 14
3.9 Health effects of arsenic 17
3.9.1 Route of entry 17
3.9.2 Acute toxicity of arsenic (III) and (V) 17
3.9.3 Chronic toxicity of arsenic (III) and (V) 18
3.9.3.1 Dermal 18
3.9.3.2 Vascular effects 18
3.9.3.3 Cancer 19
3.9.4 Characteristics of arsenicosis 19
3.9.5 Treatment 20
3.10 Mitigation of arsenic in drinking water 20
3.10.1 3-Kolshi filter 21
3.10.2 SONO filter 21
3.10.3 Arsenic bio-sand filter (Kanchan Arsenic Filter) 22
3.10.3.1 Arsenic removal unit 22
3.11 Testing and investigation 23
3.11.1 Hydride Generator Atomic Absorption Spectrophotometer 23
3.11.2 Blanket tubewell testing 23
3.12 Biological properties of arsenic 24
3.13 Microbial arsenic tolerance mechanisms 26
3.13.1 Chromosomal-determined resistance 27
3.13.2 Plasmid-mediated resistance 27
3.14 Minimum inhibitory concentration 28
3.15 Plasmid profiling 28

CHAPTER IV: MATERIALS AND METHODS **29-38**
4.1 Materials 29
4.2 Methods 29
4.2.1 Study period 29
4.2.2 Study area 29
4.2.3 Study sites 29
4.2.4 Study population 30
4.2.5 Tools for data collection and analysis 30
4.2.6 Collection of samples for water analysis 30
4.2.6.1 Sample collection from tubewell 30
4.2.6.2 Sample collection from effluent water (filter) 31
4.2.7 Study of physico-chemical parameters of water samples 31
4.2.7.1 Temperature 31
4.2.7.2 pH 31
4.2.7.2 Measurement of arsenic concentration by kit method 32
4.2.7.3 Measurement of total arsenic concentration by AAS 32
4.2.8 Physical examination for skin manifestations of chronic arsenic ingestion 32
4.2.9 Microbial examination water sample 33
4.2.9.1 Total coliform count 33
4.2.9.1.1 Enumeration of coliforms 34
4.2.9.2 Enrichment 34
4.2.9.2.1 Detection of *Salmonella* and *Shigella* spp. 34
4.2.9.2.2 Detection of *Vibrio cholerae* 34
4.2.9.3 Isolation and identification of enteric bacteria 34
4.2.9.4 Enumeration of arsenic tolerant bacteria 35
4.2.9.5 Isolation and identification of arsenic tolerant bacteria 35
4.2.9.6 Minimum inhibitory concentration (MIC) 36
4.2.10 Extraction of plasmid DNA 36
4.2.11 Electrophoresis of plasmid DNA from arsenic tolerant *E. coli* 37

4.2.12 Determination of size of DNA by semi log plot 37
4.2.13 Decontamination of Ethidium Bromide 37
4.2.14 Data analysis 37

CHAPTER V: RESULTS 39-54
5.1 Physico-chemical parameters of water 39
5.1.1 Temperature 39
5.1.2 pH 39
5.1.3 Arsenic 40
5.1.3.1 Arsenic concentration in different VDCs/Municipality above and below NDWQS 40
5.1.3.2 Arsenic concentration before and after filtration 41
5.1.3.3 Relation of arsenic concentration with age of tubewell 42
5.1.3.4 Relation of arsenic concentration with depth of tubewell 44
5.1.4 Clinical manifestations of arsenicosis patient 46
5.1.4.1 Types of arsenicosis among people 46
5.1.4.2 Age and sex-wise distribution of arsenicosis cases 47
5.1.4.3 Sex and stage-wise distribution of arsenicosis cases 48
5.1.4.4 Distribution of arsenicosis vs. duration of tubewell use 48
5.2 Bacteriological analysis of water 49
5.2.1 Total coliform count 49
5.2.2 Isolation and identification of enteric bacteria by MF technique 50
5.2.3 Isolation and identification of arsenic tolerant bacteria by PCA 51
5.2.4 Colony count in PCA in different concentration of As (V) 51
5.2.5 Minimum inhibitory concentration of arsenic tolerant bacteria 52
5.3 Plasmid analysis 53
5.3.1 Profiles of plasmids from arsenic tolerant *E. coli* 53

CHAPTER VI: DISCUSSION AND CONCLUSION 55-63
6.1 Discussion 55

6.1.1 Physico-chemical parameters of water 55
6.1.2 Age of installation of tubewell 56
6.1.3 Depth of tubewell 57
6.1.4 Health affects 58
6.1.5 Microbial quality of water 59
6.1.6 Enteric bacteria isolated from water samples 60
6.1.7 Arsenic tolerant bacteria isolated from PCA 61
6.1.8 Colony count in PCA in different concentration of As (V) 61
6.1.9 Minimum inhibitory concentration of arsenic tolerant bacteria 62
6.1.10 Plasmid of arsenic tolerant and sensitive *E. coli* 62
6.2 Conclusion 62

CHAPTER VII: SUMMARY AND RECOMMENDATION **64-67**
7.1 Summary 64
7.2 Recommendations 67

CHAPTER VIII: REFERENCES **68-76**

LIST OF ABBREVIATIONS

AAS	:	Atomic Absorption Spectrophotometer
APHA	:	American Public Health Association
As (III)	:	Trivalent Arsenic
As (V)	:	Pentavalent Arsenic
As	:	Arsenic
ATP	:	Adenosine Tri-Phosphate
BFD	:	Black Food Disease
CBS	:	Central Bureau of Statistics
CFU	:	Colony Forming Unit
CIM	:	Composite Iron Matrix
DNA	:	Deoxyribonucleic Acid
DMAA	:	Dimethyl Arsinic Acid
DoHS	:	Department of Health Services
DOL	:	Department Of Irrigation
DWQIP	:	Drinking Water Quality Improvement Program
DWSS	:	Department of Water Supply and Sewage
ENPHO	:	Environment and Public Health Organization
EtBr	:	Ethidium Bromide
ETPAM	:	Environment Technologies Programs for Arsenic Mitigation
FFF	:	Filters for Families
FINNIDA	:	Finnish International Development Agency
GIS	:	Geographic Information System
H_2S	:	Hydrogen Sulphide
HGAAS	:	Hydride Generation Atomic Absorption Spectrophotometer
IARC	:	International Agency for Research on Cancer
IDWSS	:	International Decade of Water Supply and Sanitation
JRCS	:	Japanese Red Cross Society

KAF	:	Kanchan Arsenic Filter
Kbp	:	Kilo base pair
LD_{50}	:	Lethal Dose-50
LF	:	Lactose Fermenting
mg/l	:	milligram per liter
µg/L	:	microgram per liter
MA	:	MacConkey Agar
MIC	:	Minimum Inhibitory Concentration
MIT	:	Massachusetts Institute of Technology
MMAA	:	Monomethyl Arsonic acid
MR	:	Methyl Red
NA	:	Nutrient Agar
NASC	:	Nepal Arsenic Steering Committee
NDWQS	:	National Drinking Water Quality Standard
NRCS	:	Nepal Red Cross Society
ppb	:	parts per billion
ppm	:	parts per million
RNA	:	Ribonucleic Acid
rpm	:	revolution per minute
RWSSSP	:	Rural Water Supply and Sanitation Support Program
SIM	:	Sulphide Indole Motility
SPSS	:	Statistical Package for Social Sciences
UNDP	:	United Nation Development Programme
UNICEF	:	United Nation Children's Fund
US-EPA	:	United State-Environment Protection Agency
VDC	:	Village Development Committee
VP	:	Voges Proskauer
WHO	:	World Health Organization

LIST OF TABLES

Table 1: Distribution of temperature of water samples

Table 2: Arsenic concentration in different VDCs/Municipality

Table 3: Arsenic concentration before filtration

Table 4: Arsenic concentration after filtration

Table 5: Arsenic concentration paired t-test analysis

Table 6: Classification of arsenic concentration by age of tubewell

Table 7: Classification of arsenic concentration by depth of tubewell

Table 8: Distribution of skin manifestation and gender

Table 9: Distribution of arsenicosis by types and sex

Table 10: Age and sex-wise distribution of arsenicosis cases among people

Table 11: Sex and stage-wise distribution of arsenicosis cases among people

Table 12: Distribution of arsenicosis cases versus duration of tubewell use

Table 13: Source wise distribution of enteric bacterial isolates of water samples

Table 14: Types of bacteria isolated from arsenic contaminated groundwater

Table 15: Colony count in Plate Count Agar in different concentrations of arsenic (V)

Table 16: Minimum inhibitory concentration of arsenic tolerant bacteria to arsenic species (As III and As V)

Table 17: Plasmid profiles of selected arsenic tolerant *E. coli*

LIST OF FIGURES

Figure 1: Kanchan™ arsenic filter cross section

Figure 2: Illustration of arsenic removal mechanism

Figure 3: The environmental and global cycle of arsenic and its compounds

Figure 4: Comparison of pH measurements of water samples with standards

Figure 5: Scattered diagram of age of installation of tubewells and arsenic concentration

Figure 6: Scattered diagram of depth of tubewells and arsenic concentration (influent water)

Figure 7: Analysis of water samples by total coliform count

LIST OF PHOTOGRAPHS

Photograph 1: Arsenic tolerant *Bacillus subtilis* isolated on nutrient agar (NA) incorporated with arsenic concentration

Photograph 2: Enumeration of arsenic tolerant bacteria in different concentrations of arsenic (V) by Pour plate method

Photograph 3: Biochemical tests of *E. coli*

Photograph 4: Colonies of total coliforms on M-endo agar

Photograph 5: Plasmid profiles of arsenic tolerant and sensitive *E. coli*

Photograph 6: Spotted melanosis on the back side of chest of an arsenicosis patient

Photograph 7: Diffused keratosis on both soles of an arsenicosis patient

Photograph 8: Keratosis on both palms of an arsenicosis patient

Photograph 9: Arsenic concentration measurement by using Wagh tech kit

Photograph 10: Water sample collection from shallow tubewell

LIST OF APPENDICES

Appendix-I	Questionnaire
Appendix-II	Maps of study area
Appendix-III	List of the equipment and materials used during the study
Appendix-IV	I. Composition and preparation of different culture media II. Composition and preparation of different biochemical media
Appendix-V	Composition and preparation of different staining and test reagents
Appendix-VI	Staining methods; methodology of biochemical tests used for identification of bacteria
Appendix-VII	Biochemical characteristics of different bacteria
Appendix-VIII	Minimum inhibitory concentration of different arsenic concentration
Appendix-IX	Protocols
Appendix-X	Number of KAF distributed in Nawalparasi District
Appendix-XI	Standard curve of known DNA bands Vs distance
Appendix-XII	Arsenic test data from different VDCs/Municipality of Nawalparasi District
Appendix-XIII	Method of application as described in Wagtech international arsenic test kit leaflet