PERSPECTIVES OF ARSENIC EXPOSURE AND ASYMPTOMATIC MICROBIAL INFECTIONS IN NAWALPARASI DISTRICT

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By

NARENDRA MADEN

Central Department of Microbiology

Tribhuvan University

Kirtipur, Kathmandu

Nepal

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RECOMMENDATION

This is to certify that **Mr. Narendra Maden** has completed this dissertation work entitled **PERSPECTIVES OF ARSENIC EXPOSURE AND ASYMPTOMATIC MICROBIAL INFECTIONS IN NAWALPARASI DISTRICT** as a partial fulfillment of Master of Science Degree in Microbiology under our supervision. To our knowledge this thesis work has not been submitted for any other degree.

……	……	……..……	……..……
Dr. A. Singh, Ph.D.	**Dr. L. Smith, Ph.D.**	**Mr. M. Maharjan**	**Ms. S. Basnyat**
Head of Department	Director	Public Health Officer	Lecturer
Central Department of Microbiology	Filters For Families	Environment and Public Health	Central Department of
Tribhuvan University	Arsenic mitigation	organization, Kathmandu	Microbiology
Kathmandu	Kathmandu	Nepal	Kathmandu
Nepal	Nepal		Nepal

Date: July 6, 2008

CERTIFICATE OF APPROVAL

On the recommendation of **Dr. Anjana Singh, Dr. Linda S. Smith, Mr. Makhan Maharjan** and **Ms. Shaila Basnyat** this dissertation work by **Mr. Narendra Maden** is approved for the examination and is submitted to the Tribhuvan University in partial fulfillment of the requirement for Master of Science Degree in Microbiology.

.....

Dr. Anjana Singh, Ph.D.

Head

Central Department of Microbiology

Tribhuvan University

Kirtipur, Katmandu

Nepal

Date:.....

BOARD OF EXAMINERS

Recommended by:

…….
Dr. Anjana Singh, Ph.D.
Supervisor

…….
Dr. Linda S. Smith, Ph.D.
Supervisor

……...
Mr. Makhan Maharjan
Supervisor

…….
Ms. Shaila Basnyat
Supervisor

Approved by:

……
Dr. Anjana Singh, Ph.D.
Head of Department

Examined by:

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

……
Dr. Shreekant Adhikari, Ph. D.
Internal Examiner

Date:July 10, 2008

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Narendra Maden

Date:……..

ABSTRACT

Exposure to arsenic through groundwater has raised significant health problem in Terai region of Nepal. Elucidating antibiotic susceptibility pattern of urinary bacteria in community setting and dermatophyte carriage in scalp has very significant public health implications which have seldom been observed in Nepal. Based on the field work in arsenic affected communities of Nawalparasi district, various perspectives of arsenic exposure and status of asymptomatic bacteriuria and dermatophyte carriage were investigated.

Households of highly arsenic affected communities in the program areas of Filter for Families (FFF) were targeted for examination of arsenical dermatological manifestations and urine, hair and scalp swab collection. Hair and urine were analyzed by hydride generation atomic absorption spectrophotometer (SOLAAR 969AA Spectrometer, Thermo Elemental, UK) for total arsenic content and similarly, urine and scalp swab were cultured for urinary bacteria and fungi.

Among 240 participants observed, gender difference in dermatological manifestation was significant, males having nearly three times higher risk than females ($p=0.01$,OR=2.82, CI= 1.23-6.41). Similarly, dermatological manifestation was higher in the older age group ($p=0.01$) and tubewell users of high arsenic level ($p=0.053$). 70% (21/30) of arsenicosis cases had mild dermatological manifestation. The most common dermatological manifestations were keratosis of sole in male (28.5%) and keratosis of palm plus keratosis of soles in females (33.3%). Exposure analysis showed that 67% (73/109) and 65.8% (77/117) of subjects had urinary and hair arsenic level above the normal level. Similarly, subjects having urinary and hair arsenic level above normal level but without arsenical dermatological manifestations were 68.8% (75/109) and 47% (55/117), respectively. Difference in urinary arsenic level was insignificant for both gender and comparison of cases and non-cases ($p>0.05$). However, hair arsenic level differed significantly for both gender ($p=0.01$) and comparison of cases and non-cases ($p=0.03$). There was significant positive correlation of both urine and hair arsenic levels to tubewell arsenic level ($r=0.27$, 0.37, $p<0.01$) and negative correlation with the age of the subjects ($r=-0.18$, $p=0.06$). Binomial logistic regression revealed tubewell arsenic level, age and gender had the strongest association with arsenicosis ($p<0.05$).

Of 118 urine samples cultured, 37.2% (44/118) showed positive growth for uropathogens, females had higher growth rate (42.2%) than males (30.7%). The highest growth rate (47%) was in age group 31-45 years. *E. coli* was the most common isolate (19.6%) and multi-drug resistance (MDR) rate was 30.4%. Norfloxacin was the most effective antibiotic for Gram negative urinary isolates (with susceptibility of 96%; n=25) and ceftriazone for Gram positive isolates (with susceptibility of 71%; n=21). Resistance to amoxycillin was higher

for both isolates (80%, 90.5%). *Pseudomonas aeruginosa* had the highest tolerance to As (V) (mean=725.71 ppm) and *Klebsiella pneumoniae* to As (III) (mean=25 ppm). Difference in mean tolerance of MDR and non-MDR isolates to As (III) and As (V) was not statistically significant ($p>0.05$).Urinary arsenic level shows significant positive correlation to tolerance of urinary bacteria to As (V) and As (III) ($r=0.34, 0.4$; $p<0.05$).

Of total 118 scalp swab cultured for fungi, 56.7% (67/118) showed positive growth. The fungal isolates comprised 8 (12%) dermatophytes and 59 (88%) non-dermatophytes. The dermatophyte carriage rate was 6.7% (8/118). Male had higher carriage rate (7.8%) than female (5.9%). The highest carriage rate (11.1%) was in age group 15-30 years. *Trychophyton tonsurans, T. schonleinii* and *Epidermatophyton floccosum* were common isolates, 25% (2/8) each. *Aspergillus* spp. was the most common non-dermatophytes isolate 28 (47.4%) There was no statistically significant difference in fungal growth in two seasons- summer and winter ($p>0.05$).

Exposure to arsenic and subsequent burden of the disease is still prevailing in highly arsenic contaminated communities of Nawalparasi district. Tube well arsenic level, age and gender have the strongest association with the disease. Multidrug resistance in uropathogens raises a challenge even in the community setting, and such drug resistance is not correlated to urinary arsenic level. Tolerance of uropathogens to As (V) and As (III) was correlated to urinary arsenic level. Carriage of dermatophytes in scalp of adolescent and older age group is a significant consideration and anthrophilic dermatophytes are more common isolates in scalp.

Key words: Arsenic; Asymptomatic bacteriuria; Dermatophyte carriage; Nawalparasi; MDR; As (V); As (III)

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ABBREVIATIONS

µg/L	:	Microgram per Liter
ABU	:	Asymptomatic Bacteriuria
ANOVA	:	Analysis Of Variance
As (III)	:	Trivalent Arsenic
As (V)	:	Pentavalent Arsenic
As	:	Arsenic
AAS	:	Atomic Absorption Spectrophotometer
BMI	:	Body Mass Index
CFU	:	Colony Forming Unit
CFSPH	:	Center for Food Security and Public Health
CI	:	Confidence Interval
CMA	:	Corn Meal Agar
DMA	:	Dimethyl Arsinic Acid
D/W	:	Distilled water
DWQIP	:	Drinking Water Quality Improvement Program
DWSS	:	Department of Water Supply and Sewerage
ENPHO	:	Environment and Public Health Organization
FFF	:	Filters for Families
GIS	:	Geographic Information System
HGAAS	:	Hydride Generation Atomic Absorption Spectrophotometer
HPLC	:	High Performance Liquid Chromatography
IARC	:	International Agency for Research on Cancer
ICP	:	Inductively Coupled Plasma
IDSA	:	Infectious Diseases Society of America
KP	:	Keratosis of Palm

KS	:	Keratosis of Sole
MDR	:	Multi-Drug Resistance
MHA	:	Mueller Hinton Agar
MIC	:	Minimum Inhibitory Concentration
MMA	:	Monomethyl Arsonic acid
MP	:	Melanosis of Palm
MS	:	Melanosis of Sole
MT	:	Melanosis of Trunk
NIS	:	National Interim Standard
NRC	:	National Research Council
OR	:	Odds Ratio
ppb	:	Parts Per Billion
ppm	:	Parts Per Million
RWSSSP	:	Rural Water Supply and Sanitation Support Program
SD	:	Standard Deviation
SGA	:	Sabouraud's Glucose Agar
SPSS	:	Statistical Package for Social Sciences
TwAs	:	Tube well Arsenic level
UTI	:	Urinary Tract Infection
VDC	:	Village Development Committee
WHO	:	World Health Organization

CHAPTER- I

1. Introduction

Globally, arsenicosis is an important non-communicable disease resulting from drinking arsenic (As) contaminated groundwater. Arsenic poisoning of drinking water affects more than 137 million people in more than 70 countries (WHO, 2005a; Wikipedia, 2008).The groundwater arsenic contamination is widely regarded as the largest current calamity of chemical poisoning in the world (Ng, 2005). In South-East Asia region (Bangladesh, India, Myanmar, Nepal and Thailand) over 10 million tube wells are in use potentially exposing between 40 to 50 million people to unsafe levels (>10μg/L) of arsenic (WHO, 2005a).

Chronic arsenic exposure at low level is known to cause characteristic skin alterations such as melanosis especially on the trunk and extremities, and keratosis of palms and soles (Ahmed *et al*., 1999; Claudia *et al*., 1998). Inorganic arsenicals have been classified as group I carcinogens based on the epidemiological data (IARC, 1987). Ingestion of inorganic arsenic is an established cause of skin cancer and studies have shown significant association of chronic arsenic exposure to the development of cancers of bladder, lung, and kidney, and liver (Milton, 2003; Claudia *et al*., 1998).The systemic non-carcinogenic effects of chronic arsenic toxicity include reproductive and developmental, neurological, respiratory, cardiovascular, hematological, gastrointestinal and endocrinological (diabetic) effects (Milton, 2003; NRC, 2001).

Groundwater arsenic contamination has raised significant public health problem in Nepal. About 50% of Nepal's population is living in 20 districts of Terai region and 90% of them are relying on groundwater as their major source of drinking water (Maharjan *et al*., 2006a; Shrestha *et al*., 2003). Recently (May 2006), the testing of 512,840 tubewells for arsenic concentration revealed that 11.5% water samples exceeded the WHO guideline value of

10µg/L and 2.4% samples exceeded the Nepal's standard of 50µg/L (Maharjan *et al*., 2006b). In Nepal, the estimated population exposed to arsenic exceeding 10 µg/L and 50µg/L are 3.19 million and 0.55 million, respectively (Ahmad, 2003).

Nawalparasi district has the highest rate of groundwater arsenic contamination. Until May 2006, more than 31,655 tubewells have been tested which revealed that 25.8% and 12.1% exceeded the WHO and Nepal Interim Standard (NIS) limit, respectively. Similarly, the highest prevalence of arsenicosis has been reported in Nawalparasi district (6.1%) (Maharjan *et al.* 2006b).

Although the reports of chronic arsenic toxicity focus on skin manifestations due to their diagnostic specificity; this element affects other systems of the body (Guha Mazumder, 2000). Subclinical effects of chronic arsenic toxicity prevail in great extent in arsenic affected communities which need to be clarified. For this and to know and evaluate various perspectives of chronic arsenic toxicity (or arsenicosis) such as the status of arsenic exposure, severity due to its health effects, as diagnostic criteria, and in monitoring of mitigation measures assessment biomarkers of arsenic exposure such as urine and hair are needed. In this regard, comparative exposure analysis of subjects living in highly arsenic affected areas needs to be evaluated in Nepal. Similarly, accumulating and exploring evidence about factors that increase the risk of arsenicosis can help not only to explain the mechanisms of development of clinical arsenicosis but also to develop specific and effective interventions.

Urinary tract infection (UTI) is among the most common bacterial infections and has become a serious health problem that affects millions of people each year (Forbes *et al.*, 2002; Stamm and Norrby, 2001). Asymptomatic bacteriuria (ABU) is a condition in which bacteria are present in the urine but there are no clinical symptoms of a urinary tract infection (Colgan *et al*., 2006). Asymptomatic bacteriuria is a strong predictor of

subsequent symptomatic urinary tract infection especially in young women (Hooton *et al*, 2000). Asymptomatic bacteriuria is common, with varying prevalence by age, gender, sexual activity, and the presence of genitourinary abnormalities. Asymptomatic bacteriuria is most common in women, and in patients with diabetes, the elderly, and in those with indwelling urinary catheters. In healthy women living in the community, the prevalence of bacteriuria increases with age (Colgan *et al*., 2006; Harvard Health Publications, 2006; Nicolle, 2003).

Detection of asymptomatic bacteriuria and its antibiogram from the community provides information about the pattern of etiology of community acquired UTI that can help in guiding empiric therapy to clinicians. The concept of co-resistance of metal and antibiotic in bacteria is emerging. Bacterial resistance to diverse metals and antibiotics are often genetically linked suggesting that exposure to toxic metals may select for strains resistant to antibiotics and vice versa (Stepanauskas *et al.,* 2006).

Dermatophytoses are among the most prevalent infections in the world (Brooks *et al*., 2004). Tinea capitis, one of the common dermatophytoses, is a significant public health problem especially in children and such infection may remain undetected. Contributing to the problem is an asymptomatic carrier stage in both children and adults (Vargo and Cohen, 1993). Any person with no sign and symptoms of tinea capitis, but with positive scalp fungal culture can be considered as asymptomatic carrier of tinea capitis (Babel and Baughman, 1989). Asymptomatic carriers are considered as major reservoir of infection. Infection or reinfection of individuals within families, communities and schools may in large part be due to these reservoirs (Vargo and Cohen, 1993).

The presence of dermatophytes plays an important role in spread and persistence of tinea capitis and it is therefore important to identify the carriers of dermatophytes (Babel and

Baughman, 1989). The prevalence of asymptomatic carriers is thought to be correlated with incidence rates of tinea capitis in the community (Frieden, 1999). Most asymptomatic carriage has been reported in children. However, it has also been demonstrated in adults (Frieden, 1999; Vargo and Cohen, 1993). So it seems very necessary to screen out all age groups for asymptomatic dermatophyte carrier.

This study was conducted to compare and evaluate perspectives of arsenic exposure and its disease burden in arsenic contaminated communities of Nawalparasi district. In addition, this study also attempted to detect the status of asymptomatic bacteriuria and tried to correlate antibiotic resistance and arsenic tolerance of urinary bacteria to arsenic exposure. Its aim was also to find out the status of asymptomatic dermatophyte carriers in all age groups in the community.

CHAPTER-II

2. Objectives

2.1 General objective

To gain a better understanding of the perspectives of arsenic exposure and asymptomatic microbial infections in arsenic affected communities of Nawalparasi district

2.2 Specific objectives

- To detect and characterize the dermatological manifestations of arsenicosis in relation to their status and demographic characteristics of study subjects
- To compare and correlate status of arsenic exposure in study subjects
- To correlate arsenic level in drinking water with urinary and hair arsenic level of study subjects
- To evaluate different risk factors of arsenicosis
- To isolate and identify etiological agents of asymptomatic bacteriuria and perform arsenic tolerance and antibiotic susceptibility testing
- To correlate arsenic tolerance in bacteria with urinary arsenic level and antibiotic resistance
- To identify asymptomatic dermatophyte carriers in study subjects

CHAPTER - III

3. Literature review

3.1 Arsenic perspectives

3.1.1 Arsenic and its groundwater contamination

Arsenic, a metalloid, is a P-Block, group IV element of the periodic table with atomic number 33 and atomic mass 74.91. It is widely distributed in the earth's crust and present at an average concentration of 2 mg/kg. It occurs in trace quantities in all rock, soil, water, organisms and air. Arsenic can exist in four valence states: –3, 0, +3 and +5. Under reducing conditions, arsenite As (III) is the dominant form whereas arsenate As (V) is generally the stable form in oxygenated environments. Elemental arsenic is not soluble in water. Arsenic salts exhibit a wide range of solubility depending on pH and the ionic environment (WHO, 2001).

Arsenic is mainly found as a component of more than 245 minerals. Some important arsenic minerals are arsenopyrite (FeAsS), realger (AsS), orpiment (As_2S_3), domeykite (Cu_3As), loellingite ($FeAs_2$), nicolite (NiAs), cobalt-glance (CoAsS), nickel-glance (NiAsS), smaltite ($CoAs_2$) and arsenolite (As_2O_3) (Azcue *et al.*,1994). Processes of arsenic mobilization from its source to groundwater are mainly either natural or anthropogenic. The extent of mobilization depends on hydro-chemical characteristics of ground water aquifers, presence of oxidized/reduced mineral phases and arsenic-rich solid phases (Saxena *et al*., 2004).

By natural process, arsenic is released in ground water as soluble form by three mechanisms.

a) Oxidation of arsenopyrites

Arsenopyrites (or pyrites) are present in subsoil strata which are laid down by rivers over the centuries. Air leaks from the standpipes or wells oxidize arsenopyrites to soluble form that contaminates water source (DWQIP, 2003). For a particular location or tubewell, factors associated with oxidation of arsenopyrites are: i) presence of pyrite in the sediments; ii) the cumulative arsenic concentration connected to decline of water table; and/or iii) yearly change in arsenic concentration and the yearly decline of groundwater table (Fazal *et al*., 2001).

b) Reduction of oxy-hydroxide

Arsenic is adsorbed onto iron or manganese oxy-hydroxides in high concentration in the alluvial sediments. Under reducing environment created by activities of dissimilatory arsenic respiring microorganisms (e.g. *Chrysiogenes* spp., *Bacillus* spp., *Desulfomicrobium* spp., *Sulfospirillum* spp., *Shewanella* spp., *Citrobacter* spp., *Sulforihydrogenibium* spp.) and by the reduction in ionic oxygen in geographical strata, arsenic undergoes dissolution and is released in subsoil water sources (DWQIP, 2003; Fazal *et al*., 2001). For a particular location, factors associated with oxy-hydroxide reduction are: i) the presence of iron or manganese oxy-hydroxides and level of organic matter in the sediments; (ii) arsenic concentration and the reducing conditions; and/or (iii) arsenic concentration and the depth of contamination (Fazal *et al*., 2001).

Second important means of arsenic contamination are anthropogenic sources which can elevate concentration of arsenic in ground water and soil. These include application of arsenical pesticides and herbicides, industrial wastewater discharge, mine tailing, landfill leaching, paint industries and manufacturing and use of wood preservatives (RWSSSP, 2004).

In Nepal the groundwater arsenic contamination is related to Siwalik source rock and statistical GIS analysis indicates correlation of both average and maximum arsenic concentration in shallow ground water from alluvial fans to Siwalik source rocks in the erosional part of watersheds, where fan sediment is produced. Areas of greatest arsenic contamination occur on alluvial fans where Siwalik rocks are the only sediment source, although areas free of arsenic contamination also occur on the same fans. On alluvial fans lacking Siwalik sediment, arsenic contamination is rare, despite the presence of secondary ferric oxyhydroxides and detrital pyrite containing arsenic (Williams *et al*., 2005).

3.1.2 Acceptable level of arsenic in drinking water

World Health Organization (WHO) has set the guideline value of arsenic content for drinking water at 10ppb or 0.01mg/L (WHO, 1995). However, guideline value set by WHO is not binding limits. Depending upon the local or national environmental, social, economic and cultural conditions, each country fixes its own national standard. Based on the standard of Bangladesh and India, Nepal has accepted 50ppb or 0.05mg/L of arsenic in drinking water as its National Interim Standard (NIS) (DWQIP, 2003).

3.1.3 Health effects of arsenic

Occurrence of high concentration of arsenic in drinking water has been recognized as has become a major public health problem in several parts of the world (RWSSSP, 2004; Vater, 2007). As environmental toxicants and carcinogens, arsenicals impose significant health impacts on both humans and animals (Ng, 2005). Chronic exposure to inorganic arsenic (As) is known to cause a wide range of adverse health effects, ranging from skin lesions to cancer. The characteristic skin alterations due to chronic arsenic exposure at low level include pigmentation changes especially on the trunk and extremities (melanosis), and thickening of the outer layer of skin (keratosis) of palms and soles (Ahmad *et al*., 1999; Cluadia *et al*., 1998). The IARC has classified inorganic arsenicals as group I carcinogens

based on the epidemiological data, however, there is inadequate evidence of its carcinogenic potential in animals (IARC, 1987). Ingestion of inorganic arsenic is an established cause of skin cancer and studies have shown significant association of chronic arsenic exposure to the development of cancers of bladder, lung, and kidney, and liver (Cluadia *et al*, 1998; Milton, 2003).Apart from skin alterations and carcinogenicity, arsenic affects all organs and systems of the body. Chronic arsenic exposure also affects gastrointestinal tract, circulatory system, liver, kidney, nervous system, respiratory system, reproductive system and endocrine system (Milton, 2003; NRC, 2001).

3.1.4 Metabolism of arsenic and its mechanism of toxicity in human body

Arsenic can enter human body through (1) oral route via drinking water or food; (2) respiratory route; (3) in small amount through skin. In humans inorganic arsenic (both trivalent and pentavalent) is metabolized in liver by two sequential chemical reactions: (1) oxidation and reduction reactions between As (V) and As (III) and (2) consecutive methylation reactions. It is likely that glutathione, cysteine and dithiothritol present in body reduce As (V) to As (III) which can accept methyl group from S-adenosylmethionine (SAM) to produce the methylarsenic species such as monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) (Cullen and Reimer, 1989; Milton, 2003). Unmethylated inorganic arsenic (5-25% of total) and methylated metabolites are primarily excreted in urine within 4-5 days (Hughes, 2006; Steinmaus *et al*., 2000). A small portion is excreted through faeces, salivation, perspiration, exaltation, lactation, skin exfoliation, and loss of hair and nails (DWQIP, 2003; Milton, 2003). About 45-75 percent of ingested arsenic is excreted from the body within seven days. Accumulated arsenic resides in tissues like skin, hair and nail where it remains bound to sulfhydryl group in keratin (WHO, 2005b).

The toxicity of arsenic compounds depends on the chemical and physical form of the compound, route of entry to the body, the dose and the duration of the exposure, dietary

levels of interacting elements and the age and gender of the exposed individuals. The toxicity of arsenic decreases in the order: Arsine > Inorganic Arsenic III > Organic Arsenic III > Inorganic Arsenic V > Organic Arsenic V > Arsonium compounds > metallic arsenic (Milton, 2003).

The mechanism of action by which inorganic arsenic causes toxicity, including cancer, is not well established. Trivalent methylated arsenics, monomethylarsonous acid (MMA (III)) and dimethylarsinous acid (DMA (III)) existing as intermediates in metabolic process of inorganic arsenic in human are more active than the parent inorganic arsenic for enzyme inhibition (Styblo *et al*., 1997), cytotoxicity (Petrick *et al.,* 2001) and genotoxicity (Mass *et al*., 2001). So it appears that biomethylation of inorganic arsenic in liver is a toxification pathway rather than a detoxification pathway.

Several modes of actions of carcinogenic effects have been proposed, e.g. genotoxicity, altered DNA repair, induction of oxidative stress, altered DNA methylation, altered cell proliferation and altered cell signaling. Arsenic can induce chromosomal aberrations, micronuclei, aneuploidy, endoduplication and gene amplification (Chakraborti *et al*., 2006; Gebel, 2001; Huang *et al*., 1995; Mahata *et al*., 2003). Arsenic has been seen to inhibit several DNA repair enzymes especially zinc finger proteins (Andrew *et al*., 2006). Reactive oxygen species produced arsenicals (arsenite, arsenate and DMA) *in vitro* and *in vivo* have been found to induce carcinogenicity, change in gene expression and genotoxicity (Yamanaka *et al*., 1993). Similarly hypermethylation of promoter of gene p53 and p16 (tumor suppressor genes) is associated with skin cancer in arsenic exposed people (Chanda *et al*., 2006).

3.1.5 Diagnosis of arsenicosis

Arsenicosis is defined as a chronic health condition arising from prolonged ingestion of arsenic above the safe dose for at least six months, usually manifested by characteristic skin lesions of melanosis and keratosis, occurring alone or in combination, with or without the involvement of internal organs (WHO, 2005b). Although arsenicosis or chronic arsenic toxicity produces varied nonmalignant manifestations as well as cancer of skin and different internal organs, dermal manifestations such as hyperpigmentation and keratosis are diagnostic (Guha Mazumder, 2000).

Manifestation of signs and symptoms of chronic arsenicosis differ in different countries. In Bangladesh, India and Nepal, skin manifestations are prime and the most common whereas in Taiwan and China it is not (Milton, 2003).

3.1.5.1 Sign/ symptoms of arsenicosis

Primary and common manifestations:

- Melanosis (spotted or diffuse)
- Keratosis (spotted or diffuse)

Other generalized manifestations:

- General weakness, conjunctivitis, chronic coughs, chronic bronchitis, gastroenteritis, and edema of leg (non pitting)

Complication:

- Ulcer, gangrene, hypertension, diabetes, nephropathy, hepatomegaly, peripheral neuropathy, skin cancer, and cancers of urinary bladder, lung, kidney and liver.

3.1.5.1.1 Pigmentation change

Pigmentation change includes diffuse or spotted blackening/darkening of the skin (melanosis) and depigmented whitish spots on the skin (leucomelanosis). Melanosis is

caused by deposition of melanin in the skin and mucous membrane due to stimulation of melanocyte. In case of arsenicosis, melanosis is usually distributed in the chest, back, upper part of the arms and thighs, palms soles and sometimes in the tongue, gums and lips. Leucomelanosis appears in between the black spots, giving a raindrop like appearance. It usually appears in thighs, chest and back. If any arsenicosis patient stops ingesting arsenic contaminated water and takes arsenic free water, he/she soon develops leucomelanosis (Milton, 2003).

3.1.5.1.2 Keratosis

This system is characterized by the hardening and thickening of palms and soles due to increased keratinisation which appears predominantly on the palms and the plantar sections of the feet. Keratosis is initially palpable but later on it usually advances to form raised, 2-4 mm wart like protuberances which can be seen by naked eye. In most cases distribution of keratosis is symmetric. Diffuse nodular keratosis occurs in severe cases (Guha Mazumder, 2000; Milton, 2003).

Except for dermatological manifestations, other symptoms and signs of chronic arsenicosis are non specific and can occur with other unrelated medical conditions. Hence, history of As exposure by drinking arsenic contaminated water and high levels of arsenic in urine and/or in hair and nails in association with dermatological manifestations help in diagnosis of chronic arsenicosis.

3.1.5.2 Diagnostic criteria of chronic arsenicosis

Following five criteria can be used as diagnostic criteria of chronic arsenicosis

1. At least 6 months exposure to arsenic levels of greater than 50ppb or exposure of high arsenic level from food and air.
2. Dermatological features characteristic of chronic arsenicosis.

3. Non carcinomatous manifestations: weakness, chronic lung disease, non cirrhotic fibrosis of liver with or without portal hypertension, peripheral neuropathy, peripheral vascular disease, non pitting edema of feet/hand.
4. Cancers: Bowen's disease, squamous cell carcinoma, basal cell carcinoma at multiple site, occurring in unexposed parts of the body.
5. Arsenic level in hair and nail above 1mg/kg and 1.08mg/kg respectively and/or arsenic level in urine, above 50µg/L (without any history of taking seafood).

(Guha Mazumder, 2000)

3.1.5.3 Dermatological criteria and grading of severity of chronic arsenicosis

Based on the type and severity of symptoms chronic arsenicosis can be divided into following grades:

Grade	Characteristics
Grade I: mild	a) Diffused melanosis. b) Suspicious spotty depigmentation/pigmentation over trunk and/or limbs. c) Mild diffused thickening of soles and palms.
Grade II: moderate	a) Definite spotty pigmentation/ depigmentation on the trunk limbs which are bilaterally distributed. b) Severe diffuse thickening (with or without wartlike nodules of the palms and soles).
Grade III: severe	a) Definite spotty pigmentation /depigmentation with few blotchy pigmented/ depigmented macular patches over trunk or limbs.

b) Pigmentation involving undersurface of tongue and/or buccal mucosa.

c) Larger nodules over thickened palms and sole occasionally over dorsal aspect of hands and feet. Diffuse verrucous lesions of the soles with cracks and fissures and keratotic horns over palms/soles.

(Guha Mazumder, 2000)

3.1.5.4 Case definition of arsenicosis

Based on diagnostic and dermatological criteria case definition strategy of the arsenicosis has been developed as following:

A) Definite

1. Criterion 1 + criterion 2 ± criterion 3 ± criterion 4+ criterion 5
2. Criterion 1 + criterion 2 (Grade II/III) ± criterion 3 ± criterion 4
3. Criterion 2 (Grade II/III) ± criterion 3 ± criterion 4 + criterion 5

B) Probable

1. Criterion 1 + criterion 2 (Grade I) ± criterion 3 ± criterion 4
2. Criterion 2 (Grade I) ± criterion 3 ± criterion 4+ criterion 5
3. Criterion 2 (Grade II/III) ± criterion 3 ± criterion 4
4. Criterion 3 + criterion 5
5. Criterion 4 + criterion 5

(Guha Mazumder, 2000)

3.1.6 Biomarkers of exposure

3.1.6.1 Urine

The concentration of total arsenic urine has been used as indicator of recent arsenic exposure because urine is the main route of excretion of most arsenic species (Buchet *et al.* 1981;Vater, 1994).The half time of inorganic arsenic is about 4 days.

An important question is whether to collect 24-hr urine samples, spot urine samples, or first-morning urine samples. Ideally, the amount of arsenic excreted over a certain period of time should be assessed. Usually that is done by measuring the excretion of arsenic in all urine produced in 24 hr. However, obtaining complete 24-hr urine collections is difficult (Johansson *et al*. 1998). It requires supervision and validation. Because of those difficulties and other problems (e.g., the risk of contamination of the urine during sampling), the first-morning urine or spot urine samples are generally collected for determination of the urinary concentration of arsenic or arsenic metabolites. To evaluate the concentration properly, especially in the case of spot urine samples, the dilution of the urine has to be considered. The urine flow is highly variable, being dependent on numerous factors, such as body size, body water content, solute intake, physical activity, and diurnal variations (Diamond, 1988). A short time after the consumption of large amounts of fluid, the urine is much diluted and has a low solute content. To compensate for the dilution, the concentration of arsenic can be related to the concentration of creatinine or the specific gravity. A disadvantage of using the creatinine-adjusted urinary arsenic measurement is that it is dependent on the muscle mass and thus is often quite different for men and women. Protein intake might also influence urinary creatinine concentrations (Guha Mazumder, 2000).

Seafood in the diet may influence urinary arsenic measurements. Certain seafoods esp particularly cold water fin fish, crustaceans, and molluscs often have high concentrations of organo arsenic compounds mainly in the form arsenobetaine that does not have known mammalian toxicity. These are rapidly excreted in the urine (Guha Mazumder, 2000;

Vahter, 1994). In effort to avoid the contribution of complex organo arsenicals in seafood, measurement of only inorganic arsenic or its metabolites, monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) should be done (Chana and Smith, 1987).

3.1.6.2 Hair and nail

Arsenic is normally found in higher concentrations on human hair and nail than in other parts of the body, because they contain large amounts of keratin that contains sulfhydryl groups that accumulate arsenic (Yamauchi and Tamamura, 1983). Arsenic levels in hair can provide useful information in chronic arsenic poisoning but undue weight should not be given to the results because only a very approximate relationship between hair arsenic concentration and arsenic toxicity has been found. There is inter and intra-hair variations in arsenic content in hairs of the same individual. Thus samples should consist of at least one gram of hair cut close to the scalp and derived from several sites on the head and the whole sample should be analyzed. External contamination of the hair by arsenic must be excluded in order to use hair arsenic concentrations to assess toxicity. Ingested arsenic and arsenic derived from external contamination are both avidly bound to the outer surface of the hair and these two sources cannot be differentiated by any known technique and thus can mislead the result (Guha Mazumder, 2000; Hindmash,1998).

3.1.6.3 Blood

Blood arsenic level can reflect exposure for only a short period following absorption which is very time dependent. However due to short half-life of arsenic in the blood compared with the half-life in the body, it is difficult to discern a relationship between blood As concentration and arsenic burden in different organs or in whole body (Guha Mazumder, 2000). Compared with urine blood is a much less sensitive biomarker of exposure to As via drinking water (NRC, 1999).

3.1.7 Analysis of arsenic in biological samples

There are many methods by which arsenic in biological samples can be analyzed both quantitatively and qualitatively, viz. atomic absorption spectrophotometry (AAS), inductive coupled plasma (ICP) mass spectrometry, neutron activation analysis, PIXE technique, high performance liquid chromatography (HPLC) etc. However, hydride generation atomic absorption spectrophometry (HG-AAS) has been the most commonly used method to analyze arsenic in biological samples like urine and hair (WHO, 2003).

3.1.8 Treatment and case management of arsenicosis patients

There is no effective therapy for arsenicosis. Patients once affected may not recover even after remediation of the As contaminated water. Chelation therapy using arsenic chelators like DMSA (Dimercaptosuccinic acid), DMPS (Dimercaptopropane succinate) and d-penicillamine has been practiced for relief of systemic clinical manifestations and reduction of arsenic stores in the body, reducing subsequent cancer risk. Similarly oral supplement of retinoids in appropriate dose to arsenicosis patients has been useful in regressing dermatological manifestations and may also offer significant promise in the chemoprevention of arsenic-related cancers (Guha Mazumder, 2000). The available case management strategies for arsenicosis patients include:1) cessation of exposure to arsenic contaminated water, 2) administration of nutritional supplements, 3) provision of non-specific therapy like keratolytic agents, 4) secondary prevention of latent effects through medical surveillance, 5) counseling and education (WHO, 2005a).

3.1.9 Arsenic investigation in Nepal

Arsenic contamination of groundwater in Terai districts was first indicated in 1999 by DWSS with support from WHO. Since then governmental and nongovernmental organizations and some researchers have tested hundreds of thousands of groundwater water samples from Terai for arsenic concentration. Until May 2006, 512,840 tubewells

have been tested and 11.5% water samples exceeded the WHO guideline value of 10µg/L and 2.4% samples exceeded the Nepal Standard of 50µg/L. The percentage of water samples exceeding10µg/L varied from 0.0% (Chitwan) to 43.3% (Morang) while it was 0.0% (Chitwan) to 12.1(Nawalparasi) for water samples exceeding 50 µg/L of arsenic (Maharjan *et al.* 2006b).

Nepal lacks extensive a population based health survey of arsenicosis in all Terai districts. Maharjan *et al*. (2006b) report from the summary of surveys on arsenicosis cases by governmental and non-governmental organizations in six arsenic contaminated districts of Terai namely Nawalparasi, Bara, Parsa, Rauthat, Rupandehi, and Kapilbastu that the prevalence of arsenicosis in these districts on an average was 2.9% and ranged from 0.7% in Kapilbastu to 6.1% in Nawalparasi (Table1).

Table 1 Gender-wise distribution of arsenicosis cases in six districts of Nepal

Gender	Nawalparasi (%)	Parsa (%)	Bara (%)	Rauthat (%)	Rupandehi (%)	Kapilbastu (%)	Total (%)
Male	213/2370 (9)	36/1548 (2.3)	28/1212 (2.3)	114/3696 (3.1)	3/318 (0.9)	7/828 (0.8)	401/9972 (4)
Female	86/2505 (3.4)	23/1493 (1.5)	20/1262 (1.6)	53/3745 (1.4)	3/337 (0.9)	4/838 (0.5)	189/10180 (1.9)
Total	299/4875 (6.1)	59/3041 (1.9)	48/2474 (1.9)	167/7441 (2.2)	6/655 (0.9)	11/1666 (0.7)	590/20152 (2.9)

Number of arsenicosis cases and number of subjects examined are on left and right side of the slash respectively, and in parentheses the prevalence rate of arsenicosis is maked.

Similarly, geological research has been done to elucidate the source and the nature of arsenic contamination in Terai districts (Bhattacharya *et al*., 2003; Gurung *et al.*, 2005).

Rahman *et al.* (2006) assessed prevalence of skin lesions due to arsenic exposure through drinking water in a population based survey in Matlab, Bangladesh, where more than 60% tube wells exceeded 50 µg/L, by screening entire population above 4 years of age (n =166,943). They found that prevalence rate of skin lesions was 0.3%. There were gender and age differences in both exposure and skin lesions. Hossain *et al*. (2005) found from an analysis of a total of 1482 arsenicosis patients through household screening living in 496 upzilas (sub-districts) of Bangladesh that melanosis was common dermatological manifestation (97%) among them and about two-thirds (68.7%) of the patients were suffering from keratosis. Rahman *et al*. (2005) found prevalence of arsenicosis in an arsenic affected village of West Bengal, India at 18.1% (n=825) where 63 % of tube wells exceeded acceptable arsenic concentration of 50µg/L (n=336). The parametric values of arsenic in hair samples were: mean 1931 µg/kg, median 1587 µg/kg, and range 535-8453 µg/kg (n=188); and those of urine samples were: mean 420 µg/L, median 244 µg/L, and range 33-2353 µg/ L (n =50). Ahmad *et al.* (2001) studied relationship between total arsenic in urine and the severity of disease manifestations in 60 subjects consisting of 30 cases and 30 controls. They found statistically significant ($p<0.001$) difference in mean urinary levels of total arsenic between the cases (163.8 ppb) and the controls (10.7 ppb). Statistically significant differences ($p< 0.001$) in duration of tubewell water use, and total arsenic in urine between cases with grade-1 and grade-2 manifestations were also observed. Watanabe *et al*. (2001) found highly significant correlation ($R^2 = 0.504$; $p < 0.001$) between urinary and tubewell arsenic level in two villages of Bangladesh suggesting that the major source of the arsenic is tubewell water. Although the levels of exposure were similar between the sexes, overall dermatological manifestations was higher in males than in females (n= 551; $p<0.005$).

Mosaferi *et al*. (2005) found significant correlation between current arsenic content of drinking water and arsenic concentration in hair ($r=0.662$, $p<0.001$) in 39 human hair samples from female subjects residing in three villages of Kurdistan province of Iran. Arsenic content of drinking water ranged from 0 to 0.455 mg/L (average: 0.18) and arsenic concentration in hair was from 0.012 to 3.41 mg/kg (average: 0.53). RWSSSP (2004) reported prevalence rates of arsenicosis in Rupandehi and Kapilbastu districts of Nepal at 0.9 % (n=655) and 0.7 % (n=1666) respectively. Skin symptoms of the arsenicosis cases were mild, and keratosis of palms and soles was the predominant manifestation accounting 35.3 % of total cases (n=48). For Rupandehi district, correlation between urinary and tubewell arsenic levels was weakly negative and insignificant ($r= -0.21$, $p =0.25$, $n=62$), however, for Kapilbastu district, correlation was weak but significant ($r= 0.25$, $p =0.028$, $n =154$). Maharjan (2004) reported prevalence of arsenicosis in three communities (Sano Kunwar, Thulo Kunwar and Goini) of Nawalparasi district at 6.9 % (n=1343) where average arsenic contamination rate was 87.6 % (n=146). Gender difference in arsenicosis was significant ($p<0.001$) with odds ratio 2.095, males having higher prevalence (9.3%) than females (4.4%). 79.6% and 20.4% patients had mild and moderate skin manifestations, respectively. He also reported that half of villagers (50.5%) were underweight and conferred that arsenic exposure worsens nutritional status and modifies occurrence or development of arsenicosis. DWQIP (2003) reported the prevalence rate of arsenicosis by dermatological examination at 14 VDCs in Nawalparasi district at 2.7% among the exposed population (n=2174). The prevalence rate was higher in males (4.2%) than in females (1.4%), and highest prevalence was in age group of above 65 years (19.2%) and lowest in 15-49 years (1.5%). Of arsenicosis symptoms, melanosis on the trunk and keratosis of palm were common. Of total hair samples (n=49) analyzed, 67.34% exceeded normal hair arsenic concentration.

3.2 Asymptomatic microbial infections

3.2.1 Asymptomatic bacteriuria

Urinary tract infection (UTI) is one of the most common bacterial infections that has become a serious health problem that affects millions of people each year (Forbes *et al.*, 2002; Stamm and Norrby, 2001). UTI usually starts as a bladder infection but often ascends to affect the kidneys and ultimately can result in renal failure or dissemination of bacteria to the blood. UTI is classified into disease categories by the site of infection: cystitis (the bladder), pyelonephritis (the kidney), and bacteriuria (the urine). When a significant number of bacteria are detected in the urine, they are indicative of a possible infection somewhere in the urinary tract (kidneys, ureters, bladder, and urethra). Asymptomatic bacteriuria (ABU) is a condition in which bacteria are present in the urine but there are no clinical symptoms of a urinary tract infection (Colgan *et al*., 2006). Asymptomatic bacteriuria is a strong predictor of subsequent symptomatic urinary tract infection especially in young women (Hooton *et al*, 2000).

3.2.1.1 Epidemiology

Asymptomatic bacteriuria is common, with varying prevalence by age, gender, sexual activity, and the presence of genitourinary abnormalities. Asymptomatic bacteriuria is most common in women, and in patients with diabetes, the elderly, and in those with indwelling urinary catheters. In healthy women, the prevalence of bacteriuria increases with age, from about 1 percent in females five to 14 years of age to more than 20 percent in women at least 80 years of age living in the community (Colgan *et al*., 2006; Harvard Health Publications, 2006; Nicolle, 2003). *E. coli* is the most common organism isolated from patients with asymptomatic bacteriuria. Infecting organisms are diverse and include Enterobacteriaceae, Gram positive cocci especially coagulase negative *Staphylococci*, *Pseudomonas aeruginosa*, *Enterococcus* spp., Group B *streptococcus* etc.

3.2.1.2 Asymptomatic bacteriuria in selected population

3.2.1.2.1 Premenopausal, nonpregnant women

Premenopausal, nonpregnant women with asymptomatic bacteriuria experience no adverse effects and usually will clear their bacteriuria spontaneously. However, these women are more likely to experience subsequent symptomatic UTI than women who do not have asymptomatic bacteriuria (Colgan *et al*., 2006; Hooton *et al*., 2000). Treatment of asymptomatic bacteriuria does not decrease the frequency of symptomatic UTI or prevent further episodes of bacteriuria. Asymptomatic bacteriuria has not been shown to be associated with detrimental long-term outcomes (e.g. hypertension, renal failure, genitourinary cancer, or decreased survival). For these reasons, the IDSA (Infectious Diseases Society of America) does not recommend screening for or treatment of asymptomatic bacteriuria in premenopausal nonpregnant women.

3.2.1.2.2 Pregnant women

The prevalence of asymptomatic bacteriuria in pregnancy varies from 4-7% (range 2-11%) and is similar to that observed in non-pregnant women. The prevalence is higher among individuals in lower socioeconomic classes, and those with a past history of asymptomatic urinary infection (Nicolle, 1994). Women with asymptomatic bacteriuria during pregnancy are more likely to deliver premature or low-birth-weight infants and have a 20- to 30-fold increased risk of developing pyelonephritis during pregnancy compared with women without bacteriuria (Kincaid-Smith and Bullen, 1965). Treatment of asymptomatic bacteriuria in pregnancy significantly decreases the risk of subsequent pyelonephritis (Smaill, 2001), improves fetal outcomes, with decreases in the frequency of low-birth-weight infants and preterm delivery (Mittendorf *et al.*, 1992; Romero *et al*., 1989).

3.2.1.2.3 Women with diabetes

Postmenopausal women with diabetes have higher risk of asymptomatic bacteriuria in relation to diabetes duration and severity (Boyko *et al*., 2005). Antimicrobial therapy for women with diabetes and asymptomatic bacteriuria is unable to delay or decrease the frequency of symptomatic UTI or the rate of hospitalization for UTI (Harding *et al*., 2002).

3.2.1.2.4 Elder individuals

Prevalence of bacteriuria is higher in elder individuals than younger, and is especially high in elder women reaching up to 6-8% in women aged 60 years, and over 20% in elderly women in the community over 80 years (Nicolle,1994). Though antimicrobial therapy decreases prevalence of asymptomatic bacteriuria for short period, it cannot prevent symptomatic episodes (Boscia *et al*., 1987).

3.2.1.2.5 Patients with spinal cord injuries

Patients with spinal cord injuries have a higher prevalence of asymptomatic bacteriuria and their treatment may show up early recurrence of bacteriuria following therapy. Screening or treating of asymptomatic bacteriuria should not be recommended in patients with spinal cord injuries (Colgan *et al*., 2006).

3.2.1.2.6 Patients with indwelling urethral catheters

Patients with chronic indwelling Foley catheters are uniformly bacteriuric, but treatment is warranted only if the patient is symptomatic (Colgan *et al*., 2006).

3.2.1.3 Diagnosis of asymptomatic bacteriuria

The presence of a significant quantity of bacteria in a urine specimen properly collected from a person without symptoms or signs of a UTI characterizes asymptomatic bacteriuria

Quantitative criteria for identifying significant bacteriuria in an asymptomatic person are: (1) at least 100,000 colony-forming units (CFUs) per mL of urine in a voided midstream clean-catch specimen; and (2) at least 100 CFUs per mL of urine from a catheterized specimen According to the IDSA guideline, the diagnosis of asymptomatic bacteriuria in women is appropriate only if the same species is present in quantities of at least 100,000 CFUs per mL of urine in at least two consecutive voided specimens (Lipsky *et al*.,1987; Nicolle *et al*, 2005).

3.2.1.4 Treatment

Antibiotic treatment for asymptomatic bacteriuria is recommended for children, people with obstruction or abnormal structure of the urinary tract, pregnant women and men about to undergo urologic or prostate surgery and people who have had a kidney transplant (InteliHealth, 2006).

3.2.1.5 Prevention

The steps recommended especially for women to help prevent a bladder infection or asymptomatic bacteriuria from recurring include: i) drinking plenty of fluids (six to eight glasses each day); ii) urinating regularly before and after intercourse and emptying bladder completely each time; iii) keeping the vaginal area clean; iv) wearing cotton underwear which allows better air circulation than nylon and avoiding tight clothes in the genital area and v) using tampons instead of sanitary pads during menstrual period (Women health Advisor, 2006).

3.2.1.6 Antimicrobial susceptibility testing

In recent years drug resistant bacteria have given rise to several serious outbreaks of infection that led to a development of national and international surveillance programmes to monitor antimicrobial resistance in bacteria. Thus obtained microbiological and

epidemiological information is then used by clinicians in selecting the most appropriate antimicrobial agent for the treatment of a microbial infection (WHO, 2004).

WHO recommends modified disc-diffusion technique of Kirby-Bauer for clinical and surveillance purposes in view of its technical simplicity and reproducibility. The method is particularly suitable for use with bacteria belonging to the family Enterobacteriaceae, but it can also be recommended as general purpose method for all rapidly growing pathogens (WHO, 2004).

3.2.1.6.1 Modified Kirby-Bauer method

In this diffusion test, paper discs, impregnated with a specified amount of an antimicrobial, are placed on agar medium (MHA) uniformly seeded with the test organism and incubated at 35ºC usually for 16-18 hours. A concentration gradient of the antimicrobial agent is formed by diffusion from the disc and the growth of the organism is inhibited at a distance from the disc that is related to the susceptibility of the organism. The diameter of the zone of inhibition around each disk is measured in millimeters and the susceptibility result thus obtained is interpreted into three categories, viz. susceptible, intermediate, and resistant for each antimicrobial agent-bacterial species combination (Forbes *et al.*, 2002;WHO, 2004).

Antimicrobial susceptibility test may be performed to guide the clinicians in selecting the best antimicrobial agent for an individual patient and/or to accumulate epidemiological information on the resistance of microorganisms of public health importance within the community (WHO, 2004).

3.2.1.3 Minimum inhibitory concentration (MIC)

It is defined as the lowest concentration of antimicrobial agent that completely inhibits visible bacterial growth. It involves challenging the organism with antimicrobial agents in a broth environment. The antimicrobial agent is tested using a range of concentration and

concentration range tested will often vary from one antimicrobial agent to another. In case of antibiotics, concentration is commonly expressed in µg of active drug/mL of broth (µg/mL) (Forbes *et al.*, 2002). Doubling dilutions, usually in the range 0.12-256 µg/mL for most antibiotics- of the antimicrobial under test are prepared in a suitable broth media such as Mueller Hinton broth. Then one loopful of logarithm phase cells is added to each dilution to result in a final cell density of around 5x 10^5 CFU/ml. After incubation at 35ºC for 18 hours the concentration in the first clear tube is read as the MIC. This test needs sterility control and growth control. MIC can also be determined by agar dilution test and by newer microdilution test which uses 96-well microtitre plates (Smith, 2006).

3.2.1.8 Arsenic resistance in bacteria

Arsenic ions are very toxic for most microorganisms. Many bacteria possess genetic determinants that confer resistance. In bacteria, these determinants are often found on plasmids, which have facilitated to study the molecular mechanisms of arsenic resistance. Chromosomal determinants of toxic metal resistances are also known in *E. coli* and *Pseudomonas aeruginosa*, and the distinction between plasmid resistances and those from chromosomal genes has blurred, because for some metals (notably mercury and arsenic), the plasmid and chromosomal determinants are basically the same (Cervantes *et al*.,1994; Saltikov and Olson, 2002; Silver and Phung,1996).The most well characterized genetic system for resistance to arsenicals is known as the *ars* operon which encodes specific efflux pumps that extrude arsenite As (III) thus lowering the intracellular concentration of the toxic ions (Cervantes,1995; Saltikov and Olson, 2002). In Gram-negative bacteria, the efflux pump consists of a two-component ATPase complex. ArsA is the membrane bound anion-translocating ATPase subunit and is associated with an integral membrane subunit, ArsB which forms anion-conducting channel. Arsenate is enzymatically reduced to arsenite by arsenate reductase, a small cytoplasmic ArsC polypeptide (Saltikov and Olson, 2002). In Gram-positive bacteria, comparable arsB and arsC genes (and proteins) are found, but arsA

is missing. Many other bacteria confer resistance to arsenite by enzymatic oxidation of more-toxic arsenite to less-toxic arsenate (Cervantes, 1995; Silver and Phung, 2005).

3.2.2 Asymptomatic dermatophyte carrier of scalp/hair

The carrier is defined as an individual who harbors in his/her body a specific organism of a disease without manifesting symptoms and thus acts as carrier or distributor of the infection (Dorland's illustrated medical dictionary, 1965). Tinea capitis is a superficial fungal infection of the scalp/hair caused by dermatophyte. Any person with no sign and symptoms of tinea capitis i.e. without black-dot lesions, obvious hair loss, scaling, crusts, postules or erythema, but with positive scalp fungal culture can be considered as asymptomatic carrier of tinea capitis (Babel and Baughman, 1989). Asymptomatic carriage is considered as major reservoir of infection. Tinea capitis is a significant public health problem especially in children and the infection may remain undetected. Contributing to the problem is an asymptomatic carrier stage in both children and adults. Infection or reinfection of individuals within families, communities, and schools may in large part be due to these reservoirs of organism (Vargo and Cohen, 1993).

3.2.2.1 Dermatophytes

The dermatophytes represent 39 closely related fungi in three imperfect genera: *Microsporum*, *Tricophyton* and *Epidermatophyton* (Fitzpatrick, 1987). These are hyaline, septate, and monomorphic fungi. The dermatophytes have the capacity to invade keratinized tissue (skin, hair, and nails) of humans and other animals to produce an infection, dermatophytosis, commonly referred to as ringworm which is among the most prevalent infections in the world (Brooks *et al*., 2004; Forbes *et al*., 2002; Weitzman and Summerbell, 1995). Infection is generally cutaneous and restricted to the nonliving cornified layers because of the inability of the fungi to penetrate the deeper tissues or organs of immunocompetent hosts. Reactions to a dermatophyte infection may range from

mild to severe as a consequence of the host's reactions to the metabolic products of the fungus, the virulence of the infecting strain or species, the anatomic location of the infection, and local environmental factors (Dei Cas and Vernes, 1986).

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Dermatophytes are identified by their colonial appearance and microscopic morphology after growth for 2 weeks at 25-30ºC on Sabouraud's glucose agar (SGA). *Trichophyton* spp. develop cylindrical smooth-walled macroconidia and characteristic macroconidia. *Microsporum* spp. tend to produce distinctive multicellular macroconidia with echinulate to roughened walls and solitary, one celled ovoidal to clavate macroconidia. *Epidermatophyton floccosum*, which is the only pathogen in this genus, produces only macroconidia, which are smooth-walled, clavate with blunt tip, two to four-celled, and formed in groups of two or three (Brooks *et al*., 2004; Hoog *et al*, 2000).

3.2.2.2 Epidemiology

Dermatophytoses are communicable diseases acquired from infected animals or birds or from the fomites they have engendered. Although they can be persistent and troublesome, they are not debilitating or life threatening. Dermatophytes are classified as geophilic, zoophilic, or anthrophilic depending on whether their usual habitat is soil, animals, or humans. Anthrophilic species cause the greatest number of human infections in human and may be difficult to eradicate. Conversely, geophilic and zoophilic dermatophytes, being less adapted to human hosts, produce more acute inflammatory infections that tend to resolve more quickly (Chmel, 1980).

Zoophilic dermatophytes usually infect animals or are associated with animals but occasionally infect humans. Geophilic dermatophytes are primarily associated with keratinous materials such as hair, feathers, hooves, and horns after these materials have

been dissociated from living animals and are in the process of decomposition. These species may cause human and animal infection (Chmel, 1980).

Transmission between hosts usually occurs by direct contact with a symptomatic or asymptomatic host, or direct or airborne contact with its hairs or skin scales (CFSPH, 2005). Infective spores found within or attached to the exterior of infected hairs and within skin scales may dissociate from skin cells in the environment and come in contact with new potential hosts. Their persistence as an environmental source of contagion for several months to years in the environment may lead to recurrent outbreaks of dermatophytosis in individuals and in institutions (Kane *et al*., 1988). Asymptomatic individuals with high spore loads may be a more important vector in the transmission of tinea capitis than symptomatic cases because they have potential to shed large number of spores over longer periods of time (as long as their carrier status remain undetected and not treated) (William *et al.*,1995). Geophilic dermatophytes, such as *Microsporum nanum and Microsporum gypseum* are usually acquired directly from the soil rather than from another host (CFSPH, 2005).

Carriage rate is usually higher in winter months than spring and autumn months, which can be attributed to higher humidity during winter months, which favours fungal growth and less frequent hair washing in this season due to cold weather conditions. Carriage rate is higher in male than in female children. Secretion of sebaceous glands in childhood favours the growth of dermatophytes and such secretions last into adolescent in males unlike females (Ali-Shtayeh *et al.,* 2002). Similarly, host susceptibility for dermatophyte infection may be enhanced by specific skin chemistry, composition of sebum and perspiration, youth, heavy exposure, hygiene, socioeconomic conditions, and genetic predisposition (Brooks *et al*., 2004; Filipello *et al.,* 1996). Ali-Shtayeh *et al.* (2002) found prevalence of asymptomatic dermatophytes carriage rate at 0.78% (n=4119) in school children in Nablus

area, Palestine. *M. canis* was the most common (65.6%; 21/32). Carriage rate was higher in winter season ranging from 1.02 to 3.01% in the different communities than in autumn (0 to 0.24%), in males (1.02%) than in female (0.51%), and in younger children (<10years: 0.9%) than in older children (>10 years: 0.6%). William *et al.* (1995) found prevalence of asymptomatic dermatophytes carriage rate at 14 %(n =224) in all black children of Philadelphia, United States of America. *T. tonsurans* was the predominant dermatophyte (96% of 125 positive culture) followed by *M. canis*.

CHAPTER-IV

4. Materials and methods

4.1 Materials

A list of materials used in this study is given in appendix III.

4.2 Methods

4.2.1 Study period

This study was conducted from June 2007 to April 2008. Field work- data collection, examination of the subjects for arsenicosis cases and urine, hair, and scalp swab sample collection were done in three successive sessions.

4.2.2 Study area

This study was conducted in highly arsenic affected communities of Nawalparasi district. Nawalparasi district is located at 83º36'-84º25'east altitude and 27º21'-27º47'north latitude; and lies in Lumbini zone of Western Development Region of Nepal. The district has area of 2162 square kilometers comprising 73 VDCs and one municipality (Ramgram). Its border districts are Chitwan and Tanahu in the east, Rupandehi and Palpa in the west, Palpa and Tanahu in the north and Bihar state of India in the south.

4.2.3 Study sites

VDCs in the program areas of FFF were selected as study sites. Thirteen communities in five VDCs (Jahada, Sarawal, Sunawal, Sukrauli and Swati) and one municipality (Ramgram) were chosen as study sites depending upon their extent of arsenic contamination and its health effects, filter distribution and accessibility from the headquarter (Parasi).These included ThuloKunwar, SanoKunwar, Kanchanaha, Ghanashyampur and Baikunthapur of Ramgram, Samaitole and Kirtipur of Sunawal,

Bankatti of Swati, Bhatalaiya of Jahada, Goini of Sarawal, and Nadawa of Sukrauli. In this study, a cluster of each village was examined since the entire village is too large to be covered. A schematic map of study area (district) and sites is given at Appendix I.

4.2.4 Study population

For this study a household was considered as sampling unit and household members as the study population.

4.2.5 Inclusion and exclusion criteria

4.2.5.1 Inclusion criteria

All members of targeted households of age ≥5 years living in the study area at least for six months were included for the dermatological examination of arsenicosis.

4.2.5.2 Exclusion criteria

1. Members of household, not available at the time of visit were excluded.
2. Members of households not living there for more than six months were excluded.
3. Members who had recently (within past three days) eaten seafood and/or fish were excluded from urine sampling.
4. Visitors or guests of household were excluded in all kinds of data collection.

4.2.6 Ethical consideration

Prior to any data collection, the purpose and procedure of this study were explained to the household head or key informant and other interested family members. The verbal consent was obtained from each participant prior to dermatological examination of arsenic related skin manifestation. Similarly, urine, hair, and scalp swab collection of any two persons-one male and another female were collected only from interested family members. Participation of the subjects in all kinds of data collection and sample collection was voluntary.

4.2.7 Data collection

4.2.7.1 Interview

Necessary demographic information (e.g. age, gender) and information about duration of tubewell use, arsenic concentration of the tubewell, and duration of use of filtered water were collected by interviewing a household head or a knowledgeable family member. Arsenic concentration of tube well of each household was obtained as a secondary data from FFF and DWSS.

4.2.7.2 Dermatological examination

All interested family members of a household who were present during the visit and were older than 5 years were enrolled for dermatological examination to determine the extent of chronic arsenicosis. In this study any person having melanosis or keratosis (after ruling out non-arsenical dermatological manifestations) with or without other manifestation of chronic arsenic toxicity with known history of arsenic exposure through drinking water was diagnosed as arsenicosis patient. Dermatological manifestations were graded as 'mild', 'moderate', and 'severe' following the grading criteria given by Guha Mazumder (2000).

4.2.7.3 Anthropometric measurement

Anthropometric measurements of body height and mass were conducted for those individuals who were willing to give urine, hair and scalp swab. Body height was measured using an anthropometer and body height using portable weighing machine. Body mass index (BMI) was calculated as weight (kg)/ height (m^2).

A format of questionnaire employed for the data collection is given at Appendix III.

4.2.8 Sample collection

Two members from a household- one male and another female were enrolled for sample collection and anthropometric measurements (body mass index). From each subject, three samples viz., urine, hair and scalp swab were collected.

4.2.9 Urine sampling, preservation, transportation and analysis

4.2.9.1 Method of sample collection and preservation

Each subject was given a detailed explanation about the purpose and method of sample collection. Then their verbal consent was obtained. The subject was asked to provide mid-stream urine into a sterile urine culture plastic tube. The sample labeled was stored is a portable ice box that was filled up with ice packs. The samples were brought to FFF's field office at Parasi and refrigerated.

4.2.9.2 Sample transportation

Urine samples were transported from Parasi to Central Department of Microbiology (CDM), Kirtipur in an ice box within three days of sample collection. The samples were then kept in a refrigerator and urine culture was done following the next day. Then the samples were deep-frozen till analysis for arsenic content in urine samples

4.2.9.3 Urine analysis

4.2.9.3.1 Urine culture

The urine was shaken gently and sucked up from the surface with a 1microliter inoculating loop. Then a loopful of urine was touched to the center of the MacConkey agar (MA) (without crystal violet and sodium chloride and with sodium taurocholate), from which the inoculum was spread in a line across the diameter of the plate.

Then the loop was drawn across the entire plate, crossing the first inoculums streak numerous times to produce isolated colonies. The urine samples were also half streaked on blood agar (BA) plates. Then the plates were incubated at 37ºC for 24 hours and colony counts were made; significant growth ($\geq 10^5$ CFUs/ ml) as well as insignificant growth ($\leq 10^5$ CFUs/ ml) were noted.

4.2.9.3.2 Identification

Bacterial isolates from the MA culture plates were subcultured on the nutrient agar and identified using staining technique and a battery of selected biochemical tests and other specific tests. The following tests were conducted.

- Gram staining,
- Catalase and oxidase tests,
- IMViC test: indole, methyl red, Voges-Proskaur, and citrate utilization tests,
- TSI (triple sugar iron), urease and motility tests,
- Oxidation/fermentation (O/F) and sugar utilization tests,
- Slide and tube coagulase tests,
- Novobiocin susceptibility testing.

The detailed procedures of these tests are given in Appendix V.

4.2.9.3.3 Antibiotic susceptibility testing

Antibiotic susceptibility testing of urine isolates was performed by standard modified Kirby Bauer disc diffusion method. Antibiotics for susceptibility testing were chosen considering their antibacterial spectrum, availability, and recent trend of use. A detailed procedure for antibiotic susceptibility testing is given at appendix VI. The interpretive chart of antibiotic susceptibility testing and the list of antibiotic discs used for this testing are given at Appendix VIII.

4.2.9.3.4 Minimal inhibitory concentration (MIC)

MIC values of As (III) to urinary bacteria were determined by tube dilution method using 200ppm As (III) working solution. However, MIC values of As (V) to urinary bacteria were determined by preparing solutions of different concentration using 4% As (V) working solution. A detailed procedure for determination of MIC values of As (III) and As (V) is given at Appendix VII.

4.2.9.3.5 Total arsenic analysis of urine

Measurement of total arsenic concentration in urine samples was done by an atomic absorption spectrophotometer (SOLAAR 969AA Spectrometer, Thermo Elemental, UK), equipped with a hydride generator (HG-AAS), in the research laboratory of ENPHO, Kathmandu, Nepal.

One ml of each sample was digested with low heat with 25 ml conc. H_2SO_4 and 5 ml HNO_3 in Kjeldhal flask in the Kjeldhal digestion unit. 5 ml HNO_3 was further added along with 0.5 ml $HClO_4$ and digested again till no colour remained. The sample was then diluted by adding 10 ml using de-ionized water and treated with prereductant NaI and HCl for 30 minute. Analysis was then carried out by HG-AAS. $NaBH_4$ solution along with HCl was passed over the air acetylene flame into quartz tube to atomize arsenic. The AAS was calibrated by using arsenic (V) standard solutions gone through the same procedures as the sample solution. Assay accuracy was ensured by inclusion of reference materials, NIES CRM No.18 (Human Urine, National Institute for Environmental Sciences, Tsukuba, Japan) with total arsenic of 0.137±0.011mg/L.The detection limit of the HG-AAS was 10µg/L for urine.

4.2.10 Hair sampling, preservation, transportation and analysis

4.2.10.1 Hair sampling, preservation and transportation

Hair samples of the subjects were collected from the areas close to the scalp by using a steel scissor. 20 to 25 hair shafts were collected from four different parts of the head, frontal, parietal, temporal, and back so that amount of the sample from each subject was about one gram. The collected hair shafts of an individual were stored in the sealed plastic bag that was properly labeled. There is no need of special consideration for the transportation of the hair samples.

4.2.10.2 Total arsenic analysis of hair

The hair sample was first washed with liquid soap and with de-ionized water. It was then washed with distilled water and finally with acetone. Then the sample was made dry. Then the washed and dried sample was weighed. A weighted amount of sample was placed in a beaker and treated with 10 ml conc. HNO_3 and kept overnight. It was then heated at low heat until dissolved. Few drops of hydrogen peroxide were added to remove color and the volume of acid was reduced to about 1 ml. It was then diluted to fixed volume (5-10ml). The solution was treated with NaI and HCl for 30 minutes. Then analysis was carried out by HG-AAS.

4.2.11 Scalp swab collection, transportation, and culture

4.2.11.1 Scalp swab collection and transportation

A commercially available sterile cotton swab was moistened with sterile water. Then the swab was vigorously rubbed and rotated over the area of scalp of the subject for about 15 seconds. The collected swab was placed inside the paper cover of the swab and stored in a plastic bag. The samples were transported from Parasi to CDM, Kirtipur within three days and cultured.

4.2.11.2 Swab culture

Scalp swab samples were inoculated on Sabouraud's glucose agar (SGA) plates (with 0.5% chloramphenicol but without cycloheximide) and incubated at 25-30ºC for upto 3 weeks. Then the colonial morphology and pigmentation of the fungal colonies were noted in every two-day for upto 2 weeks. Initial growth of dermatophytes from the SGA was subcultured onto corn meal agar (CMA) for better sporulation.

4.2.11.3 Identification of dermatophytes and other molds

Dermatophytes as well as other fungi were identified by i) examination of morphology by adhesive (scotch) tape preparation using lactophenol cotton blue and observing under the microscope for characteristic shape and arrangement of spores; ii) observation of colonial morphology and pigmentation of the fungal colonies; and iii) observation of growth rate of the colonies.

4.2.12 Statistical analysis

All data entries, descriptive analyses, cross tabulation, and statistical analyses were performed by using SPSS version 15. Associations between or among attributes were examined by Chi-square ($\chi 2$) test. Correlations between two continuous variables were calculated as Pearson correlation coefficient. Mean differences for continuous variables were measured by double mean test (t-test) and analysis of variance (ANOVA). Extent of association of different variables to arsenicosis was determined and modally fitted using binomial logistic regression.

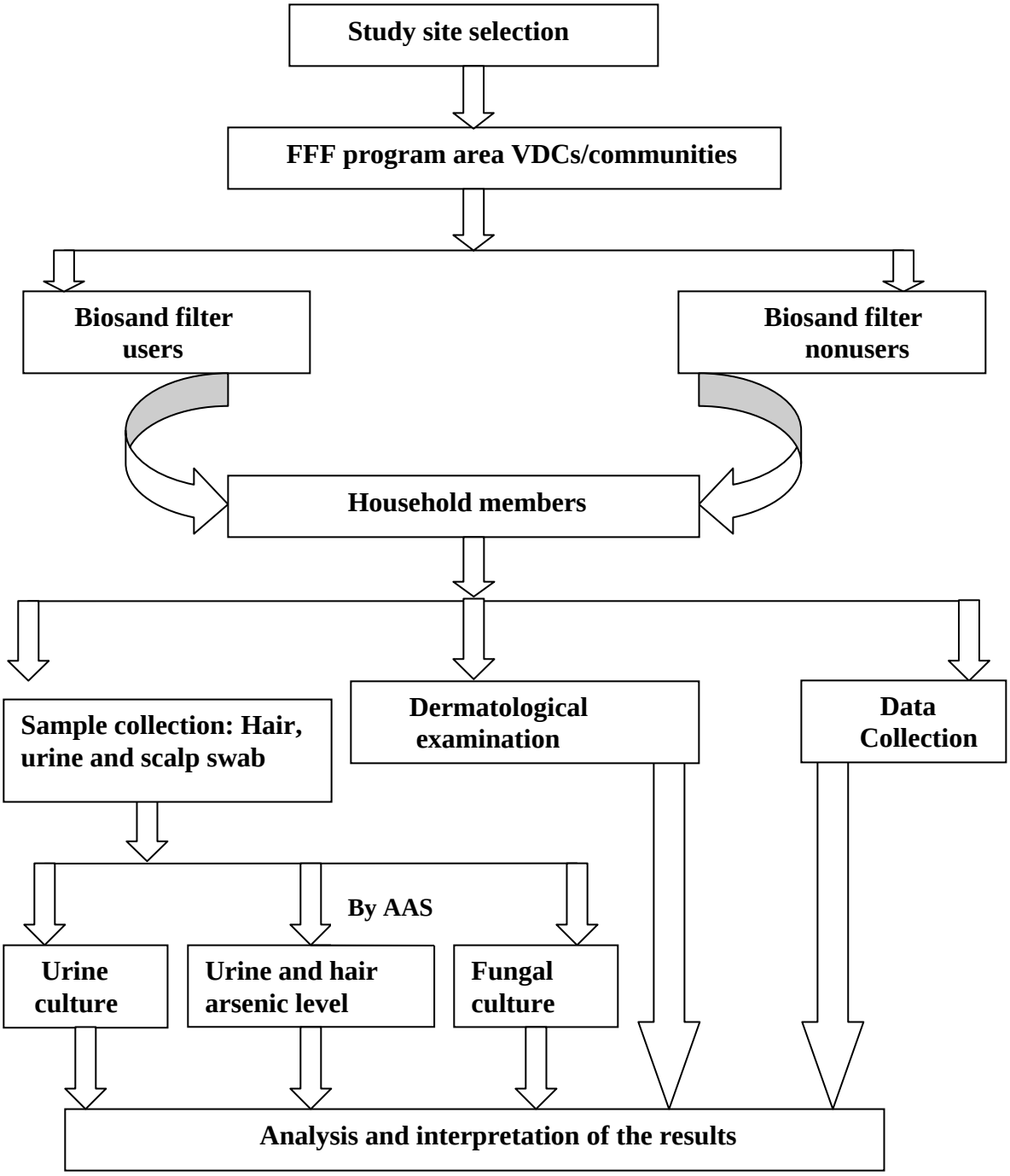


Figure I: Flow sheet of methodology

CHAPTER-V

5. Result

240 subjects were examined for arsenical dermatological manifestation. 116 (48.3%) were male and 124 (51.7%) were female. Among the total subjects, 30 (12.5%) had at least one type of arsenical dermatological manifestation. Gender difference in dermatological manifestation was significant at 0.01 level (p=0.01), male having nearly three times higher risk than female (OR=2.82, 95%, CI 1.23-6.41) considering the confounders such as sunlight exposure, and genetic difference in methylation of arsenic (Table 2).

Table 2 Gender difference in dermatological manifestation

	Dermatological manifestation			Significance	Odds ratio
Gender	Yes	No	Total	(p-value)*	(OR)
Male	21	95	116(48.3%)	0.01	2.82
Female	9	115	124(51.7%)		
Total	30(12.5%)	210(87.5%)	240(100%)		

**calculated by chi-square test*

The percentage of arsenicosis cases was highest (28.5%) in age group >60 years followed by 24.3% in age group 46-60 years and lowest (4.2%) in <15 years. There was significant difference in distribution of arsenicosis cases in different age groups at 0.01 level (p=0.01) (Table 3).

Table 3 Distribution of age in arsenicosis cases

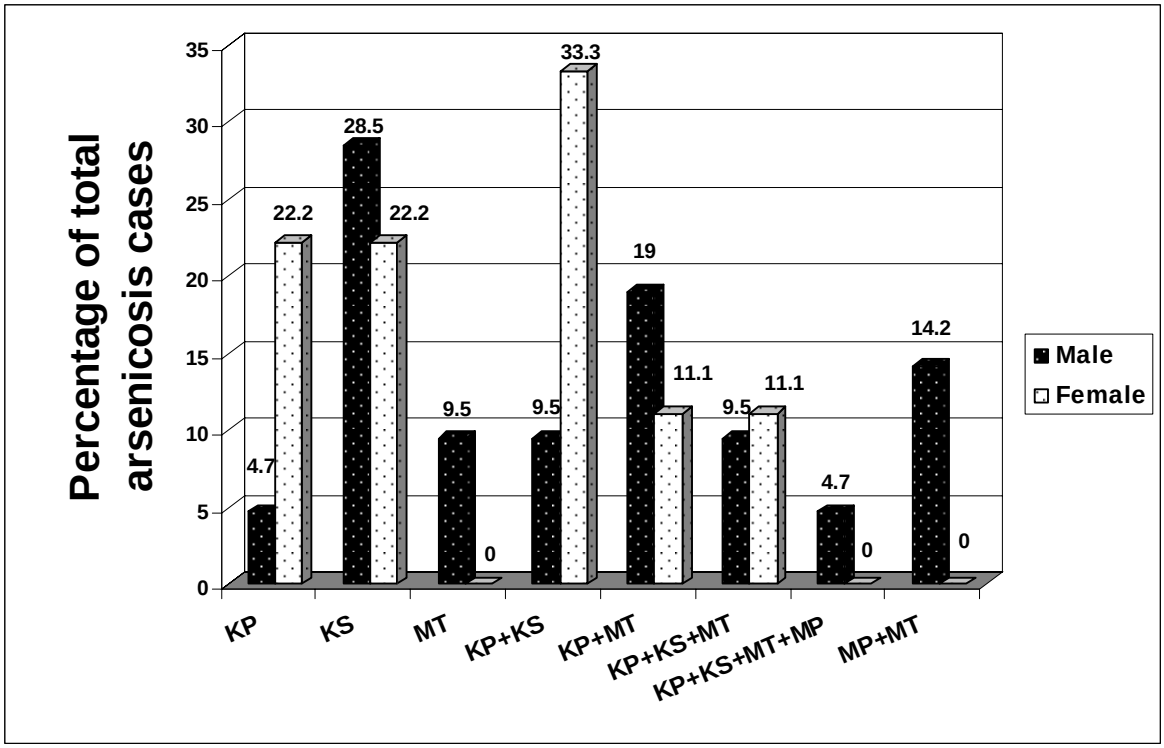
Age group	Male	Female	Total	Percentage	Significance (p-value)
<15	19(1%)	28(1%)	47(2%)	4.2%	0.01
15-30	42(4%)	35(2%)	77(6%)	7.8%	
31-45	25(5%)	36(3)	61(8%)	13.1%	
46-60	24(7%)	17(3%)	41(10%)	24.3%	
>60	6(4%)	8(0%)	14(4%)	28.5%	
Total	116(21%)	124(9%)	240(30%)	12.5%	

The percentage of arsenicosis cases was highest (32.2%) among the subjects who were drinking water from arsenic concentration group >400 µg/L, followed by 12.5% in 101-200µg/L As group. No arsenicosis cases were found from arsenic concentration group <50 µg/L. There was marginally significant difference in distribution of arsenicosis cases in different arsenic concentration groups ($0.05 > p < 0.1$) (Table 4).

Table 4 Distribution of arsenicosis cases and tube well arsenic concentration

As group (µg/L)	Male	Female	Total	Percentage of arsenicosis cases	Significance (p-value)
< 50	1(0%)	2(0%)	3(0%)	0%	0.053
50-100	31(4%)	38(1%)	69(5%)	7.2%	
101-200	11(1%)	5(1%)	16(2%)	12.5%	
201-300	22(3%)	27(1%)	49(4%)	8.1%	
300-400	15(2%)	16(0%)	31(2%)	6.4%	
>400	36(11%)	36(6%)	72(17%)	32.1%	
Total	116(21%)	124(9%)	240(30%)	12.5%	

Among 21 (70%) male arsenicosis cases, keratosis of sole (KS) was the most common dermatological manifestation (28.5%) followed by keratosis of palm and melanosis of trunk (KP+MT) (14.2%). Among 9 (30%) female arsenicosis cases, keratosis of palm plus keratosis of soles (KP+KS) was the most common (33.3%) followed by keratosis of soles (KS) and keratosis of palm (KP) 22.2% in each (Figure 2).



KP=Keratosis of palm, KS=keratosis of sole, MP=melanosis of palm, MT=melanosis of trunk

Figure 2: Distribution of dermatological manifestations of arsenicosis by gender

Of the total 30 arsenicosis cases, 21 (70%) had mild dermatological manifestation, 7 (23%) moderate and 2 (6.7%) severe (Table 5).

Table 5 Distribution of severity of dermatological manifestation

	Male	Female	Total (%)
Mild	15	6	21(70)
Moderate	4	3	7(23.3)
Severe	2	0	2(6.7)
Total	21	9	30(100)

Of the total 109 subjects, 73 (67%) had their urinary arsenic level exceeding the normal level (50 µg/L). The distribution of subjects within (≤50ppb) and exceeding the normal urinary arsenic level (>50ppb) was not statistically significant (p>0.05). Of total 117 subjects, 77 (65.8%) had their hair arsenic level exceeding the normal level (>1ppm). Similarly, the distribution of subjects within (≤1ppm) and exceeding the normal hair arsenic level (>1ppm) was not significant (p>0.05) (Table 6).

Table 6 Distribution of subjects who have normal or exceeding levels of arsenic in urine and hair

Urinary arsenic level in µg/L (or ppb)	Male	Female	Total (%)	Significance (p-value)
≤50	13	23	36 (31)	
>50	35	38	73 (67)	0.24
Total (%)	48 (44)	61 (56)	109 (100)	
Hair arsenic level in mg/kg (or ppm)	Male	Female	Total (%)	0.48
≤1	16	24	40 (34.2)	
>1	36	41	77 (65.8)	
Total (%)	52 (44.4)	65 (55.6)	117 (100)	

Among 109 individuals, 75 (68.8%) individuals had urinary arsenic level higher than 50µg/L without dermatological manifestation. Similarly, 55 (47%) individuals had hair arsenic level higher than 1ppm without dermatological manifestation (Table 7).

Table 7 Distribution of subjects by dermatological manifestation above the normal levels of arsenic in urine and hair

	Dermatological manifestation		
	Yes	No	Total subjects
Urinary As level: >50µg/L	16(14.6%)	75(68.8%)	109
Hair As level: >1mg/kg	22(18.8%)	55(47%)	117

The mean urinary arsenic level of males was higher (126.02 µg/L, n=48) than in females (96.83, n=61). However, this difference was not statistically significant (p=0.21). Similarly, the mean hair arsenic level of males was higher (2.348mg/kg, n=52) than of females (1.530mg/kg, n=65). This difference was statistically significant at 0.01 level (Table 8).

Table 8 Gender wise distribution of urinary and hair arsenic levels

		Urinary arsenic level (µg/mL)					Significance (p-value) *
Gender	N	Mean	SD	Median	Max.	Min.	
Male	48	126.02	137.7	69	663	24	
Female	61	96.83	91.82	65	583	13	0.21
		Hair arsenic level (mg/kg)					
Gender	N	Mean	SD	Median	Max.	Min.	
Male	52	2.348	2.199	1.580	9.433	0.02	0.01
Female	65	1.530	1.028	1.229	4.705	0.08	

** calculated by t test*

The mean urinary arsenic level of 25 arsenicosis cases of varying arsenic concentration in drinking water was 90.12 µg/L with standard deviation (SD) 73.20, median 63 µg/L and range 303 (max.-min.); and those of 30 randomly assigned non-arsenicosis cases was mean 105.46 µg/L with SD 92.31, median 65 µg/L and range 400 µg/L. However, there was no statistical significant difference in mean urinary arsenic level of these two groups (p=0.49). Similarly the mean hair arsenic level of 25 arsenicosis cases was 2.875 mg/kg with SD 2.439, median 2.291mg/kg, and range 9.183 mg/kg, and those of non-arsenicosis cases was 1.683 mg/kg with SD 1.485, median 1.204 mg/kg and range 6.927mg/kg. There was statistically significant difference in mean hair arsenic level of these groups at 0.05 level (p=0.03) (Table 9).

Table 9 Urinary and hair arsenic levels of arsenicosis cases and non-cases

	Urinary arsenic level (µg/L)						Significance (p-value)
	N	Max.	Min.	Mean	SD	Median	
Cases	25	333	30	90.12	73.20	63	
Non-cases	30	413	13	105.46	92.31	65	0.49
	Hair arsenic level (mg/kg)						
	N	Max.	Min.	Mean	SD	Median	
Cases	25	9.433	0.251	2.875	2.439	2.291	
Non-cases	30	7.192	0.265	1.683	1.485	1.204	0.03

Moderately positive correlation was obtained between urinary arsenic level and tube well arsenic level (r =0.27) significant at 0.01 level and between hair arsenic level and tube well arsenic level (r = 0.37) significant at 0.01 level. Similarly, a moderately positive correlation was also obtained between urinary arsenic level and hair arsenic level (r =0.26) significant at 0.01 level. There was almost no significant correlation between hair arsenic level and age of the subjects (r =0.01, p =0.87), however, there was weakly negative correlation between urinary arsenic level and age of the subjects (r =-0.18, p =0.061) (Table 10).

Table 10 Correlation coefficients (Pearson's) of different variables

Arsenic level	Tubewell arsenic level	Hair arsenic level	Age of subjects	Significance (p-value)
Urine	0.27			0.008
Hair	0.37			0.000
Urine		0.26		0.005
Hair			0.01	0.87
Urine			-0.18	0.061

Among the seven independent variables such as gender, age, BMI, exposure duration, urinary arsenic level, hair arsenic level and tube well arsenic level applied for potential significance to arsenicosis by binomial logistic regression, tubewell arsenic level, gender and exposure duration had the strongest association with arsenicosis with p-values 0.000, 0.008 and 0.037, respectively (Table11).

Table 11 Evaluation of different risk factors of arsenicosis

Variables	Significance
Gender	0.008
Age (45yrs[a])	0.28
BMI (18.5[b])	0.507
Exposure duration (10yrs[c])	0.037
Urinary Arsenic level (50ppb[d])	0.831
Hair Arsenic level (1ppm[e])	0.179
Tube well arsenic level (400ppb[f])	0.000

[a] Older *versus* younger age using 45 years as a cut point.
[b] cut off value for malnutrition (underweight).
[c] High *versus* low exposure duration using 10 years as a cut point.
[d] cut off value for normal urinary arsenic level.
[e] cut off value for normal hair arsenic level.
[f] High *versus* low arsenic level using 400ppb as a cut point.

Stepwise backward strategic method was used to build up the binomial logistic regression model. Seven independent categorical variables, gender, age, BMI, exposure duration, urinary arsenic level, hair arsenic level and tube well arsenic level were used. The outcome or dependent variable was dichotomous, dermatological arsenicosis symptom- yes and no. Final model following Parsimonious model included three variables viz. gender, age and tubewell arsenic level. The logistic regression models were obtained as: ln (P/1-P)=-3.669+0.981×gender, ln (P/1-P)=-3.669+1.549× age and ln (P/1-P)=-3.669+1.591 ×TwAs, respectively. The fitness of the variables was significant at 0.01 level and the strength of the relationship was 21.4% (R^2 value= 0.214) (Table 12).

Table 12 Binomial logistic regression model for arsenicosis

Variables	B	S.E.	Significance	Exp(B)	R^2 value
Gender	0.981	.444	0.027	2.67	0.214
Age (45years)	1.549	.452	0.001	4.7	
TwAs level (400ppb)	1.591	.442	0.000	4.9	
Constant (α)	-3.669	.414	0.000		

Model : ln (P/1-P)= α+βx
Equations:
1. ln (P/1-P)= -3.669+1.591 ×TwAs
2. ln (P/1-P)= -3.669+1.549× Age
3. ln (P/1-P)= -3.669+0.981×Gender

TwAs= Tubewell arsenic level, P= probability of getting arsenicosis symptom, 1-P= probability of not getting arsenicosis symptom, β=coefficient, x=dependent variable, α=constant

Of 118 urine samples cultured, 44 (37.2%) were positive for growth of uropathogens. Larger number of urine samples contained uropathogens in females (42.2%) than in males (30.7%). The highest positive growth (47%) was found in age group 31-45 years followed by 38.8% in age group 15-30 years (Table 13).

Table 13 Growth pattern of urinary bacteria

Age group	Male	Female	Total (%)
<14	2/5	1/4	3/9(33.3)
15-30	7/15	7/21	14/36(38.8)
31-45	3/12	13/22	16/34(47.0)
46-60	4/17	7/13	11/30(36.6)
60+	0/3	0/6	0/9(0)
Total (%)	16/52 (30.7)	28/66(42.2)	44/118(37.2)

Of total 46 urinary isolates, 9 (19.6%) were *Escherichia coli*, 8 (17.3%) were *Pseudomonas aeruginosa*, 7 (15.2%) were *Staphylococcus aureus* and *Staphylococcus saprophyticus* each, 4 (8.7%) were *Staphylococcus epidermidis* and *Acinetobacter* spp. each, and so on.

The antibiotics used during antibiotic susceptibility testing for Gram positive urinary isolates were amoxicillin, cotrimoxazole, ciprofloxacin, ceftriazone, norfloxacin, erythromycin, cloxacillin, cephalexin and vancomycin. Similarly, antibiotics used during the susceptibility testing for Gram negative urinary isolates were amoxycillin cotrimoxazole, ciprofloxacin, ceftriazone, norfloxacin, ofloxacin, nitrofurantoin, gentamycin and amikacin. The percentage of multi-drug resistant (MDR) (≥3 drug resistance), two drug resistant, one drug resistant and no drug resistant isolates were 30.4%, 37%, 30.4% and 2.2% respectively. *Acinetobacter* spp. and *Staphylococcus epidermidis* were the most drug resistant isolates, for each of them, 3 out of 4 isolates were MDR (Table 14).

Table 14 Drug resistance pattern of urinary isolates

Urinary isolates	No drug resistance	1 drug resistance	2 drug resistance	≥ 3 drug resistance	Total (%)
Escherichia coli	1	3	4	1	9(19.6)
Klebsiella pneumoniae	0	1	0	0	1(2.2)
Citrobacter fruendii	0	1	0	0	1(2.2)
Enterobacter aerogenes	0	0	2	0	2(4.3)
Staphylococcus aureus	0	4	1	2	7(15.2)
Staphylococcus saprophyticus	0	2	3	2	7(15.2)
Staphylococcus epidermidis	0	1	0	3	4(8.7)
Acinetobacter spp.	0	0	1	3	4(8.7)
Pseudomonas aeruginosa	0	2	3	3	8(17.3)
Enterococcus faecalis	0	0	3	0	3(6.6)
Total (%)	1(2.2)	14(30.4)	17(37.0)	14(30.4)	46(100)

Among 25 Gram negative urinary isolates, 96% (24/25) were susceptible to norfloxacin followed by ciprofloxacin to which 78% isolates were susceptible. 80% (20/25) of Gram negative isolates showed resistance to amoxicillin (Table 15).

Table 15 Antibiotic susceptibility profile of Gram negative urinary isolates

Antibiotics	Number	Susceptible (%)	Intermediate (%)	Resistant (%)
Amoxycillin	25	2(8)	3(12)	20(80)
Cotrimoxazole	25	8(32)	8(32)	9(36)
Ciprofloxacin	25	19(76)	4(16)	2(8)
Ceftriazone	25	8(32)	16(64)	1(4)
Norfloxacin	25	24(96)	0(0)	1(4)
Ofloxacin	18	12(66.6)	3(16.7)	3(16.7)
Nitrofurantoin	11	8(72.7)	3(27.3)	0(0)
Gentamycin	11	11 (100)	0(0)	0(0)
Amikacin	8	7(87.5)	1(12.5)	0(0)

Among 21 Gram positive urinary isolates, 76.1% (16/21) were susceptible to ciprofloxacin and Ceftriazone. 90.5% (19/21) of Gram positive urinary isolates were resistant to amoxycillin (Table 16).

Table 16 Antibiotic susceptibility profile of Gram positive urinary isolates

Antibiotics	Number	Susceptible (%)	Intermediate (%)	Resistant (%)
Amoxycillin	21	2(9.5)	0(0)	19(90.5)
Cotrimoxazole	21	10(47.6)	7(33.3)	4(19.1)
Ciprofloxacin	21	16(76.1)	3(14.2)	2(9.7)
Ceftriazone	21	16(76.1)	2(9.7)	3(14.2)
Norfloxacin	21	15(71.4)	4(19.1)	2(9.7)
Erythromycin	21	11(52.3)	5(23.8)	6(23.9)
Cloxacillin	21	5(23.8)	4(19.1)	12(57.1)
Cephalexin	21	11(52.3)	3(14.2)	7(33.5)
Vancomycin	2	2(100)	0(0)	0(0)

Minimum inhibitory concentration (MIC) determination of all 46 isolates for arsenic (V) and arsenic (III) tolerance was performed, and it showed that *P. aeruginosa* (8 isolates) had the highest tolerance to arsenic (V) with mean 725.71 ppm, median 840 ppm and standard deviation 215.93, and the mean tolerance to arsenic (III) was 7.89 ppm. Similarly, *K. pneumoniae* (1 isolate) had the highest tolerance to arsenic (III) with mean 25 ppm followed by *Acinetobacter* spp. (4 isolates) with mean 10.93 ppm and SD 3.12. The overall

(mean of the means) tolerance of all isolates to arsenic (V) and arsenic (III) were 509.67 ppm and 10.46 ppm respectively, with fold toxicity of 48.72 (Table 17).

Table 17 MIC of arsenic species to urinary isolates

Urinary isolates	N	As species	Mean	SD	Median	Min.	Max.	Fold toxicity
E. coli	9	As (V)	600	600	158.74	400	840	76.04
		As(III)	7.89	6.25	6.46	3.125	25	
P. aeruginosa	8	As (V)	725.71	840	215.93	360	920	87.12
		As(III)	8.33	6.25	3.22	3.125	12.5	
K. pneumoniae	1	As (V)	600	--	--	--	--	24.00
		As(III)	25	--	--	--	--	
Acinetobacter spp.	4	As (V)	320	320	138.56	200	440	29.27
		As(III)	10.93	12.5	3.12	6.25	12.5	
Citrobacter fruendii	1	As (V)	400	400	--	--	--	128.00
		As(III)	3.125	3.125	--	--	--	
Enterobacter aerogenes	2	As (V)	680	680	113.13	600	760	54.40
		As(III)	12.5	12.5	--	--	--	
S. aureus	7	As (V)	371.42	360	50.14	280	440	55.51
		As(III)	6.69	6.25	2.8	3.125	12.5	
S. epidermidis	4	As (V)	346.66	360	61.1	280	400	47.55
		As(III)	7.29	6.25	4.77	3.125	12.5	
S. saprophyticus	7	As (V)	480	480	110.27	360	600	38.40
		As(III)	12.5	12.5	6.84	6.25	25	
Enterococcus faecalis	3	As (V)	573	680	333.06	200	840	55.04
		As(III)	10.41	3.125	12.62	3.125	25	
Total	46	As (V)	509.67					48.72
		As(III)	10.46					

The mean arsenic tolerances of non MDR and MDR isolates to As (III) were 9.179 ppm and 8.928 ppm, respectively. This difference was not statistically significant (p=0.87). Similarly the mean arsenic tolerances of non MDR and MDR isolates to As (V) were 533.75 ppm and 458.57 ppm, respectively. This difference was also not significant (p=0.28) (Table 18).

Table 18 Tolerance of MDR and non MDR isolates to arsenic species

	As (III) tolerance						Significance (p-value)
	N	Mean	SD	Median	Min.	Max.	
Non MDR	32	9.179	6.778	6.25	3.125	25	0.87
MDR	14	8.928	3.848	9.37	3.125	12.5	
	As (V) tolerance						
Non MDR	32	533.75	201.82	520	200	920	0.28
MDR	14	458.57	216.89	420	100	920	

One way ANOVA analysis of tolerance to arsenic species by three dominant isolates, i.e. *E. coli*, *P. aeruginosa* and *S. saprophyticus* showed that there was significant mean difference among these three isolates for tolerance to As (V) species (p=0.03) but not to As (III) species (p=0.41). Post ad hoc analysis of significant variable, i.e. tolerance to As (V), showed significant mean difference in between S. *saprophyticus* and *P. aeruginosa* at 0.05 level (Table 19).

Table 19 ANOVA of predominant isolates to arsenic tolerance

		Mean square	Significance (p-value)
As (V) tolerance	between groups	97028.57	0.036
	within groups	24930.61	
As (III) tolerance	between groups	31.00	0.410
	within groups	33.32	
For As (V)tolerance (Post ad hoc test)			
(I) Grouping variables	(J) Grouping variables	Mean difference (I-J)	Significance (p-value)
S. saprophyticus	*P. aeruginosa*	-227.14	0.042
P. aeruginosa	*S. saprophyticus*	227.14	0.042

Moderately positive correlation (r=0.34) was obtained between urinary arsenic level and tolerance of urinary bacteria to As (V) which was significant at 0.05 level. Similarly, a moderately positive correlation (r=0.4) between urinary arsenic level and As (III) tolerance of urinary isolates was significant at 0.01 level. Correlation between tolerance of As (V) and As (III) of the isolates was weakly positive and insignificant (r=0.16, p=0.26) (Table 20).

Table 20 Correlation of arsenic tolerance of urinary isolates to urinary arsenic

	N	Tolerance to As (V)	Tolerance to As (III)	Significance (p-value)
Urinary arsenic level	46	0.34		0.020
Urinary arsenic level	46		0.40	0.006
Tolerance to As(V)	46		0.16	0.26

From 118 scalp cultures for fungi, 8 (6.7%) dermatophytes were isolated. Males had higher carrier rate (7.8%) than females (5.9%). The highest carrier rate (11.1%) was found in age

group 15-30 years followed by 10% in age group 46-60 years. The overall carriage rate was 6.7% (Table 21).

Table 21 Distribution of growth of dermatophytes

Age group	Male	Female	Total (%)
<14	0/4	0/5	0/9(0)
15-30	0/15	4/21	4/36(11.1)
31-45	1/12	0/22	1/34(2.9)
46-60	3/17	0/13	3/30(10)
>60	0/3	0/6	0/9(0)
Total (%)	4/51(7.8)	4/67(5.9)	8/118(6.7)

Of total 67 (56.7%) positive growth, 8 (12%) were dermatophytes and 59 (88%) were non-dermatophytes. Of total 8 isolates of dermatophytes, 2 (25%) were *E. floccosum, T. schonleinii*, and *T. tonsurans* each, and 1 (12.5%) was *M. cookie* and *M. nanum*, each. There was no statistically significant difference in growth of dermatophytes in two seasons-summer and winter (p=0.72). Among non-dermatophytes, *Aspergillus* spp. was the most common 28 (47.4%), followed by *Mucor* spp. 9 (15.2%). Seasonal difference of growth of non-dermatophytes was not statistically significant (p= 0.63) (Table 22).

Table 22 Fungal isolates and their pattern of growth

Fungus	N	Overall percentage	Summer (n=50)	Winter (n=68)	Significance (p-value)
Positive growth	67	56.7			
Dermatophytes					
E. flocossum	2(25%)	2.9	1	1	
M. nanum	1(12.5%)	1.45	0	1	
M. cookei	1(12.5%)	1.45	0	1	0.72
T. schonleinii	2(25%)	2.9	2	0	
T. tonsurans	2(25%)	2.9	1	1	
Total	8(100%)	11.9	4(8)	4(5.8)	
Non-dermatophytes					
Aspergillus spp.	28(47.4%)	41.7	15	13	
Alternaria spp.	6(10.1%)	8.9	3	3	
Penicillium spp.	5(8.4%)	7.4	3	2	
Rhizopus spp.	7(11.8%)	10.4	0	7	0.63
Mucor spp.	9(15.2%)	13.4	2	7	
Curvularia spp.	4(6.7%)	5.9	0	4	
Total	59(100%)	79.1	23(46)	36(53)	
No growth	51	43.3			

Note: four samples had co-growth of dermatophytes as well as non-dermatophytes.

CHAPTER- VI

6. Discussion and conclusion

6.1 Discussion

This study was carried to examine the effects of arsenic exposure by examining arsenic-related dermatological manifestations and status of arsenic exposure in risk population. In addition, the study extended to expand our understanding of the status of asymptomatic microbial infections in communities of Nawalparasi district.

Thirty participants (12.5%) among the total (240) had at least one type of arsenical dermatological manifestation. Gender difference in dermatological manifestation was significant (p=0.01), male having nearly three times higher risk than female (OR=2.82). This finding is concordant with findings of Maharjan (2004), and Watanabe *et al,* (2001). Watanabe *et al,* (2001) considered such gender difference as a fact of differential population coverage and higher cumulative exposure in male. However, in this study, there was nearly equal coverage rate between sexes (48.3% male and 51.7% female) and the approximate average exposure duration was only slightly higher in males than in females (male: 9.87 years; female: 8.77 years), considering the age of the current tube well used by the household. Other confounding factor, sunlight exposure has been found to be associated with dermatologic conditions such as melanosis and keratosis (Memon *et al.,* 2000). However, dermatological manifestations found in this study were common in least sunlight exposed areas of the body such as soles, palms and trunk rather than sunlight exposed areas of skin. So this gender difference cannot be explained by differential population coverage or concept of cumulative exposure and sunlight exposure. Gender difference in arsenic intake, genetic methylation and polymorphism, role of sex hormones etc. could be the most

plausible explanations for this gender difference in dermatological manifestation (Lindberg *et al.,* 2008; Yu *et al.*, 2000).

The burden of the disease was higher in older age group. The subjects of old age group, >60 years and 46-60 years had higher percentage of arsenicosis cases. This finding is similar to a large sample study reported by Maharjan *et al* (2006a) in Nawalparasi district where the highest prevalence of arsenicosis (32/143 i.e., 22.4%) was found in age group ≥65 years. Such a higher occurrence of arsenicosis in older age groups might be due to higher cumulative exposure, general health degradation due to aging and longer duration of subclinical stage of the disease to manifest as overt dermatological symptoms. Because the sample size for age group of 60 and over was very small compared to other groups a high percentage of 28.5% might be artifact. In contrary to the finding of this study, Maharjan (2004) reported higher prevalence in age group 30-39 and 40-49 years in a community based study and Rahman *et al.* (2006) from Bangladesh, in age group 30-44 years.

The burden of the disease was higher among population having higher tubewell arsenic level with the highest percentage (32.2%) among the subjects who were drinking water from arsenic concentration group >400 µg/L, followed by 12.5% in 101-200 µg/L As group. Similar to this finding, Guo *et al.*, (2006) found increased risk of getting arsenicosis especially melanosis with the increase of arsenic concentration in water. In this study, among the exposed population (>50ppb), there was no relative increase in the of arsenicosis cases with the tube well arsenic concentration increased. This may be due to unequal distribution of observed subjects in different tube well arsenic concentration or difference in individual susceptibility.

The most common dermatological manifestation in males and females were keratosis of sole (KS) and keratosis of palm and keratosis of soles (KP+KS), respectively. Maharjan *et*

al. (2006a) found most of cases with combined manifestations of pigmentation changes and keratosis in three communities of Nawalparasi district in agreement with findings of Rahman *et al* (2006) in Bangladesh. Melanosis and leucomelanosis appear in unexposed areas of the body like chest, back and especially in thighs. In this study observation of such body sites were limited because of participants' hesitation to examination. This may explain decreased finding of pigmentation especially leucomelanosis compared to others. However, melanosis was comparatively more common combined with keratosis especially in moderate and severe cases.

Dermatological symptoms were mostly mild as detected by Maharjan *et al.* (2006a). However, in this study, two severe cases were also detected and such finding indicates the aggravation of arsenicosis cases over time. There are also substantial differences in opinions concerning the diagnosis of dermatological manifestation from early to mild cases which might need laboratory support or special attention for confirmation or the evaluation of a professional dermatologist.

WHO has set normal urinary arsenic level at 50 μg/L and hair arsenic level at 1mg/kg. Urinary arsenic level is most commonly used as an indicator of recent arsenic exposure. Among 109 urine samples from all examined subjects, 73 (67%) had urine arsenic level exceeding the normal level. This implies that exposure is still a major problem in the study area despite the arsenic mitigation efforts. Males were slightly more exposed than females though they used the same tubewell. This indicates gender difference in arsenic intake or gender related quantitative differential arsenic excretion. Arsenic exposure indication by hair arsenic level showed that males had higher quantitative exposure than females. This may be due to their longer duration of exposure. Gender difference of urinary and hair arsenic level within normal and exceeding the level was not statistically significant.

There was very high number of study subjects, 75 (68.8%) who did not have arsenical dermatological manifestation yet had urinary arsenic level exceeding the normal level. Of the subjects whose hair arsenic levels were elevated above normal, 55 (47%) did not have dermatological manifestation. This indicates a significant recent as well as long duration arsenic exposure. Since symptoms of arsenicosis are often insidious and varied in nature and take usually 5-7 years to develop dermal manifestation, these subjects could be considered as subclinical affected. Most of them are likely to develop symptoms of arsenicosis and other arsenic-related diseases.

The mean urinary arsenic level of males was higher than females. However, this difference was not statistically significant. There was large inter-individual variation of urinary arsenic level among the individuals sharing the same tubewell as observed by Watanabe *et. al*. (2001). It has been reported that levels of MMA in urine has association with arsenical dermatological manifestation (Yu *et al*., 2000). Such gender difference in urinary arsenic levels may contribute to differential percentage of MMA and subsequent dermatological manifestation. The mean hair arsenic level of males was higher (2.348mg/kg, n=52) than female (1.530mg/kg, n=65). The amount of arsenic in hair is usually associated with the severity of disease (Hindmarsh, 1998). So it is presumable that males having more pronounced burden of the disease can have more amount of arsenic in their hair. While deriving such interpretation, however, higher arsenic level due to long duration exposure and external contamination should not be underemphasized.

Of all 30 subjects with dermatological manifestations only 25 gave urine sample whereas 79 subjects who did not have dermatological manifestation all gave urine sample. To maintain appropriate comparison, 30 subjects were selected by simple random sampling from 79 subjects and considered them as randomly assigned non-arsenicosis cases. The mean urinary arsenic level of arsenicosis cases (n=25) was lower (90.12 μg/L) than those

of randomly assigned non-arsenicosis cases (105.46 µg/L, n=30). This could be due to the result of general awareness of subjects to arsenic exposure and its health effects or due to the individual or exposure variation in them. However, the mean hair arsenic level of arsenicosis cases was higher (2.875 mg/kg) than those of non-arsenicosis cases (1.683 mg/kg) with significant statistical difference. This is concordant with the general notion that hair arsenic is good indicator of arsenic toxicity (Hindmarsh, 1998).

Moderately positive correlation was obtained between urinary arsenic level and tube well arsenic level (r=0.27) significant at 0.01 level. This finding is concordant with findings of Karagas *et al*., (2001), Meza *et al.* (2004) and RWSSSP (2004). Positive correlation indicates excretion of arsenic in urine as a function of exposure to arsenic in drinking water and proves predominant source of arsenic exposure in the study areas is through drinking water. A moderately positive correlation was also observed between hair arsenic level and tube well arsenic level (r=0.37) significant at 0.01 level, concordant to findings of Mosaferi *et al*. (2005) and Spallholz *et al*. (2005), indicating hair arsenic concentration can be used as proxy for chronic arsenic ingestion through water. Similarly, a moderately positive correlation was also obtained between urinary arsenic level and hair arsenic level (r =0.26) significant at 0.01 level indicating them as parallel biomarkers of arsenic exposure from drinking water. There was almost no correlation between hair arsenic level and age of the subjects (r=0.01, p=0.87). However, there was weakly negative correlation between urinary arsenic level and age of the subjects (r =-0.18, p=0.06) which testifies to the decrease in the functionality of the urinary system with the increase in the age and longer arsenic exposure (Pandey *et al*., 2007).

Like many other non-communicable diseases causation of arsenicosis is multi-factorial and evaluation of these causative factors becomes crucial for cure and treatment of the disease as well as priority grading for intervention programmes. Among the seven variables, gender, age, BMI, exposure duration, urinary arsenic level, hair arsenic level and tube well

arsenic level selected for potential significance to arsenicosis by binomial logistic regression, tubewell arsenic level, age of subjects and gender had comparatively higher significant association to arsenicosis (p-values 0.000, 0.001 and 0.027 respectively). Logistic model included three variables, tubewell arsenic level, age and gender deducted by backward stepwise strategic method following Parsimonious model. The fitness of the variables was significant at 0.01 level and the strength of the relationship was 21.4% (R^2 value= 0.214).

Of 118 urine samples cultured, 44 (37.2%) were positive for growth of uropathogens. In concordant to the findings of Colgan *et al*. (2006) and Nicolle (2003) asymptomatic bacteriuria was more common in females (42.2%) than in males (30.7%). Positive growth was found to increase from age group younger than 14 years to age group 31-45 years which followed the general conception of asymptomatic bacteriuria that its prevalence increases as age increases. In this study, since the number of elderly subjects was low, the lower positive growth in such group may not explain that such trend is really low.

Hooton *et al.* (2000) reported that in sexually active women the most important behavioral risk factors were the same as those identified for symptomatic bacteriuria that are sexual intercourse and use of spermicide. Less than 10 percent of episodes of asymptomatic bacteriuria progressed to symptomatic urinary tract infection in which the organism isolated when the women was asymptomatic was also isolated at the time of the symptomatic infection. There are some methodological discrepancies about urine culture for asymptomatic bacteriuria concerning the use of single culture or multiple (two or three) consecutive culture (Nicolle, 2000). Basing the diagnosis only on a single urine culture increases the observed prevalence of asymptomatic bacteriuria. Similarly transient bacteriuria is common, especially after intercourse. In this study since it was impossible to culture within 24 hours of midstream urine sample collection obtained from normal

community people, significant as well as insignificant growth of urinary bacteria in nonselective MacConkey agar was considered. This led to the increased percentage of positive growth and all growth positive subjects cannot be considered as diagnosed cases of asymptomatic bacteriuria.

Of total 46 urinary isolates *E. coli*, 9 (19.6%) was the most common followed by *P. aeruginosa*, 8 (17.3%) and 7 (15.2%) were *S. saprophyticus* and *S. aureus* each. There was no predominant growth of single isolate. Many hospital based studies report *E. coli* as predominant isolate from urine. Since this study is community based, the findings do not suffer from the biases arising from geographical variation of study subjects as observed in hospital based study. Raveh *et. al*. (2006) reported that *P. aeruginosa* and *Enterococcus* spp. were more associated with asymptomatic bacteriuria. This study is concordant to such finding.

The percentage of multi-drug resistant (MDR) (≥3 drug resistance), two drug resistant, one drug resistant and no drug resistant urinary isolates were 30.4%, 37%, 30.4% and 2.2% respectively. This shows that drug resistance is also emerging problem in community setting. Such drug resistance may be spontaneous or induced. Since history of antibiotic use by community people was not traced in this study, drug induced resistance could not be inferred. *P. aeruginosa* and *Acinetobacter* spp. have inherent drug resistance to many antibiotics (Forbes *et al*., 2002). *S. epidermidis* and *Acinetobacter* spp. were the most drug resistant isolates, for each of them, 3 out of 4 isolates were MDR.

Gram negative urinary isolates were *E. coli, K. pneumoniae, Citrobacter fruendii, Enterobacter aerogenes, P. aeruginosa* and *Acinetobacter* spp. Among 25 Gram negative urinary isolates, 96% (24/25) were susceptible to norfloxacin followed by ciprofloxacin to which 78% isolates were susceptible. 80% (20/25) of Gram negative isolates showed

resistance to amoxicillin. Gram positive urinary isolates were *S. aureus, S saprophyticus, S. epidermidis* and *Enterococcus faecalis*. Among 21 Gram positive urinary isolates, 76.1% (16/21) were susceptible to ciprofloxacin and Ceftriazone. 90.5% (19/21) of Gram positive urinary isolates were resistant to amoxycillin. Resistance to antibiotics by microorganisms is significantly determined by the environment in which they are present or the geographic region (Forbes *et al*., 2002; Gupta *et al*., 2001). Recommendations for empiric therapy for community acquired urinary tract infection hinge on knowledge of antimicrobial susceptibility patterns in the geographical region of the practitioner. This study shows that norfloxacin and ciprofloxacin for Gram negative urinary isolates and ceftriazone and ciprofloxacin for Gram positive isolates can be the best choice of antibiotics that may be practiced for empiric therapy to cure community acquired urinary tract infection in the study geographic areas. In compare to them, amoxycillin does not seem to be good choice for antibiotic prescription by clinical practitioners.

Minimum inhibitory concentration (MIC) determination by tube dilution of all 46 isolates for As (V) and As (III) tolerance was performed. There was variability in tolerance to arsenic species among urinary isolates. The variability was higher for As (V) than As (III). *P. aeruginosa* had the highest tolerance to As (V) with mean 725.71 ppm, median 840 ppm and standard deviation 215.93, and the mean tolerance to As (III) was 7.89 ppm. Similarly, *K. pneumoniae* (1 isolate) had the highest tolerance to As (III) with mean 25 ppm followed by *Acinetobactor* spp. (4 isolates) with mean 10.93 ppm and SD 3.12. The overall (mean of the means) tolerance of all isolates to As (V) and As (III) were 509.67 ppm and 10.46 ppm respectively. The crude fold toxicity for As (III) also differed for different species and mean fold toxicity for all isolates was 48.72ppm. Since the growth environment regarding arsenic species in urine differed for these urinary bacteria according to individual urinary arsenic level, their tolerance resulting from genetic expression of resistance genes may have varied. All the urinary isolates are facultative anaerobes and tolerance of resistance in

them is brought about by the expression of *ars* operon by which intracellular arsenic level is effluxed through trans-membrane protein (Cerventes, 1995).

It can be presumed that, in subjects continually excreting arsenic species in urine due to arsenic exposure from drinking water, urinary arsenic species may have produced selective pressure to urinary bacteria present transiently or persistently in urine which could result co-resistance. The mean arsenic tolerances of non MDR and MDR isolates to As (III) were 9.179 ppm and 8.928 ppm, respectively. This difference was not statistically significant ($p=0.87$). Similarly the mean arsenic tolerances of non MDR and MDR isolates to As (III) were 533.75 ppm and 458.57 ppm, respectively. This difference was also not significant ($p=0.28$). Thus it can be said that MDR in urinary isolates is not due to arsenic species present in the urine.

One way ANOVA analysis of tolerance to arsenic species was carried out to find whether urinary isolates (genera) differed in tolerance to arsenic species. For this, three dominant isolates, i.e. *E. coli*, *P. aeruginosa* and *S. saprophyticus* were chosen and it showed that there was significant mean difference among these three isolates for tolerance to As (V) species ($p=0.03$) but not to As (III) species ($p=0.41$). Post ad hoc analysis of significant variable, i.e. tolerance to As (V), significant mean difference was in between *S. saprophyticus* and *P. aeruginosa* ($p<0.05$). Such finding might indicate that these isolates 'ars B protein which pumps out As (III) form cytoplasm might have functioned equally irrespective of level of arsenic exposure. As (V) generally enter bacterial cells through phosphate transporter and it is converted intracellularly to As (III) by arsenate reductase (ars C protein). The differential tolerance As (V) among these isolates indicates the difference either in uptake of As (V) or in expression of *ars C* gene.

Moderately positive correlation was obtained between urinary arsenic level and tolerance of urinary bacteria to As (V) which was significant at 0.05 level. Similarly, a moderately positive correlation between urinary arsenic level and As (III) tolerance of urinary isolates was significant at 0.01 level. This indicates that arsenic tolerance among urinary isolates is a function of urinary arsenic level. Correlation between tolerance of As (V) and As (III) of the isolates was weakly positive but statistically insignificant ($r=0.16$, $p=0.26$). This is an indirect indication that resistance/tolerance to arsenic species in urinary isolates is a related mechanism.

Ilkit and Demirhindi, (2008) report that the prevalence of asymptomatic carriage differs from region to region with a rate of 0.1-49%. This study shows lower carriage rate (8/118, 6.7%) compared to finding of Chigozie *et al*. (2006) from children in Nigeria and Williams *et al*. (1995) from Black children in America. Most carriage has been observed in children especially among those between 4 and 8 years of age; however it has also been reported in adults (Ali-Shtayeh *et al*., 2002). In this study, specific age group like children were not selected rather tried to find out carriage rate in all age groups of the study subjects. So the carriage rate was low but was considerable. Age group <14 years had no dermatophyte positive culture. Since only 9 subjects were present in this category, this result seems artifact. Male had higher carriage rate (7.8%) than female (5.9%). The highest carriage rate (11.1%) was found in age group 15-30 years followed by 10% in age group 46-60 years. Such carriers are important from epidemiological point of view because they can act as source and reservoir of the tinea capitis especially for children in which the burden of the disease is high.

T. tonsurans, T. schonleinii and *E. floccosum* were the commonest (25%) dermatophytes isolated. Others were *M. cookie* and *M. nanum*, (12.5%). Anthropophilic dermatophytes, *T. tonsuran, T. schonleinii*, *E. floccosum* and *T. violaceum* have been found with high rates of

asymptomatic carriage in concordant to Ilkit and Demirhidi, (2008). Especially, *T. tonsurans* has been reported as a commonest isolate by Ilkit and Demirhidi, (2008), Friedlander *et al*., (1999) and Williams *et al*. (1995). *E. floccosum* is usually associated with ringworm infection of nails, however, its asymptomatic carriage and sporadic infection of scalp has been reported (Ali-Shtayeh *et al.,* 2002). Unlike the finding of Ali-Shtayeh *et al.,* (2002), there was no statistically significant difference in growth of dermatophytes in two seasons- summer and winter (p=0.72). In winter, due to higher humidity fungal growth is favored. Lower carriage rate in winter (5.8%) than summer (8%) may be due to lower sample size, unequal observation of subjects and community variation in dermatophyte carriage.

Nearly 80% of fungal isolate from scalp swab culture were non-dermatophytes (molds). This growth was due to the fact that anti-mold agent, cycloheximide, was not incorporated in primary screening medium- Sabouraud's agar. *Aspergillus* spp. was the commonest 28 (47.4%), followed by *Mucor* spp. 9 (15.2%). These molds are predominant in environment. Such a high recovery rate in scalp indicates their environmental contamination especially through agricultural practices and infrequent hair washing. Mold carriage rate was slightly higher in winter (53%) than in summer (46%) and this can be attributed to higher humidity in winter which favors fungal growth and less frequent hair-washing due to cold weather condition.

6.2 Conclusion

Arsenicosis is multifactorial and association of each factor with the disease varies in strength and specificity. Tube well arsenic level, age and gender have the strongest association with the disease. Exposure to arsenic and subsequent burden of the disease is still prevailing in highly arsenic contaminated communities of Nawalparasi district. So it can be concluded that only mitigation practices cannot solve these problems. Considering

correlation analyses, urinary and hair arsenic level can act as proxy for recent and chronic arsenic ingestion through drinking water. From arsenic level analysis, it can be concluded that hair arsenic level is better biomarker for case definition and urinary arsenic level the better exposure indicator.

Asymptomatic bacteriuria is more prevalent in reproductive age and in female. Multidrug resistance raises a challenge even in the community setting; however, such drug resistance is not imparted by arsenic in urine. Urinary isolates vary in great extent in tolerance to arsenic species and arsenic (III) has much higher toxicity than arsenic (V). Such tolerance is significantly correlated with arsenic content in urine. Carriage of dermatophytes in scalp of adolescent and older age group is a significant consideration and anthrophilic dermatophytes are more common isolates in scalp.

CHAPTER- VII

7. Summary and recommendation

7.1 Summary

This study was carried to examine the effects of arsenic exposure by examining arsenic-related dermatological manifestations and status of arsenic exposure in risk population. In addition, the study extended to expand our understanding of the status of asymptomatic microbial infections in communities of Nawalparasi district.

This study included on field data collection and arsenical dermatologic examination of the subjects, urine, hair and scalp swab sample collection of two subjects (one male and another female) from a household. Urinary bacteria were isolated and identified, and their antibiotic susceptibility testing as well as arsenic tolerance test was done at the laboratory of Central Department of Microbiology, Kirtipur. Dermatophytes (as well as molds) were isolated and identified. Total arsenic analysis of hair and urine was done by Atomic Absorption Spectrophotometer with Hydride Generation at the laboratory of Environment and Public Health Organization, Kathmandu.

- Of total 240 participants of the dermatological examination indicative of arsenicosis, 116 (48.3%) were males and 124 (51.7%) were females. 30 (12.5%) among the total participants had at least one type of arsenical dermatological manifestation. Gender difference in dermatological manifestation was significant at 0.01 level ($p=0.01$), male having nearly three times higher risk than female (OR=2.82, 95% CI 1.23-6.41).

- The percentage of arsenicosis cases was higher in older age groups, 28.5% in age group >60 years followed by 24.3% in age group 46-60 years and lowest (4.2%) in childish age group <15 years. There was significant difference in distribution of arsenicosis cases in different age groups at 0.01 level (p=0.01).

- The percentage of arsenicosis cases was highest (32.2%) among the subjects who were drinking water from As concentration group >400 µg/L, followed by 12.5% in 101-200µg/L As group. No arsenicosis cases were found from As concentration group <50 µg/L. There was marginally significant difference in distribution of arsenicosis cases in different As concentration groups ($0.05 > p < 0.1$)

- Among 21 (70%) male arsenicosis cases, keratosis of sole (KS) was the most common dermatological manifestation (28.5%) followed by keratosis of palm and melanosis of trunk (KP+MT) (14.2%). Among 9 (30%) female arsenicosis cases, keratosis of palm and keratosis of soles (KP+KS) was the most common (33.3%) followed by keratosis of soles (KS) and keratosis of palm (KP) 22.2% in each. Of total 30 arsenicosis cases, 21 (70%) had mild dermatological manifestation, 7 (23%) moderate and 2 (6.7%) severe.

- Of total 109 subjects, 73 (67%) had their urinary arsenic level exceeding the normal level (50 µg/L). The distribution of subjects within and exceeding the normal urinary arsenic level was not statistically significant (p=0.24). Of total 117 subjects, 77 (65.8%) had their hair arsenic level exceeding the normal level (1mg/kg). Similarly, the distribution of subjects within and exceeding the normal hair arsenic level was not significant (p=0.48).

- Among 109 individuals, 75 (68.8%) individuals had urinary As level >50μg/L without dermatological manifestation. Similarly 55 (47%) individuals had hair As level >1ppm without dermatological manifestation.

- The mean urinary arsenic level of male was higher (126.02 μg/L, n=48) than female (96.83, n=61). However, this difference was not statistically significant (p=0.21). Similarly, the mean hair arsenic level of male was higher (2.348mg/kg, n=52) than female (1.530mg/kg, n=65). This difference was statistically significant at 0.01 level.

- The mean urinary arsenic level of 25 arsenicosis cases was 90.12 μg/L with standard deviation (SD) 73.20, median 63 μg/L and range 303 (Max.-Min.); and those of 30 randomly assigned non-arsenicosis cases was mean 105.46 μg/L with SD 92.31, median 65 μg/L and range 400 μg/L. However, there was no statistical significant difference in mean urinary arsenic level of these two groups (p=0.49). Similarly the mean hair arsenic level of 25 arsenicosis cases was 2.875 mg/kg with SD 2.439, median 2.291mg/kg, and range 9.183 mg/kg; and those of non-arsenicosis cases was 1.683 mg/kg with SD 1.485, median 1.204 mg/kg and range 6.927mg/kg. There was statistically significant difference in mean hair arsenic level of these groups at 0.05 level (p=0.03).

- Moderately positive correlation was obtained between urinary arsenic level and tube well arsenic level (r=0.27) significant at 0.01 level and between hair arsenic level and tube well arsenic level (r=0.37) significant at 0.01 level. Similarly, a moderately positive correlation was also obtained between urinary arsenic level and hair arsenic level (r =0.26) significant at 0.01 level. There was almost no significant correlation between hair arsenic level and age of the subjects (r=0.01,

p=0.87), however, there was weakly negative correlation between urinary arsenic level and age of the subjects (r =-0.18, p =0.061).

- Among the seven variables, gender, age, BMI, exposure duration, urinary arsenic level, hair arsenic level and tube well arsenic level selected for potential significance to arsenicosis by binomial logistic regression, tubewell arsenic level, age and gender had the strongest association with arsenicosis with p-values 0.000, 0.001 and 0.027, respectively.

- Stepwise strategic method built up the binomial logistic regression model with three variables- gender, age and tubewell arsenic level. Their models were obtained as: ln (P/1-P) = -3.669+0.981×gender, ln (P/1-P) = -3.669+1.549 × age and ln (P/1-P) = -3.669+1.591 ×TwAs, respectively. The fitness of the variables was significant at 0.05 level and the strength of the relationship was 21.4% (R^2 value= 0.21.4).

- Of 118 urine samples cultured, 44 (37.2%) were positive for growth of uropathogens. Larger number of urine samples contained uropathogens in females (42.2%) than in males (30.7%). The highest positive growth (47%) was found in age group 31-45 years followed by 38.8% in age group 15-30 years.

- Of total 46 urinary isolates, 9 (19.6%) were *E. coli*, 8 (17.3%) were *P. aeruginosa*, 7 (15.2%) were *S. aureus* and *S. saprophyticus* each, 4 (8.7%) were *S. epidermidis* and *Acinetobacter spp.* each, and so on. The percentage of multi-drug resistant (MDR) (≥3 drug resistance), two drug resistant, one drug resistant and no drug resistant isolates were 30.4%, 37%, 30.4% and 2.2% respectively. *Acinetobacter*

spp. and *S. epidermidis* were highly drug resistant, for each bacterium, 3 out of 4 isolates were MDR.

- Among 25 Gram negative urinary isolates, 96% (24/25) were susceptible to norfloxacin followed by ciprofloxacin to which 78% isolates were susceptible. 80% (20/25) of Gram negative isolates showed resistance to amoxicillin.

- Among 21 Gram positive urinary isolates, 76.1% (16/21) were susceptible to ciprofloxacin and Ceftriazone. 90.5% (19/21) of Gram positive urinary isolates were resistant to amoxycillin.

- *P. aeruginosa* (8 isolates) had the highest tolerance to As (V) with mean 725.71 ppm, median 840 ppm and standard deviation 215.93, and the mean tolerance to As (III) was 7.89 ppm. Similarly, *K. pneumoniae* (1 isolate) had the highest tolerance to As (III) with mean 25 ppm followed by *Acinetobactor* spp. (4 isolates) with mean 10.93 ppm and SD 3.12. The overall (mean of the means) tolerance of all isolates to As (V) and As (III) were 509.67 ppm and 10.46 ppm respectively, with fold toxicity of 48.72.

- The mean arsenic tolerances of non MDR and MDR isolates to As (III) were 9.179 ppm and 8.928 ppm, respectively. This difference was not statistically significant ($p=0.87$). Similarly the mean arsenic tolerances of non MDR and MDR isolates to As (III) were 533.75 ppm and 458.57 ppm, respectively. This difference was also not significant ($p=0.28$).

- *E. coli*, *P. aeruginosa* and *S. saprophyticus* showed that there was significant mean difference among these three isolates for tolerance to As (V) species (p=0.03) but not to As (III) species (p=0.41). Significant mean difference was in between *S. saprophyticus* and *P. aeruginosa*.

- Moderately positive correlation (r=0.34) was obtained between urinary arsenic level and tolerance of urinary bacteria to As (V) significant at 0.05 level. Similarly, a moderately positive correlation (r=0.40) between urinary arsenic level and As (III) tolerance of urinary isolates was significant at 0.01 level. Correlation between tolerance of As (V) and As (III) of the isolates was weakly positive and insignificant (r=0.16, p=0.26).

- From 118 scalp culture for fungi, 8 (6.7%) dermatophytes were isolated. Male had higher carriage rate (7.8%) than female (5.9%). The highest carriage rate (11.1%) was found in age group 15-30 years followed by 10% in age group 46-60 years. The overall carriage rate was 6.7%.

- Of total 67 (56.7%) positive growth, 8 (12%) were dermatophytes and 59 (88%) were non-dermatophytes. Of total 8 isolates of dermatophytes, 2 (25%) were *E. floccosum, T. schonleinii*, and *T. tonsurans* each, and 1 (12.5%) was *M. cookie* and *M. nanum*, each. There was no statistically significant difference in growth of dermatophytes in two seasons- summer and winter (p=0.72). Among non-dermatophytes, *Aspergillus* spp. was the most common 28 (47.4%), followed by *Mucor* spp. 9 (15.2%) and so on. Seasonal difference of growth of non-dermatophytes was not statistically significant (p= 0.63).

7.2 Recommendations

- Since arsenic exposure still exists in the communities of Nawalparasi district, it is important to make people aware of it and provide an effective mitigation measures or arsenic free safe options with regular monitoring.
- Case management and treatment strategies for the arsenicosis patients are highly recommended.
- Priority grading system considering various risk factors for arsenicosis should be developed in any kind of intervention program related to arsenic exposure and its health effects.
- Systematic dose response study considering various factors such as water intake, nutritional status, associated infection, and genetic factors should be carried out.
- Collaborative research work with national or international research agencies is highly recommended for the search of better biomarker diagnostic of arsenic related toxicity and carcinogenesis.
- Community based study should be carried out for screening the antibiotic resistance in uropathogenic bacteria prevailing in the specific geographic area.
- Co-resistance study of urinary bacteria to arsenic in the urine and antibiotics should be done on molecular basis.
- Study relating to modification to arsenic species by persistent growth of urinary bacteria in urine and its effects on urinary bladder should be carried out.
- In Nepal, systematic community based study to reveal the prevalence of asymptomatic dermatophyte carriage in general population as well as in specific age group, e.g. children need to be carried out.

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