CHAPTER-I

1. INTRODUCTION

The word water is associated with life and soul, as well as gentleness and quietness. It is a precious gift of nature as it is the basis of life. Without water, life on earth cannot be sustained. The whole world is facing a water crisis and it is increasingly clear that safe water is connected to economic development. Arsenic contamination in drinking water is an epidemic not only in Nepal but also of global concern. Nepal is grappling with the mass poisoning of at least 2.2 million people in the Terai. Groundwater is used for drinking water and is contaminated with naturally occurring inorganic arsenic. Over 90% of the Terai population draws groundwater from tubewells for drinking, household use, and irrigation. Improved health however, is impossible to achieve without adequate water supplies, safe drinking water and basic sanitation. So, clean, protected and safe water is an absolute need for a healthy productive life.

The exploitation of groundwater to replace the use of surface water was instrumental in reducing the incidence of water born diseases, and made an important contribution to the improvement of public health in many developing countries. However, the improvement in water quality has been negatively affected by the presence of arsenic in groundwater, which is now widely recognized as a threat to public health in many countries in South and South East Asia. Although the primary and more immediate threat to public health in most areas undoubtedly remains diarrhoeal disease caused by poor microbiological water quality, poor hygiene and lack of adequate sanitation, in the medium to long term exposure to arsenic must be addressed to avoid chronic health effects in exposed populations (World Bank, 2005).

Water, although an absolute necessity for life can be a carrier of many water borne diseases such as typhoid, cholera, hepatitis, dysentery and other diarrhoeal related diseases. UNICEF estimates that over 80% of the world diseases are water borne.

Children bear the greatest health burden associated with poor water and sanitation. Diarrhoeal diseases attributed to poor water supply, sanitation and hygiene account for 1.73 million deaths each year and contribute over 54 million disability adjusted life years, a total equivalent to 3.7% of the global burden of disease. This place diarrhoeal disease due to unsafe water, sanitation and hygiene as the 6th highest burden of disease on a global scale, a health burden that is largely preventable (WHO, 2002). An outbreak of waterborne epidemic in Nepal is not uncommon especially in rainy season. Each and every summer, water borne epidemics hit different parts of the country including Nawalparasi district, the study area. Annual report from DoHS (2004/2005) showed that there were 3,952 cases of typhoid, 13,971 cases of diarrhoeal diseases, 7,384 cases of intestinal worms, 595 cases of jaundice and infectious hepatitis in Nawalparasi district.

Besides these water borne diseases, the chemical quality of water is generally a far lower priority, as the majority of the impact of poor chemical quality is chronic rather than acute. However, there are some exceptions to this and some chemicals, notably iron, nitrate and recently arsenic are often included in routine monitoring of water supplies (Speets, 2001). With the identification of massive arsenic toxicity caused by consumption of arsenic-contaminated tubewell water in Bangladesh and in West Bengal, arsenic contamination was identified in Nepal on 1999; in the Terai region (Maharjan *et al.*, 2006).

In the field area for this study, ENPHO and NRCS study team observed arsenicosis cases and tested the tubewell water for arsenic from Kunwar and Gohini village. Very high proportions of tube wells in both villages were contaminated with arsenic with values more than 500 ppb. For example in Kunwar 14 out of 16 tubewells were found to have high arsenic content (>50 ppb) (Shrestha, 2003). More than 20% of the adult population in Kunwar village and more than 8% in Sarawal village (Gohini) had arsenicosis related to dermatosis some with advanced clinical stages (Maharjan *et al.*, 2005). A community based study revealed overall prevalence of arsenicosis was 6.9% among part of the population older than 15 years (Ahmad *et al.*, 2004; Maharjan, 2004). The prevalence

rate of arsenic contamination (greater than 50 ppb) in Nawalparasi district was found to be 25.7% (Maharjan *et al.*, 2006).

Arsenic is toxic to bacteria, as well as to other domains of life. Arsenic uptake by bacteria is mediated by phosphate transporters and is generally pumped back out of the cell by an efflux pump (Nies and Silver, 1995). The heavy metal resistant microorganisms, bacteria in particular, are known to employ a variety of mechanisms for adaptation to the presence of toxic heavy metals. Among them metal sorption, mineralization, uptake and accumulation, extra cellular precipitation and enzymatic oxidation or reduction to less toxic form and efflux of heavy metals from the cell have been reported (Mergeay, 1991; Nies, 1999; Urrutia and Beveridge, 1993).

Plasmids confer extra chromosomal drug resistance. R factors are a class of plasmids that contain genes that code for drug resistance against one and often several antimicrobial drugs (Brooks *et al.*, 2004; Chakraborty, 2003; Forbes *et al*., 2002). Heavy metal tolerance in the environment is that it may contribute to the maintenance of antibiotic resistances genes by increasing the selective pressure of the environment. Many have shown that a correlation exists between metal tolerance and antibiotic resistance in bacteria because of the likelihood that resistance genes to both (antibiotics and heavy metals) may be located closely together on the same plasmid in bacteria and are thus more likely to be transferred together in the environment. Arsenic resistance in bacteria especially heterotrophic bacteria is mostly plasmid mediated and plasmid profiling of such bacteria helps to detect presence or absence of such resistance (Spain and Alm, 2003).

In Nepal, water borne epidemics are regular phenomenon due to poor water quality and sanitation facilities where the poor and marginalized people are mostly affected. The emergence of arsenic contamination in drinking water is known to result in adverse health outcomes causing serious consequences in terms of morbidity and mortality. This study reveals the status of groundwater quality in Nawalparasi District and is believed to

be helpful in taking necessary steps for its improvement. This study also aims to isolate and identify arsenic tolerant bacteria from arsenic contaminated groundwater and their plasmid profiling. There is growing evidence that microorganisms can be used as a cheap alternative to more costly physical and chemical remediation of arsenic contamination. These tolerant organisms can then be used for the study of their potential in bioaccumulation and bioremediation of heavy metal contamination, as well as transformation and mobilization of arsenic in groundwater. The drinking water supply in most of the rural areas and municipalities of Nepal is usually inadequate in terms of overall coverage, quantity of water and of course poor water quality. Besides this, improperly disposed sewage near water source contaminates groundwater by pathogenic and non-pathogenic bacteria. Therefore, drinking water quality assessment has always been crucial with reference to public health importance. Plasmid profiling being an epidemiological marker can be used to study the arsenic resistance mechanism in arsenic contaminated groundwater. Consequently, the outcome of this study may be useful for researchers, health planners and concerned people.

CHAPTER-II

2 OBJECTIVES

2.1 General Objective

To assess arsenic tolerant bacteria from arsenic contaminated groundwater in Nawalparasi district.

2.2 Specific Objectives

- To isolate and identify the arsenic tolerant bacteria in arsenic contaminated groundwater from Nawalparasi district.
- To determine arsenic concentration from groundwater and effluent water from Kanchan arsenic filter.
- To identify coliform and pathogenic bacteria in arsenic contaminated groundwater.
- To assess minimum inhibitory concentration of isolated organisms in arsenate and arsenite.
- To study relationship between arsenic concentration of influent water with age and depth of the tubewell.
- To study health effects due to chronic arsenic ingestion among the arsenicosis patients.
- To perform plasmid profiling of arsenic tolerant and arsenic sensitive *E. coli*.

CHAPTER-III

3. LITERATURE REVIEW

3.1 Arsenic, its sources and occurrences in the environment

Arsenic is a metalloid which is widely distributed throughout the Earths crust, most often as arsenic sulfide or as metal arsenates and arsenites found in rocks, soil, natural waters, and organisms. Being a transitional reactive element, it forms chemical and organic complexes together with other metals including iron, carbon, sulphur and oxygen. Dominant natural arsenic bearing rocks include realgar (AsS), orpiment (As_2S_3), lollingite ($FeAs_2$), and arsenopyrite (FeAsS), which are the most common arsenic ore mineral, formed primarily under high temperature conditions in the earth's crust. Arsenic concentrations are generally low in igneous rocks and higher in metamorphic and some sedimentary rocks. Soil that contains an average of 4-6 mg/kg of arsenic and arsenate [As (V)], the oxidized form of arsenic, is common in soil situated above the water table. Arsenite [As (III)] is generally found in soil and sediment below the water table where ground water is in a reduced state (NASC/UNICEF, 2005).

Processes of arsenic mobilization from its source to groundwater are either natural or anthropogenic. By natural process, arsenic is released in groundwater as soluble form by three mechanisms:

a) Oxidation of arsenopyrites or pyrites

These substances laid down into the subsoil strata over the centuries by rivers get oxidized to soluble form due to air leaks from standpipes or wells contaminating water source. Similarly aeration of any aquifer containing pyrites releases arsenic as soluble form (DWQIP, 2003).

b) Reduction of oxy-hydroxide

In alluvial sediments, arsenic is assumed to be present with high concentrations in grains as a coating of iron or manganese oxy-hydroxides. The organic matter deposited with the sediments reduces the arsenic-bearing oxy-hydroxides and releases arsenic into groundwater (DWQIP, 2003; Fazal *et al*., 2001). Under the same reducing environment, dissimilatory arsenic respiring microorganisms such as *Chrysiogenes* spp., *Bacillus* spp., *Desulfomicrobium* spp., *Citrobacter* spp., *Sulforihydrogenibium* spp. etc can reduce arsenic (V) to potentially more mobile and hazardous arsenic (III) causing its release into groundwater (Lear *et al*., 2007).

c) Desorption of arsenic by phosphate

Phosphate concentration increment from fertilizers could endorse desorption of arsenic from iron and manganese-oxides or hydroxides in the form of arsenate (AsO_4) which boost up the arsenic concentration in groundwater (DWQIP, 2003).

Groundwater arsenic contamination also increases from the anthropogenic sources. The application of arsenical pesticides on land, industrial wastewater discharge, mine trailing, landfill leaching, leaches of arsenic chemical used in manufacturing of wood preservatives and paints may elevate concentration of arsenic in groundwater and soil (RWSSSP, 2004).

In Nepal, the aquifers with arsenic contamination are found in sediments derived from Siwalik source rock. Statistical GIS analysis indicates correlation of both average and maximum arsenic concentration in shallow groundwater from tubewells in alluvial fans of the Siwalik source. Areas without Siwalik sediments arsenic contamination is rare, despite the presence of secondary ferric oxy-hydroxides and detrital pyrite containing arsenic (Williams *et al*., 2005).

3.2 History of guideline development

The first version of international standards for drinking water included arsenic in the category of toxic substances and established 200 ppb as the allowable concentration in drinking water (WHO, 1958). In updated standards of 1963, WHO lowered the allowable concentration to 50 ppb (WHO, 1963). The WHO continued its review work to lower the guideline value for arsenic in drinking water by establishing a guideline value (provisional) of 10 ppb in 1993 (WHO 1993). This provisional Guideline Value of 10 ppb has been adopted as the national standards for drinking water by a number of countries based on concern regarding its carcinogenicity in humans. However many developing countries have retained the previous WHO guideline value of 50 ppb as their national standard (Panthi *et al*., 2006).

3.3 Global scenario of arsenic poisoning of groundwater

Arsenic poisoning of groundwater used for drinking and irrigation is a global issue, as it provides 25 to 40 percent of the world's drinking water. Arsenic poisoning with the risk of harmful human exposure occurs at numerous locations across the Americas, Asia (most notably West Bengal and Bangladesh) and also central Europe. Recent studies have reported that arsenic-rich minerals prevalent in the Himalayas and their gradual transport and deposition in the alluvial deltas below, followed by microbially mediated arsenic solubilization, are the major mechanism of arsenic mobilization into aquifers within the region. Similar conditions for the development of arsenic enriched groundwater are present within the Red River and Mackong River deltas of Southeast Asia where elevated concentrations of arsenic have also recently been reported (Lear *et al*., 2007).

Arsenic pollution has been reported recently in the USA, China, Chile, Bangladesh, Taiwan, Mexico, Argentina, Poland, Canada, Hungary, New Zealand, Japan and India. The largest population at risk among the 21 countries with known groundwater arsenic contamination is in Bangladesh, followed by West Bengal in India.

3.4 National scenario of arsenic poisoning of groundwater

Ground water accessed via tube wells is the major source of water in the Terai Region of Nepal. The total area of the Terai is 33,401 sq. km, which is 23% of the total area of the country. Approximately 48% (13 million) of Nepal's total population is living in Terai region and 90% of them are relying on groundwater as their major source of drinking water. Until March 2007, 646,834 tubewells have been tested and found that 10.4% water samples exceeded the WHO guideline value of 10 ppb while 2.3% samples exceeded even Nepal standard of 50 ppb. The percentage of all tubewells exceeding 50 ppb varies from 0.9% of the wells in Saptari to 11.7% in Nawalparasi. Similarly, the percentage of tubewells exceeding the WHO guideline ranges from 5.4% in Saptari to 23.8% in Nawalparasi District. Out of 652 tested VDCs/Municipalities, the majority (approx. 72%) are moderately vulnerable and 39 (approx. 6%) are highly vulnerable to arsenic contamination in groundwater. Nearly 1% of the population in severely affected areas has showed symptoms of arsenic poisoning (NASC/UNICEF, 2005).

Until 1970s most rural people of Terai region of Nepal obtained and consumed water from dug-wells, rivers and ponds. These waters were consumed directly without any treatment. Because they were contaminated, epidemics of cholera, typhoid and other water borne diseases were very common in this region. To alleviate this problem, agencies and individuals installed a considerable number of shallow tubewells. This succeeded in reducing the number of death from water borne diseases. However it unfortunately contributed to the increase in arsenic concentration at levels higher than the safe limit for drinking purpose. In 1999, the first study on arsenic in Nepal was carried out by DWSS with support from WHO in three districts of the eastern Terai (Jhapa, Morang and Sunsari). Out of 268 tubewell water samples tested, 9% of the samples exceeded 10 ppb, the WHO limit, and 0.7% exceeded 50 ppb, the National Drinking Water Quality Standard, indicating the possibility of arsenic contamination in groundwater in the Terai (Sharma, 1999). Following this study the Nepal Red Cross Society (NRCS), supported by the Japanese Red Cross Society (JRCS), tested approximately 1,900 water samples from eight Terai districts in early 2000. The findings

showed that 22% of the samples exceeded 10 ppb and 3% of the samples exceeded 50 ppb (NASC/ENPHO, 2005).

Out of 20 arsenic affected districts, Nawalparasi is one of the hot spot of arsenic contamination in tubewells in the Terai. In the Terai belt, the eastern part that is influenced by the Narayani River has a very low arsenic concentration that increases just south of the Bhabar zone in the central part of the Nawalparasi district. The influence of the major river system, the Narayani, is clearly observed by decreasing concentrations of arsenic in the eastern and southern regions. 16 VDCs in Nawalparasi have maximum value above 100 ppb, although the highest value in the district is relatively low, 571 ppb (Kansakar, 2003). Maharjan e*t al.* (2004) reported prevalence of arsenicosis in three communities (Sano Kunwar, Thulo Kunwar and Gohini) 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%). Of the majority of patients, 79.6% demonstrated mild and 20.4% moderate skin manifestations. Maharjan e*t al.* (2004) reported half of villagers (50.5%) were underweight and conferred that arsenic exposure worsens nutritional status.

ENPHO and NRCS study team observed arsenicosis cases and tested the tubewell water for arsenic from Kunwar and Gohini village. More than 20% of the adult population in Kunwar village and more than 8% in Sarawal village had arsenicosis related to dermatosis some with advanced clinical stages (Maharjan *et al.*, 2005). Very high proportions of tubewells in both villages were contaminated with arsenic with values more than 500ppb. For example in Kunwar 14 out 16 tubewells were found to have high arsenic content (Shrestha *et al.*, 2003). A community based study revealed that overall prevalence of arsenicosis was 6.9% among part of population that was older than 15 years (Ahmad *et al*., 2004). The prevalence rate of arsenic contamination (greater than 50 ppb) in Nawalparasi district was found to be 25.7% (Maharjan *et al*., 2006).

3.5 National drinking water quality standards for arsenic in drinking water

National Drinking Water Quality Standard (NDWQS) -2062 provides guidelines for all government and non-government agencies for formulating, designing and implementing arsenic programmes. The policy has established permissible arsenic concentration values of 50 ppb for drinking water. The policy focuses on immediate attention to be given by stakeholder agencies in identifying the existing “arsenic hot spots” and carrying out more testing on hot spots to better understand the extent and magnitude of arsenic contamination. The policy also highlights health survey and health care issues as well as communication with communities.

3.6 VDCs vulnerability to arsenic

Based on the number of tubewells that have the concentration of arsenic exceeding the WHO guidelines and the NDWQS, the regions were divided into four vulnerability classes:

a) Low vulnerability to arsenic: the percentage of tubewells exceeding the WHO guideline is Zero.

b) Moderate vulnerability to arsenic: the percentage of tubewells exceeding the WHO guideline lies between 1 and 25.

c) Moderately high vulnerability to arsenic: the percentage of tubewells exceeding the WHO guideline lies between 26 and 50.

d) High vulnerability to arsenic: the percentage of tubewells exceeding the WHO guideline is above 50 (NASC/UNICEF, 2005).

3.7 Physico-chemical parameters of water

The ordinary consumer judges the water quality by its physical characteristics like taste color and odour. The provision of drinking water that is not safe but also pleasing in appearance, taste and odour is matter of high priority (Park, 2005).

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3.7.1 pH

pH measurement is one of the most important and frequently used tests in water chemistry (APHA, 1998). pH less than 7.0 may cause corrosion and encrustation in the distribution system. The disinfection with chlorine is less effective if pH exceeds 8.0 (WHO, 1993). The pH value of drinking water from any source should be within range 6.5-8.5 (Trivedy and Goel, 1986). Microorganisms generally cannot tolerate extreme pH values. Under highly alkaline or acidic conditions, some microbial cell components may be hydrolyzed or enzymes may be denatured. There are, however, some acidophilic and alkaliphilic bacteria that tolerate or even require pH conditions for growth (Atlas and Bartha, 2005).

3.7.2 Temperature

The temperature is important factor as it affects the chemistry and biological processes. A rise in temperature of the water leads to reduction in solubility of gases than changes taste and odour (Trivedy and Goel, 1986). High water temperature enhances the growth of microorganisms and may also increase in addition to taste, odour, colour and corrosion problem. Water in the temperature range of 7°C to 11°C has pleasant taste and is refreshing. Thus, cool water is generally more palatable than warm water (WHO, 1993). All microorganisms have a characteristic optimal growth temperature at which they exhibit their highest growth. Microorganisms also have minimal growth temperatures below which they are metabolically inactive and upper temperature limits beyond which they fail to grow (Atlas and Bartha, 2005).

3.7.3 Arsenic

Arsenic is a metalloid element present naturally in the earth's crust. It forms chemical and organic complexes together with other metals, carbon and oxygen. Due to several geo-physical events and natural chemical reactions, especially the oxidation and reduction processes, several arsenic compounds in soluble forms get released inside the earth crust that contaminates the groundwater. According to US Environment Protection Agency, arsenic has been classified as a group A carcinogen, also known as human

carcinogen (EPA, 1998). The toxicity of arsenic depends upon its chemical form; arsenite (As^{3+}) is more toxic than arsenate (As^{5+}). Methylated arsenic is less toxic than inorganic arsenic. Chronic arsenic poisoning may result from the accumulation of arsenic compounds in the body through arsenic contaminated drinking water and foods (APHA, 1998).

3.8 Microbiological parameters of water

Water intended for drinking must be free from agents of water borne disease. However, it's impracticable to test every pathogen, including bacteria, viruses and parasites that might be present in drinking water, since the methods are often difficult, expensive and time consuming. For this reason, in routine testing, microbial indicators of water quality i.e. the normal intestinal organisms as indicators of faecal pollution are used, since their presence shows that pathogens could also be present. Coliform organisms (total coliforms and faecal coliforms) are often used as indicator organisms of faecal pollution for monitoring and assessing the microbial quality of public water supplies.

3.8.1 Coliform bacteria

The term "coliform organism" refers to a Gram negative, oxidase negative, non-sporing rods capable of growing aerobically on agar medium containing bile salts and able to ferment lactose within 48 hours at 35-37°C with the production of both acid and gas (Cheesbrough, 1993). Coliform organisms have long been recognized as a suitable microbial indicator of drinking water quality, largely because they are easy to detect and enumerate in water. Coliform bacteria belong to the genera *Escherichia*, *Citrobater*, *Enterbacter* and *Klebsiella*. The levels of coliform organisms present in the drinking water should not exceed the maximum permissible value of less that one cell per 100 ml of water set by the WHO (WHO, 1993).

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3.8.2 Some intestinal pathogens found in contaminated drinking water

A. *Escherichia coli* pathogenic strains

E. coli is present in large numbers in the normal intestinal flora of humans and animals, where it generally causes no harm. However, in other parts of the body, *E. coli* can cause serious disease, such as urinary tract infections, bacteraemia and meningitis. A limited number of enteropathogenic strains can cause acute diarrhoea. Several classes of enteropathogenic *E. coli* have been identified on the basis of different virulence factors, including enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EVEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC). EHEC serotypes, such as *E. coli* O157:H7 and *E. coli* O111, cause diarrhoea that ranges from mild and non-bloody to highly bloody, which is indistinguishable from haemorrhagic colitis (WHO, 2004).

B. *Klebsiella* spp.

Klebsiella spp. is gram negative, non-motile bacillus that belongs to the family Enterobacteriaceae. The outermost layer of *Klebsiella* spp. consists of a large polysaccharide capsule that distinguishes the organisms from other members of the family. *Klebsiella* strains can be found in the intestinal tract of human and animals and also in plants, soil and water. *Klebsiella* spp. is excreted in the feces of many healthy humans and animals, and they are readily detected in sewage-polluted water (WHO, 2004).

C. *Citrobacter* spp.

This genus belongs to the family Enterobacteriaceae. *Citrobacter* spp. may be isolated from human or animal feces, from various clinical specimens and from food, water, sewage, soil etc. (Singleton *et al*., 2001). *Citrobacter* spp. may be opportunist pathogen. *C. freundii* and *C. diversus* have been associated with cases of human diarrhoea (WHO, 2004).

D. *Enterobacter* spp.

Unlike most other Enterobacteriaceae, *Enterobacter* spp. Grow best at 30°C rather than at 37°C. They occur mainly in water, sewage, soil, meat, plants and vegetables. Some species also occur in human and animal feces, and some can be opportunistic human pathogens (Pelczar *et al.*, 1998)

E. *Salmonella* spp.

Salmonella spp. belongs to same family, the Enterobacteriaceae. They are motile, gram negative bacilli that do not ferment lactose, but most produce hydrogen sulfide or gas from carbohydrate fermentation. *Salmonella* are excreted in the feces of infected humans or animals. *Salmonella* infections typically cause four clinical manifestations: gastroenteritis (ranging from mild to fulminant diarrhoea, nausea and vomiting), bacteraemia or septicaemia (high spiking fever with positive blood cultures), typhoid fever/enteric (sustained fever with or without diarrhoea) and a carrier state in persons with previous infections. In regard to enteric illness, *Salmonella* spp. can be divided into two fairly distinct groups: the typhoidal species/serovars (*S. typhi* and *S. paratyphi*) and the remaining non-typhoidal species/serovars (WHO, 2004).

F. *Shigella* spp.

Shigella spp. is non spore forming, non-motile, gram negative rod, which grows in the presence or absence of oxygen. There are four species: *S. dysenteriae, S. flexneri, S. boydii* and *S. sonnei. Shigella* spp. can cause serious intestinal diseases, including bacillary dysentery. All species can produce severe disease, with the exception of illness due to *S. sonnei* that is usually relatively mild and self-limiting. In the case of S. *dysentriae,* clinical manifestations may proceed to an ulceration process, with bloody diarrhoea and high concentrations of neutrofils in the stool (WHO, 2004).

G. *Proteus* spp.

Proteus spp. is actively motile, gram negative, non-capsulated lactose non fermenting members of the family Enterobacteriaceae which hydrolyze urea rapidly (WHO, 2004).

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Proteus spp. is found widely distributed in soil, polluted water, intestine of healthy man and animals. *Proteus mirabilis* is the commonest species of *Proteus* in human infection which cause urinary tract infection. It is often found in domiciliary patients with diabetes, and the patients with structural abnormalities of the urinary tract (Collee *et al*., 1996).

H. *Pseudomonas aeruginosa*

P. aeruginosa is a member of the family Pseudomonadaceae and is a polarly flagellated, aerobic, gram negative rod. When grown in suitable media, it produces the non-fluorescent bluish pigment pyocyanin. Many strains also produce the fluorescent green pigment pyoverdin (WHO, 2004). *P. aeruginosa* is a common environmental organism and can be found in feces, soil, water and sewage. It is a classical opportunistic pathogen with innate resistance to many antibiotics and disinfectants and cause skin infection especially at burn sites and wounds. It can also cause respiratory infection, otitis external, nosocomial eye infection and septicemia (Cheesbrough, 2000).

I. *Vibrio* spp.

Vibrio spp. are small, curved (comma shaped), gram negative bacteria with a single polar flagellum. *Vibrio cholerae* is the only pathogenic species of significance from freshwater environments. While a number of serotypes can cause diarrhoea, only O1 and O139 currently cause the classical cholera symptoms in which a proportion of cases suffer fulminating and severe watery diarrhoea. Strains of *V. cholerae* O1 and O139 that cause cholera produce an enterotoxin (Cholera toxin) that alters the ionic fluxes across the intestinal mucosa, resulting in substantial loss of water and electrolytes in liquid stools (WHO, 2004).

The *V. cholerae* serogroup O1 antigen has determinants that make possible further typing; the main serotypes are Ogawa and Inaba. Two biotypes of epidemic *V. cholerae* have been defined, classic and EI Tor. The EI Tor biotype produces a hemolysin, gives positive results on the Voges-Proskauer test, and is resistant to polymyxin B. *V. cholerae*

O139 (classical biotype) is very similar to *V. cholerae* O1 EI Tor biotype (Brooks *et al*., 2004).

3.9 Health effects of arsenic

Exposure to arsenic through drinking water has become a major public health problem affecting especially many Asian and Latin American countries, including Nepal (RWSSSP, 2004; Vater, 2007). Even at low concentration, arsenic can produce devastating human health effects. The toxic character of arsenic mainly depends upon its chemical form. The most toxic form is arsine gas, followed by inorganic trivalent compounds, organic trivalent compounds, inorganic pentavalent compounds, organic pentavalent compounds and elemental arsenic. Both the WHO and EPA have classified inorganic arsenic as a toxin and carcinogen (Ngai, 2001).

3.9.1 Route of entry

Arsenic can be found in different environmental media. Possible routes of entry include inhalation of arsenic contaminated air, ingestion of arsenic containing food and water, and skin contact. The ingestion of arsenic containing food and/or water is the most important route of entry. Of the many food categories, fish and shellfish contain the highest level of arsenic. Fortunately, over 90% of the arsenic is inorganic form, which is only very mildly toxic. In contrast, for arsenic contaminated drinking water, most of the arsenic is in the more toxic inorganic form. Therefore, arsenic in drinking water is of the most concern (Pandey, 2004).

3.9.2 Acute toxicity of arsenic (III) and (V)

Ingestion of large doses of arsenic usually results in symptoms within 30 to 60 minutes, but may be delayed when taken with food. Acute arsenic poisoning usually starts with a metallic or garlic like taste, burning lips and dysphagia. Then, violent vomiting and hematemesis may occur. After the initial gastrointestinal problems, multi-organ failures may occur, followed by death. Arsenic (III) and (V) behave differently in acute poisoning. Arsenic (III) binds and inactivates sulfhydryl-containing enzymes necessary

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for proper body functions. On the other hand, arsenic (V) elicits toxicity by mimicking phosphate and interfering with ATP production in the mitochondria (Pandey, 2004).

Acute poisoning has a mortality rate of 50-75% and death usually occurs within 48 hours. A lethal does will vary with the arsenic form, but 0.2-0.3 g of arsenic trioxide is usually fatal for adult humans. However, in the context of drinking water supply, acute poisoning is less common than chronic exposure (Pandey, 2004).

3.9.3 Chronic toxicity of arsenic (III) and (V)

Chronic exposure to low level of arsenic has long since been linked to adverse health effects in human. There are contradictory beliefs on the relative chronic toxicity of As (III) and (V). On one hand, As (III) should be more toxic than (V), as an extension of acute toxicity data. On the other hand, some believe that chronic toxicity at low arsenic levels, as found in most groundwater, is influenced only by total arsenic concentration, not speciation. The toxicity of arsenic decreases in the order: Arsine> Inorganic As (III)> Organic As (III)> Inorganic As (V)> Organic As (V)> Arsonium compounds> metallic arsenic (Milton, 2003).

3.9.3.1 Dermal

Initially, chronic exposure to arsenic causes skin changes such as hyperpigmentation and keratosis. Hyperpigmentation is an alteration in color resulting in spots on the skin and keratosis is a hardening of skin bulges, usually found in palms and soles. Following hyperkeratosis and hyperpigmentation, cancer may occur. After 10 years of exposure, cancer of the skin may develop (Pandey, 2004).

3.9.3.2 Vascular effects

Exposure to arsenic has been linked to various vascular diseases affecting both the large (cardiovascular) and small blood vessels (peripheral vascular). Blackfoot disease (BFD) in parts of Taiwan is an example of peripheral vascular disease. BFD is characterized by

coldness and numbness in the feet, followed by ulceration, black discoloration and subsequently dry gangrene of the affected parts (Pandey, 2004).

3.9.3.3 Cancer

In addition to skin cancer, arsenic exposure in drinking water causes lung, bladder and kidney cancer that may appear after 20 or more years. Studies have consistently shown high mortality risks from lung, bladder and kidney cancers among populations exposed to arsenic via drinking water. Moreover, the risk of cancer for these sites increases with increasing exposure (Pandey, 2004).

3.9.4 Characteristics of arsenicosis

The disease caused by arsenic is known as arsenicosis. The characteristic of arsenicosis which are as follows:

Symptoms of arsenicosis

a) Primary stage or stage I
- **Blackening of some parts of the body or whole body (Melanosis)**
- **Thickening or roughness of the palms and soles (Keratosis)**
- **Redness of the conjunctiva (Conjunctivitis)**
- **Inflammation of the respiratory tract**
- **Nausea and vomiting (Gastroenteritis)**

b) Secondary stage or stage II
- **White intermittent dots within the black area (Leukomelanosis or rain drop syndrome)**
- **Nodular growth on the palms and soles (Hyperkeratosis)**
- **Swelling of the feet and legs (Non-pitting oedema)**
- **Peripheral neuropathy**
- **Liver and kidney disorders**

c) Tertiary stage or stage III
- **Gangrene of the distal organs or the parts of the body**
- **Cancer of the skin, lungs and urinary bladder**
- **Kidney and liver failure**
- **Death**

(Source: Mazumder, 1998)

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3.9.5 Treatment

The basic treatment is to supply the patient with drinking water that is free from arsenic. If the patient suspects to be exposed to arsenic, the patient needs to continue lots of sulfur. Sulfur can eliminate some of the arsenic from the body. Fiber can also help to leach the arsenic by attaching to it and washing it out. Examples of fiber containing food are whole grains, cereals, fruits and vegetables. Chelation therapy is an option if patient has arsenic poisoning. Chelation therapy is a series of injections of EDTA and its objective is to provide the patient with chemical to which arsenic binds strongly, so it could then be excreted in urine. Besides these methods providing multivitamin tablets and improving nutritious food may be of benefit to patients. Vitamin A, B complex, C, D and E are known to be beneficial in the differentiation of various tissues, particularly the skin (Smith *et al*., 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 and 5) counseling and education (WHO, 2005a).

3.10 Mitigation of arsenic in drinking water

Arsenic mitigation on a household level faces several difficulties. An appropriate system for arsenic removal should be efficient, cheap, socially accepted, user friendly, locally available and operated without the use of chemicals. In some cases, arsenic removal technologies are often limited to small study areas and therefore do not contribute to regional progress in arsenic mitigation (USEPA, 2000).

Safe tube wells, improved dug wells and improved bio-sand filters are the most popular mitigation options being implemented in Nepal. As of December 2005, various organizations and implementing agencies have distributed over 4,700 improved bio-sand filter units in the most affected regions of the Terai districts (NASC/UNICEF, 2005).

The other options for the supply of safe water is the development of deep tubewells, artesian system, distribution of treated, organism free surface water, by harvesting rain water. An alternative option for groundwater supply is treatment of arsenic contaminated tubewell water to make it safe for consumption. Many of the technologies for arsenic removal rely on few basic chemical processes, like: oxidation/reduction, precipitation, adsorption, and biological removal process that is understood. It is important to point out that boiling does not remove arsenic from water. This is regarded as an alternative for the transitional period until a "permanent" solution is found (WHO, 2004).

Due to the severity of health problems caused by arsenic poisoning in many regions of the world, several different types of filters have been in use. In Nepal, plastic or cement bio-sand filters are being used for four years. This is basically bio-sand filter that is a modified form of 3-Kolshi filter. Information about this filter and other two filters is given below.

3.10.1 3-Kolshi filter

In a three 3-Kolshi (3-layer) water filtration system, the “Kolshi” (top, middle, and bottom) are placed on top of each other in a steel or bamboo frame. This simplifies its maintenance. The first layer consists of the mixture of cast iron turnings and sand. The second layer, “Kolshi” contains wood charcoal and sand as active ingredients. This layer also has holes that are made to allow free flow of water into junction nozzles connected to the outside. These nozzles can be easily altered to adjust flow rate (Munir *et al*., 2001).

3.10.2 SONO filter

SONO filter is a prototype of 3-Kolshi. SONO distribution center developed composite iron matrix (CIM) as the main active arsenic and pathogen removal material. The SONO filter was developed by a team led by Prof. Hussam and Dr. Munir in 2001. It retains all the excellent features of a 3-Kolshi system with stability and long life use (Hurd, 2001).

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3.10.3 Arsenic bio-sand filter (Kanchan Arsenic Filter)

Massachusetts Institute of technology (MIT), in collaboration with Environment and Public Health Organization (ENPHO) and Finnish International Development Agency (FINNIDA) has developed the arsenic bio-sand filter. A bio-sand filter has been introduced in the Terai region previously for removal of iron and bacteriological contamination (Pandey, 2004). This filter removes more than 95% of arsenic and 99% of iron (Shrestha *et al.,* 2004). The combination of physico- chemical and biological processes in the KAF can give up to 86% efficiency on bacterial removal (Figure 1). However, the bacterial removal efficiency may be low (only 50-60%) during the period immediately after filter installation. It normally takes a period of one to two weeks for a biological layer to develop to maturity in a new filter. The removal efficiency increases with the growth of biological layer (ENPHO, 2003).

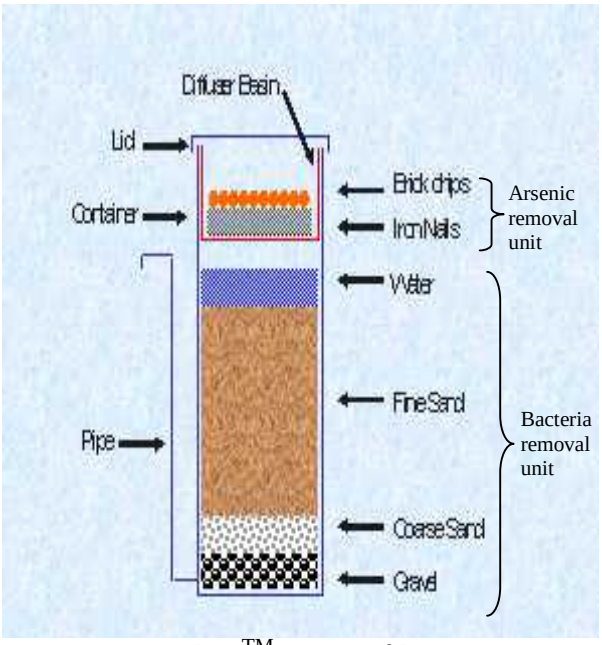

Figure 1 Kanchan^{TM} arsenic filter cross section (ENPHO)

3.10.3.1 Arsenic removal unit

In arsenic bio-sand filter, the iron nails are exposed to air and water, and rust quickly, producing ferric hydroxide particles. When arsenic contaminated water is poured into the filter, arsenic is quickly adsorbed onto the surface of the ferric hydroxide particles. These arsenic loaded ferric hydroxide particles are trapped on top of the fine sand layer. Most of the arsenic is already adsorbed on to the ferric hydroxide, and almost all ferric hydroxide is trapped on the top of fine sand layer. As a result, arsenic is effectively removed from the water (ENPHO, 2003). Figure 2 illustrates the arsenic removal mechanism (Ngai and Walewijk, 2003).

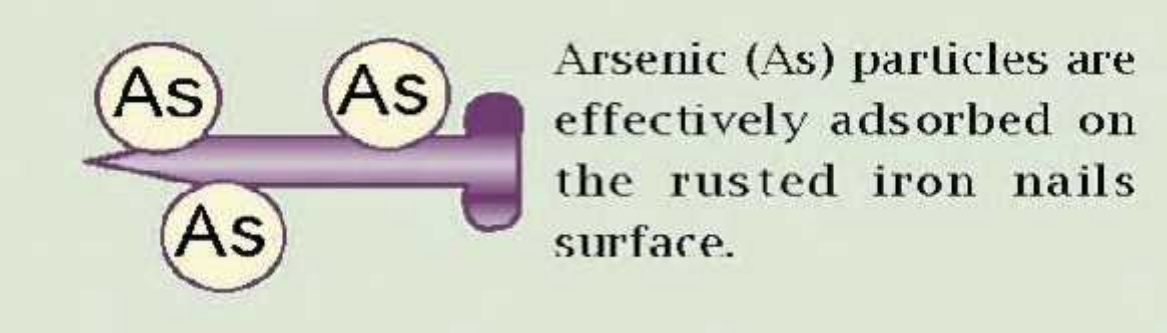

Figure 2: Illustration of arsenic removal mechanism (Source: ENPHO)

3.11 Testing and investigation

3.11.1 Hydride Generator Atomic Absorption Spectrophotometer

Arsenic contamination in ground water can be detected in the field or in the laboratory with a choice of testing methods depending on the nature and purpose of the analysis. The "Interim Nepal Guidelines and Policies for Arsenic in Drinking Water" has recommended the use of lab testing with specific preference for the atomic absorption spectrophotometer (AAS) method as the most reliable. However, the majority of tests undertaken in Nepal use field test methods because of low cost, good results and mobility in the field.

3.11.2 Blanket tubewell testing

There are several brands of field test kits in use in Nepal, but for the blanket testing the Wagtech kit is preferred because of its portability and simplicity in use as well as low cost in comparison to other field tests. This kit is able to detect arsenic concentration levels from 0.01 to 0.5 mg/l. This test kit underwent extensive field testing in Bangladesh. Wagtech field test kits are based on the mercuric bromide stain principle where As (V) is reduced to As (III) by stannous chloride. As (III) is allowed to react with hydrogen ions, generated by the reaction of HCl and Zn granules to form arsine gas. Released arsine gas produces a yellow-brown stain on mercuric bromide paper. However, due to several limitations such as the quantity of arsenic present in test water, strength of bromide paper, amount and quality of reagents used, technique adopted as well as

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presence of other inorganic and organic matters in test water, the field test kits are incapable of yielding quantitative results in samples with low arsenic concentration (below 0.1 mg/l) (Wagtech international, UK).

3.12 Biological properties of arsenic

Arsenic is a ubiquitous and abundant element. Its toxicity has been known for many centuries and arsenic salts were often used for poisoning. Arsenic toxicity is highly dependent on its oxidation state: trivalent arsenicals are at least 100 times more toxic than the pentavalent derivatives (Cervantes *et al*., 1994). Toxicity of arsenite [As (III)] is due to its binding to protein sulfhydryl groups (Gebel, 2000). Thus, As (III) inhibits enzyme reactions requiring free sulfhydryl groups, leading to membrane degradation and cell death. As (V), on the other hand, is a toxic analog for inorganic phosphate in phophorylating metabolism (Cervantes *et al.,* 1994). In general, methylated species, MMAA and DMAA, are less toxic forms than inorganic As (V) and As (III) because of their low solubility and reduced affinity with tissue. In this sense the methylation acts as a detoxification mechanism (Leonard, 1991). Arsenite and arsenate are interconverted to an extent by biological redox reactions and arsenite can be methylated by bacteria, fungi and algae. The environmental and global cycle of arsenic and its compounds is summarized in Figure 3 (Cullen and Reimer, 1989).

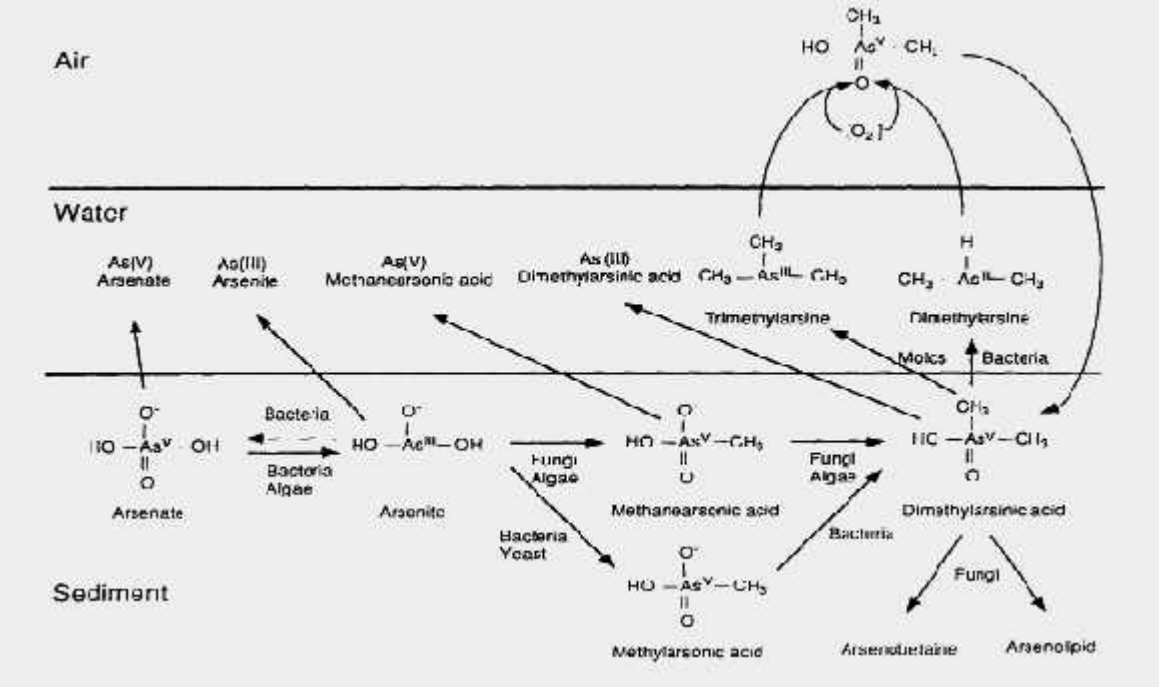

Figure 3 The environmental and global cycle of arsenic and its compounds (Cullen and Reimer, 1989)

Microorganisms in the environment are continuously exposed to metallic anions and cations. Some of these ions are taken up as essential nutrients (i.e. magnesium, potassium, copper and zinc), whereas others, with no known biological function, exert toxic effects on microbial cells (i.e. mercury, lead, cadmium, arsenic and silver). In bacteria, heavy metal resistance genes are usually located on plasmids or transposons (Silver and Misra, 1998).

Microbial interactions with metals may have several implications for the environment. Microbes may play a large role in the biogeochemical cycling of toxic heavy metals. Also they can contribute cleaning up or remediating metal-contaminated environments. Some bacteria have evolved mechanisms to detoxify heavy metals and some even use them for respiration. There is also evidence of a correlation between tolerance to heavy metals and antibiotic resistance, a global problem currently threatening the treatment of infections in animals and humans (Spain and Alm, 2003).

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Oxidation of As (III) represents a potential detoxification process that allows microorganisms to tolerate higher level of arsenite. Several examples of bacterial oxidation of arsenite to arsenate were being reported as early as 1918. Philips and Taylor (1976) isolated *Alcaligens* strains able to oxidize arsenite with oxygen as a final electron acceptor. *Alcaligens faecalis*, resistant to the toxic effects of 0.01 M sodium arsenite, was isolated from raw sewage and shown to be capable of oxidizing arsenite to arsenate. Paknikar and Mokashi (2002) isolated *Microbacterium lacticum* from municipal sewage to develop a microbially assisted process for the removal of arsenic from contaminated groundwater. It was found that *M. lacticum* has the highest MIC value for As (III) amongst the bacterial species and it was also able to retain the As (III) oxidizing capacity at 50 mmol/l concentration. Other bacterial transformations of arsenic include arsenic methylation and demethylation but these processes are not considered as mechanisms of resistance. Cyanobacteria seem to be naturally resistant to arsenate. Cyanobacteria are believed to tolerate arsenate by possessing resistance mechanisms that include both efflux systems and intracellular metabolic pathways.

3.13 Microbial arsenic tolerance mechanisms

In high concentrations, heavy metal ions react to form toxic compounds in cells (Nies, 1999). To have a toxic effect, however, heavy metal ions must first enter the cell. Because some heavy metals are necessary for enzymatic functions and bacterial growth, uptake mechanisms exist that allow for the entrance of metal ions into the cell. There are two general uptake systems – one is quick and unspecific, driven by a chemiosmotic gradient across the cell membrane and thus requiring no ATP, and the other is slower and more substrate- specific, driven by energy from ATP hydrolysis (Nies and Silver, 1995).

To survive under metal-stressed conditions, bacteria have evolved several types of mechanisms to tolerate the uptake of heavy metal ions. These mechanisms include the efflux of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, and reduction of heavy metal ions to a less toxic state (Nies, 1999). Genetic determinant for arsenic resistance in bacteria can be either chromosomal or

plasmid encoded as occurs with other arsenic resistance (Cervates *et al*., 1994; Saltikov and Olson, 2002; Silver and Phung, 1996).

3.13.1 Chromosomal-determined resistance

Chromosomally determined arsenate-resistance generally results from effects on phosphate transport. Arsenate is taken up by the phosphate transport systems of bacteria. Some bacteria possess two distinct phosphate transport pathways: one systems takes up both phosphate and arsenate at similar rates (named the Pit, Pi transport, system in *E. coli*), whereas the other one (the Pst, for phosphate-specific transport system) is highly specific for phosphate and transports arsenate poorly. Mutants defective in the Pit pathway are usually arsenate-resistant (Cervates *et al*., 1994).

3.13.2 Plasmid-mediated resistance

Bacterial resistance to arsenic ions governed by plasmids was first discovered by Novick and Roth (1968). A group of *S. aureus* β-lactamase plasmid determines resistance to heavy metals. Arsenic resistance plasmids confer tolerance to both arsenate and arsenite as well as to antimony (III) (Novick and Roth, 1968).

Chen *et al.* (1986) proposed a model for the plasmid-mediated mechanisms of the efflux of arsenate and arsenite in gram-negative bacteria. The nucleotide sequence of a fragment of DNA containing the *ars* operon found in gram negative bacterium on plasmid R773. In gram negative bacteria that encodes for the efflux of arsenate and arsenite. Three *arsA*, *arsB*, and *arsC* genes were found to be encoding for the proteins ArsA, ArsB and ArsC respectively. ArsA is a protein with ATPase activity and thus is involved in translocation of the metal ions across the cell membrane. ArsB interacts with ArsA on the inner membrane of the cell, and the two proteins form the arsenite pump. ArsC is a smaller protein that alters the specificity of the arsenite pump to allow for the efflux of arsenate. Thus, ArsC is only required for tolerance to arsenate, ArsA and ArsB are required for tolerance to both species of arsenic.

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The arsenic resistance determinants from both *E. coli* and *Staphylococcus* spp. have been cloned and sequenced. Several mechanisms for resistance to arsenic have been identified. The *ars* operons from *S. aureus* plasmid pI258 and *S. xylosus* plasmid pSX267 consist of three genes, *arsR, arsB* and *arsC*. In both bacteria, regulation of *ars* operons is done by the *arsR* gene which encodes a dimeric transacting repressor and the operon can be induced by arsenate, arsenite, antimonite and bismuth *in vivo* (Chen *et al.*, 1986).

Microorganisms utilize As (V) as a terminal electron acceptor for anaerobic respiration by dissimilatory reduction process. Dissimilatory reduction has been observed in several bacteria, such as *Bacillus arsenicoselenatis, B. selenitireducens, Pseudomonas* spp., *Desulfotomaculum auripigmentum* etc. (Ahmann *et al*., 1994; Macur *et al*., 2001; Oremland *et al*., 2000; Stolz and Oremland, 1999).

3.14 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 concentrations 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). MIC refer to the smallest concentration necessary to inhibit growth; thus lower MIC values indicate more toxic metals and higher indicate less toxicity (Spain and Alm,, 2003).

3.15 Plasmid profiling

Arsenic resistance determinants were initially found on bacterial plasmids. Bacterial resistance to both arsenate and arsenite ions were governed by plasmids. Most mechanisms studied involve the efflux of metal ions outside the cell, and genes for this general type of mechanism have been found both on chromosomes and plasmids (Spain and Alm, 2003).

CHAPTER-IV

4 MATERIALS AND METHODS

4.1 Materials

A list of materials, chemicals, equipment, media and reagents required for the study is presented in Appendix-III

4.2 Methods

4.2.1 Study period

This study was conducted from June 2007 to January 2008. Field survey for data collection, water sample collection was done in three periods- on June, September and December\January.

4.2.2 Study area

This study was conducted in highly arsenic affected area of Ramgram municipality and Sunwal, Sarawal, Swathi, Sukrauli, Jahada and Pratappur VDCs of Nawalparasi district. Nawalparasi district is located in Lumbini zone of the Western Development Region of Nepal which covers 2,162 sq. km. comprising 73 VDCs and one municipality (Ramgram). It is bounded by Palpa and Tanahun districts on the north, Rupandehi and Palpa districts on the west, Chitwan and Tanahun districts on the east and Chitwan district and Utter Pradesh state of India on the south. Study area was selected as more vulnerable and arsenic concentration exceeds greater than the WHO standard guideline (>10 ppb). A schematic map of study area is given at appendix II.

4.2.3 Study sites

Study sites were selected in the program areas of Filters for families (FFF). Out of 73 VDCs, the study was conducted in seven highly arsenic contaminated area of Nawalparasi District. These are Ramgram Municipality (Thulo Kunwar-12, Padatikar-13, Baikanthapur-8, Ghanshyampur-8, Pachagaon-12 and Kanchanaha-13), Sarawal (Gohini-

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2), Swathi (Mini Bankatti-5), Sunwal (Kirtipur-2), Jahada (Bhatauli-9), Pratappur (Pratappur-2) and Sukrauli VDC (Nadawa-8). In this study, a cluster of each village was examined since the entire village is too large to be covered.

4.2.4 Study population

For this study, households were chosen that were determined to have water with arsenic levels that were above WHO guidelines and presence of filters distributed by different agencies. The tubewells were selected on the basis of blanket testing data of FFF-2006, FINNIDA/RWSSSP and NRCS (Appendix-X).

4.2.5 Tools for data collection and analysis

A set of structured questionnaires were filled and used as primary data (Appendix-I). The questionnaire was designed to interview contact person or the water supply system (like care taker) or any individual from one of the households using that system. Information regarding the location, age and depth of the tube wells; sanitary conditions etc. were included in the questionnaire. Arsenicosis patient was diagnosed for the presence/absence of skin manifestation like melanosis and keratosis as well as by the known history of arsenic exposure through drinking water. Dermatological manifestations were graded as mild, moderate and severe following the grading criteria given by Mazumder (1998) on the basis of severity of chronic arsenic toxicity.

4.2.6 Collection of samples for water analysis

4.2.6.1 Sample collection from tube well

For the sample collection from tubewell, the hand pump was operated continuously for five minutes. The sample was collected aseptically by allowing the pump to flow directly into the sterile bottle and cap of the bottle was placed carefully. The bottle was labeled with the sample code number.

4.2.6.2 Sample collection from effluent water (filter)

As above process, the filter was operated continuously for five minutes in order remove contamination. The sample was collected aseptically by allowing the water from the filter to flow directly into the sterile bottle and replaced the bottle cap carefully. Then the bottle was labeled with the sample code number.

Five samples were collected from each site; three from tubewell (detection of arsenic concentration, water quality test and arsenic tolerant bacteria from influent water) and two from bio-sand filter i.e. after filtration (detection of arsenic concentration and water quality test from effluent water). Only one sample was collected from those household without filter.

4.2.7 Study of physico-chemical parameters of water samples

Analysis of most of the physico-chemical parameters of water were done by following "Standard methods for the examination of water and wastewater". The temperature and pH of water samples were recorded at the site during sampling period. Water samples were collected in a pre-acid washed polyethylene bottles at least 5 minutes. A distance of half kilometer was maintained between the two sampling points. The preservative (1% concentrated HCl) were used in the samples, carried in an ice-box and kept at 4°C until arsenic analysis (APHA, 1998).

4.2.7.1 Temperature

Temperature was determined with the help of a standard mercury thermometer graduated up to 50°C. Soon after collection of the sample, thermometer bulb was immersed into the water and noted the reading.

4.2.7.2 pH

Hydrogen ion concentration in the sample was measured with the help of a pH meter (HANNA Company) by inserting the electrode into the water sample.

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4.2.7.3 Measurement of arsenic concentration by kit method

Measurement of total arsenic concentration in water samples was conducted Wagtech kit (UK) for blanket tubewell testing on the spot. The method of application was done as described in kit leaflet which was given in Appendix-XIII

4.2.7.4 Measurement of total arsenic concentration by AAS

Water samples was conducted by an atomic absorption spectrophotometer (SOLAAR 969AA Spectrometer, Thermal Elemental, UK), equipped with a flow injection hydride generator (HG-AAS), in the research laboratory of ENPHO, Kathmandu, Nepal. Arsenic level in water samples was determined, following pre-reduction with 5% (w/v) KI and 5% (w/v) ascorbic acid in 10% (v/v) HCl. The accuracy of assay was ensured by including standard reference material, NIST SRM 1640, with 26.76±0.41 µg/kg arsenic. The detection limit (DL) of the HG-AAS was 3µg/l for arsenic in water. The obtained values fell within the certified ranges, and none of the samples produced values below detection limit.

4.2.8 Physical examination for skin manifestations of chronic arsenic ingestion

Health workers conducted physical examination of household members who were available during the visit for sample collection and identifying arsenic-related skin manifestations. Examination was made following "A Field Guide for Detection, Management and Surveillance of Arsenicosis Cases", WHO technical publication no. 30, SEARO, New Delhi, 2005. In this study, arsenicosis is defined as a chronic health condition arising from prolonged ingestion of arsenic above the national standard (>50 ppb) for at least six months, manifested by characteristic skin lesions of melanosis and keratosis, occurring alone of in combination. The patients with keratosis were further subcategorized into mild, moderate or severe on the basis of thickening of the skin and appearance of papules or nodules according to the above mentioned WHO’s field guideline.

4.2.9 Microbial examination water sample

The water sampling was done in an aseptic condition in sterile bottles and brought to laboratory of Parasi hospital within 8 hrs and refrigerated. Water samples were transported from Parasi to Central Department of Microbiology (CDM), Kirtipur in an ice box storing the samples at 4°C within three days of sample collection. For the isolation and identification of bacteria, the sample was processed for the enumeration of total coliform bacteria by membrane filtration technique and initial screening was conducted on arsenic enriched plate count agar to facilitate enumeration as well as screening of the arsenic tolerant bacteria. This would help to assess the difference in the microbial population with respect to water depth (below 50 ft and above 50 ft). Their colonial, morphological and biochemical properties following Bergey's manual of systematic bacteriology plus Biolog kits would identify purified isolates.

4.2.9.1 Total coliform count

In this study, total coliforms were enumerated by the membrane filtration (MF) technique as described by APHA (1998).

Standard total coliform membrane filter procedure

First of all, sterile filter holder with stopper was assembled on the filter flask. Using sterile blunt-edged forceps, a sterile membrane filter of pore size 0.45 µm (grid side up) was placed over the porous disc in such a way that it overlapped the entire circumference of sintered filterable area. The sterile funnel was securely placed on the filter base. The sample of water was well mixed by inverting the bottle several times, and then 100 ml of the water sample was poured into the funnel. The sample was slowly filtered under partial vacuum by using electric vacuum by using electric vacuum pump. The funnel was removed and the membrane was directly transferred, keeping its upper side upwards, onto a plate of M-Endo agar with the help of sterile forceps. Care was taken not to entrap air bubbles between the membrane and the medium. Then it was incubated for 24 hrs at 37°C in inverted position. After proper incubation total colony forming unit (CFU) were counted. For this, all sheen producing colonies were counted.

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4.2.9.1.1 Enumeration of coliforms

The typical coliform colony has a pink to dark-red colour with a metallic surface sheen. The sheen area may vary in size from a small pinhead to complete coverage of the colony surface. Atypical coliform colonies can be dark red or nucleated without sheen. Colonies that lack sheen may be pink, red, white or colorless and are considered to be non-coliform (APHA, 1995). All sheen producing colonies are isolated and identified.

4.2.9.2 Enrichment

Enrichment was done for only 30 samples due to lack of lab facility in the study area.

4.2.9.2.1 Detection of *Salmonella* and *Shigella* species

5 ml of influent water sample was enriched by inoculating into 45 ml Selenite F broth and mixed thoroughly. It was then incubated at 37°C for overnight, and a loopful of the upper part of the broth was sub-cultured on a selective enteric medium, SS agar. The plate was incubated at 37°C for 24 hours (Collee *et al.*, 1996).

4.2.9.2.2 Detection of *Vibrio cholerae*

5 ml of water sample was enriched by inoculated into 45 ml of 1% alkaline peptone water. It was then incubated at 37°C for 6 to 8 hours. Then a loopful of the enrichment broth was streaked on TCBS agar medium plate. The plate was incubated at 37°C for 24 hours (Collee *et al.*, 1996).

4.2.9.3 Isolation and identification of bacteria

Metallic sheen producing colonies and all colonies with different characteristics from M-Endo agar, SS agar and TCBS agar were streaked onto MA and NA and further subculture was done in NA to get pure culture. Bacteria isolated on NA were identified on the basis of their colonial characteristic, morphological characteristics and biochemical properties. Identification was carried out following Bergey's Manual of Systematic Bacteriology, 1994. Biochemical tests for identification of isolates were given in Appendix VII.

4.2.9.4 Enumeration of arsenic tolerant bacteria

Enumeration of the arsenic tolerant bacteria was done by pour plate method using PCA supplemented with different arsenic concentration of 0 ppm, 25 ppm, 50 ppm, 100 ppm and 200 ppm. This method is based upon the principle that when material containing microorganisms is cultured, each viable microorganism will develop into a colony; hence the number of colonies appearing on the plates represents the number of living organisms present in the sample. This method involves spreading the sample on pre-prepared PCA plate and counted the colonies developed after incubation for 24 hrs to 48 hrs at 30°C.

i. Pour plate technique

For the technique, Petri-plates were labeled from 0, 25, 50, 100, 200 ppm respectively. 1 ml from the water sample without dilution was kept in the Petri plates marked for corresponding concentrations. Then 1ml of respective arsenic concentration [As (V)] was poured into labeled Petri plates. The melted and cooled plate count agar medium was added to the inoculated plates. Plates were shaken gently for uniform distribution of organisms and arsenic, and kept for solidification. After the solidification, the plates were incubated at 30°C at inverted position for 24-48 hours. The colonies developed after incubation was counted with Colony Digital Counter and recorded the respective colony forming units per dilution. The count exceeding 300 colonies were reported too numerous to count (TNTC).

4.2.9.5 Isolation and identification of arsenic tolerant bacteria

The selected colonies from pour plate technique were streaked in NA plate containing same arsenic concentration and incubated at 30°C for 24-48 hours. The isolated and distinct colonies on this nutrient agar were sub-cultured repeatedly for purification. The pure cultures were also maintained in as stock culture for further analysis and stored in refrigerator at 4°C. The distinct colonies observed after the streaking technique were encoded according sample source. After encoding, each of the isolated colonies on NA was identified on the basis of their colonial characteristics, morphological characteristics and biochemical properties.

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The composition and preparation of biochemical media and reagents used in the biochemical test are mentioned in the Appendix-IV. The procedure for performing biochemical tests are mentioned in Appendix-VI.

4.2.9.6 Minimum inhibitory concentration (MIC)

MIC was determined by the ability of the organisms to survive in desired concentration of arsenic. To determine MIC, the isolated bacteria were transferred in nutrient broth containing arsenic of different concentration ranging of 0, 10, 20, 50, 100 and 200 ppm incubated for 48 hours at 30°C. The MIC was determined by observing the presence and absence of growth in the broth media. Then the MIC was determined as the lowest concentration, which inhibits the visible growth in the test tube. As (III) is more toxic than As (V), tube dilution method starting from 200 ppm was performed. MIC values of As (III) to arsenic tolerant bacteria were determined by tube dilution method using 50 ppm As (III) working solution. However, MIC values of As (V) to arsenic tolerant 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 shown in APPENDIX-VIII.

4.2.10 Extraction of plasmid DNA

The plasmid DNA was extracted from the overnight culture (mid log phase) of arsenic tolerant and sensitive (for control) *E. coli*, grown in Luria Bertani broth, supplemented with proper arsenic concentration. The plasmid was extracted according to protocols described by Sambrook and Russel, (2001) and using commercial kits procured from Bangalore genei, India. The isolates were examined for the plasmids by alkaline lysis method. For this GeNei^{TM} Spin Mini Prep Kit was used to isolates plasmid DNA from *E. coli* cultures. The kit uses Silica Gel membrane based column. Bacterial cells were harvested, lysed by alkaline lysis and adsorbed onto silica membrane. After washing, purified DNA was then eluted using a small volume of tris buffer. The detailed protocols are mentioned in appendix. The composition of Luria Bertani broth is mentioned in

appendix-IV. The preparation of solutions for extraction of plasmid is mentioned in appendix-IV.

4.2.11 Electrophoresis of plasmid DNA from arsenic tolerant and sensitive *E. coli*

The electrophoresis was done in standard agarose in 70 volts for 3 hours. Then the agarose slab was visualized under the UV transilluminator. The detailed protocol for electrophoresis is mentioned in appendix-IX.

4.2.12 Determination of size of DNA by semi log plot

Making standard curve: Using an AutoCAD, the distance traveled from the well (in mm) by each illuminated band was measured. The number of base pairs (of marker DNA) of each illuminated band versus the distance traveled was plotted on semi-log paper. No. of base pairs was plotted on the log scale, and mm migrated on the linear scale. The best line through these points was drawn to generate a standard curve. From this curve the size of each band was determined. (Appendix-X)

4.2.13 Decontamination of ethidium bromide

The procedure for decontamination of ethidium bromide remaining in the agarose slab is mentioned in the Appendix VIII.

4.2.13 Data analysis

Analysis of data was done by using statistical tools like MS-excel, SPSS and AutoCAD.

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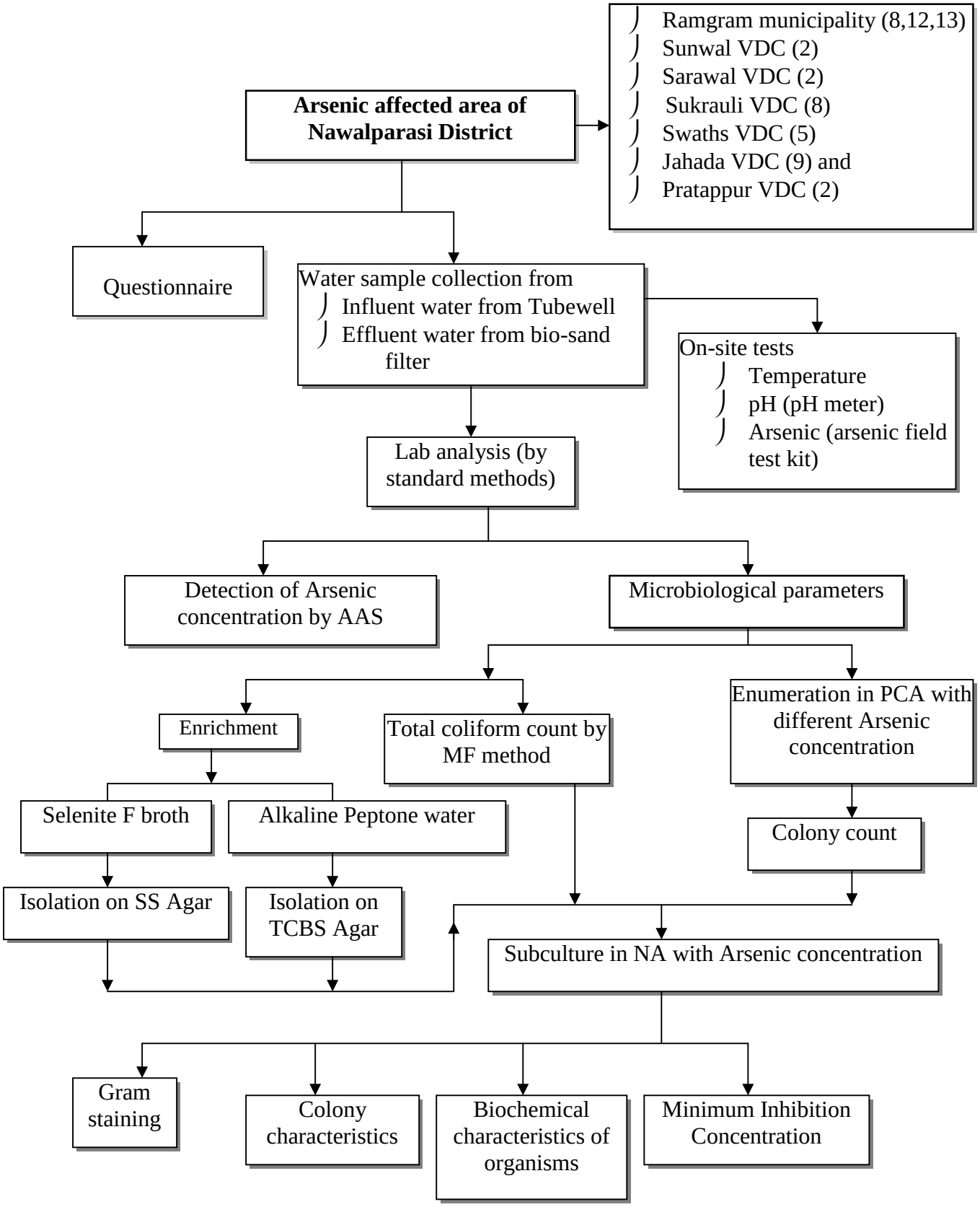
FLOWCHART: METHODS OF IDENTIFYING ARSENIC TOLERANT BACTERIA



CHAPTER-V

5 RESULTS

A total of eighty six water samples were collected from one municipality (Ramgram-8, 12, 13) and six VDCs ward (Sunwal-2, Sarawal-2, Sukrauli-8, Swathi-5, Jahada-9 and Pratappur-2). These samples were collected from groundwater i.e. shallow tubewell present in different arsenic contaminated area. All water samples were analyzed for physico-chemical and microbiological parameters to assess the drinking water quality.

5.1 Physico-chemical parameters of water

The analysis of physico-chemical parameters of collected water samples was considered as one of important tool for drinking water quality assessment and hence various parameters were tested.

5.1.1 Temperature

The temperature of water samples showed a remarkable variability ranging from 17°C to 34°C. The minimum temperature was recorded in the month of December in tubewell water collected from Baikanthapur (Ramgram municipality), while the maximum temperature (34°C) was recorded in the month June from Shivanagar (Ramgram municipality). The average temperature from different sites was found 26.07°C.

Table 1 Distribution of temperature of water samples

S.N.	Source	Temperature (°C)		
		Minimum	Maximum	Average
1	Shallow tubewell water	17	33	26.07

5.1.2 pH

A notable variation in pH value was observed. The pH value of tubewell ranged from 6.3 to 7.9. The average pH of tubewell was 7.06. A comparison of pH value with WHO

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standard revealed that 88.37% of samples were within standard, 11.63% below the standard and 0% above the standard. The results are shown in figure 4.

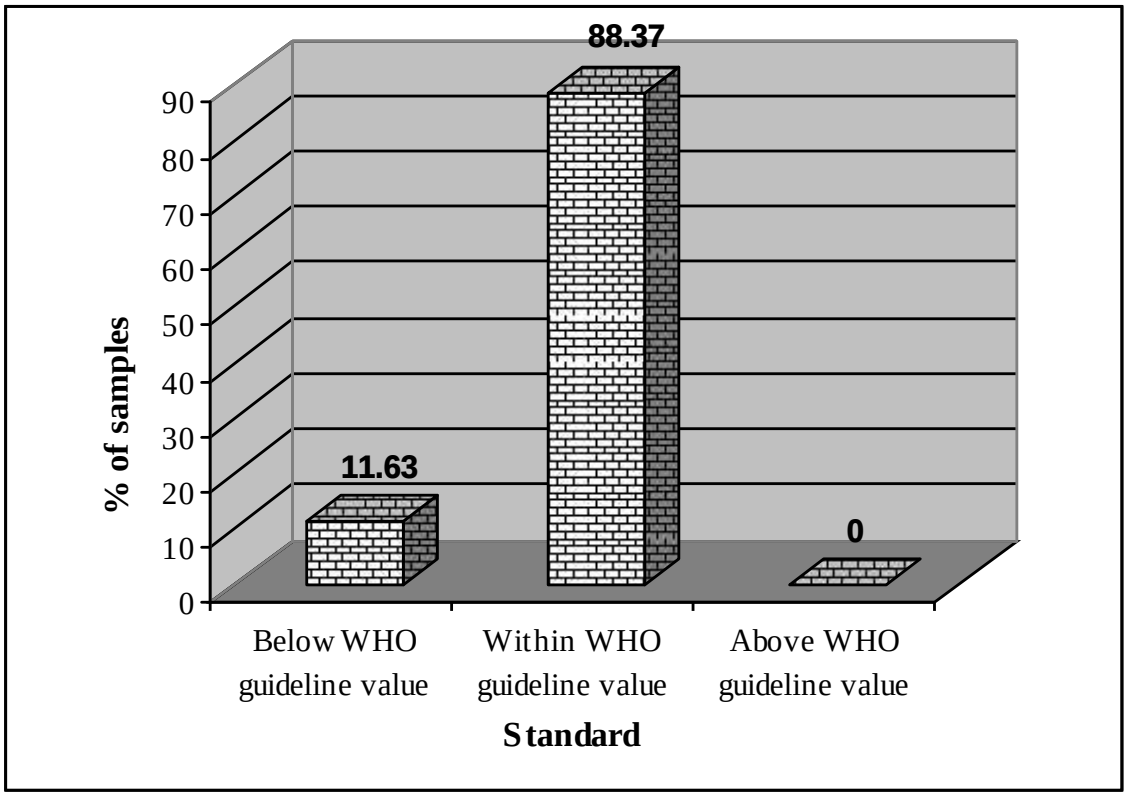

Figure 4 Comparison of pH measurements of water samples with standards

5.1.3 Arsenic

5.1.3.1 Arsenic concentration in different VDCs/Municipality above and below National Drinking Water Quality Standards (NDWQS)

Among seven different places, 38 (44.19%) samples were collected from Ramgram Municipality and among them 29 (54.7%) samples were found above National Drinking Water Quality Standard (50ppb). Results shows that in Sarawal, Pratappur, Sukrauli also arsenic concentration was above NDWQS except Sunwal, Jahada and Swathi VCDs. Most of samples were taken from that place where bio-sand filter was distributed to check its current condition; efficiency of filter and arsenicosis symptoms was high to find out socio-economic impact of arsenic poisoning.

Table 2 Arsenic concentration in different VDCs/Municipality

VDCs/Municipality	<50 ppb	>50 ppb	Total
Ramgram Municipality	9 (27.27)	29 (54.7)	38 (44.19)
Sunwal	3 (9.09)	0 (0.00)	3 (3.49)
Sarawal	2 (6.06)	10 (18.87)	12 (13.95)
Pratappur	1 (3.03)	3 (5.66)	4 (4.65)
Sukrauli	7 (21.21)	7 (13.20)	14 (16.28)
Jahada	4 (12.12)	1 (1.88)	5 (5.81)
Swathi	7 (21.21)	3 (5.66)	10 ((11.63)
Total	33 (38.37)	53 (61.63)	86

5.1.3.2 Arsenic concentration before and after filtration

Arsenic concentration was detected before filtration i.e. from shallow tubewell, artesian well and after filtration i.e. effluent from filter. This was done to find out the efficiency of filter that was used for arsenic removal in different parts of Nawalparasi district. Table 3 shows that 61.63% tubewell was found above National Drinking Water Quality Standard and 89.53% tubewell was found above WHO guidelines. Table 4 shows that 30.16% filter (19 filtered water) shows above NDWQS whereas 68.25% filter (43 filtered water samples) shows above WHO guidelines. In 23 households there was no filter, of them people of Sunwal VDC were not using bio-sand filter. They were using water from rehabilitated dug well and some were drinking from same shallow arsenic tubewell. It was not found of constructing safe shallow tubewell instead of arsenic contaminated shallow tubewell water. As shown in table 5, arsenic concentration paired t-test statistics shows that there was significant difference before and after filtration ($p<0.05$).

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Table 3 Arsenic concentration before filtration

Arsenic concentration before filtration i.e. from Tube well etc.	NDWQS		WHO Guidelines		Total samples
	<50 ppb	>50 ppb	<10 ppb	>10 ppb	
Total	33	53	9	77	86
Percentage	38.37	61.63	10.47	89.53	

Table 4 Arsenic concentration after filtration

Arsenic concentration after filtration of filtered water	NDWQS		WHO Guidelines		No Filter	Total Samples
	<50 ppb	>50 ppb	<10 ppb	>10 ppb		
Total	44	19	20	43	23	63
Percentage	69.84	30.16	31.75	68.25		

Table 5 Arsenic concentration paired t-test analysis

Arsenic concentration (ppb)	Mean	Std. deviation	p-value
Before filtration	144.11	156.072	<0.05
After filtration	54.34	77.403	

5.1.3.3 Relation of arsenic concentration with age of tubewell (installation in year)

The difference between the year of installation of the tube well and year the water was sampled is the age of the tube well or the usage period. From the table 6, it was found 16 (30.19%) tubewells exceed arsenic concentration above NDWQS in tubewell age between 6-10 years followed by 14 (26.42%), 12 (22.64%), 10 (18.87%), 1 (1.88%) tubewells above NDWQS in tubewell age 0-5 years, 16-30 years, 11-15 years and 31-50 years respectively. The tubewells were of different ages from recently installed to 40

years old. In the six VDCs and one municipality, 57 (66.28%) tubewells were constructed within the last 10 years and 29 (33.72%) of the tube wells were constructed in the last 10-40 years. Here, zero age means water sample was analyzed in same year of installation of the tubewell. For the relationship of arsenic with tubewell age, moderately positive correlation was obtained between arsenic concentration and age of tubewells (r=0.23, p=0.83) (Figure 5).

Table 6 Classification of arsenic concentration by age of tubewell

Well age	**0-10 ppb**		**11-50 ppb**		**>50 ppb**		**Total**	
	No	**%**	**No**	**%**	**No**	**%**	**No**	**%**
0-5	5	55.56	8	33.33	14	26.42	27	31.4
6-10	2	22.22	12	50.00	**16**	**30.19**	**30**	**34.88**
11-15	0	0.00	1	4.17	10	18.87	11	12.79
16-30	1	11.11	2	8.33	12	22.64	15	17.44
31-50	1	11.11	0	0.00	1	1.88	2	2.32
>50	0	0.00	1	4.17	0	0.00	1	1.62
Total	9	100	24	100	53	100	86	100

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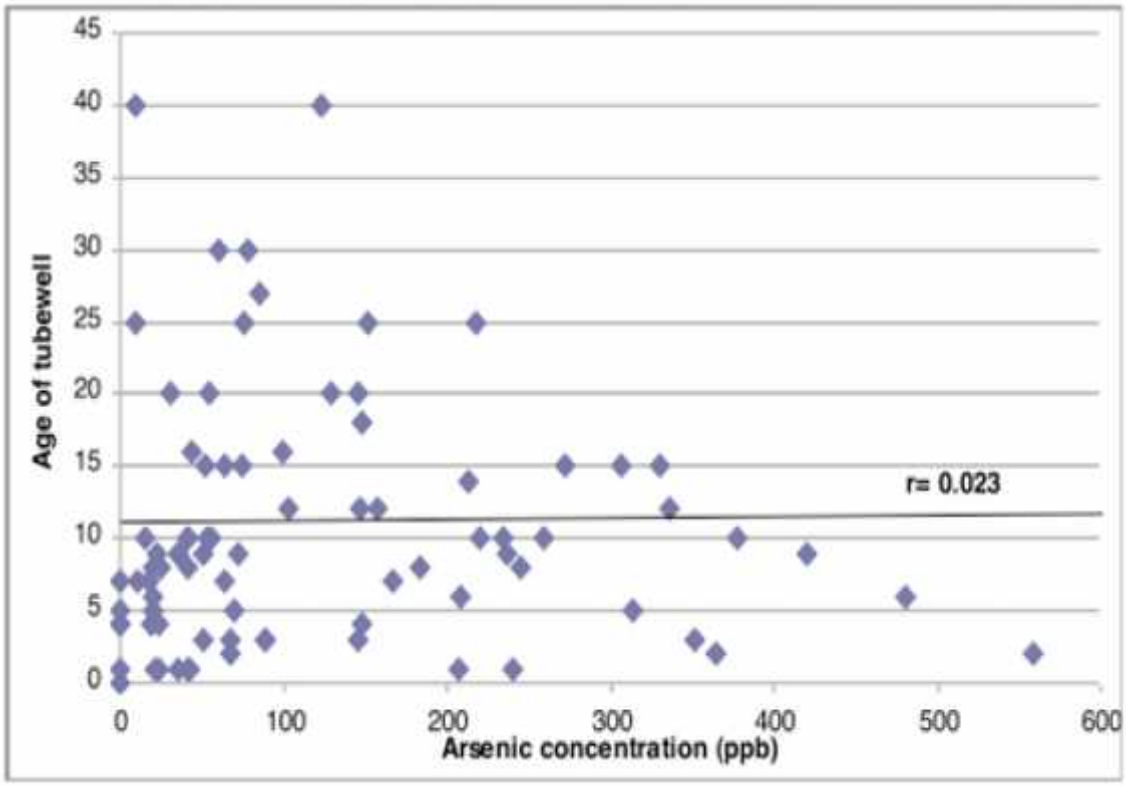

Figure 5 Scattered diagram of age of installation of tubewells and arsenic concentration

5.1.3.4 Relation of arsenic concentration with depth of tubewell

The relationship of the depth to arsenic concentration was shown in figure 6. The highest percentage of arsenic tested tubewells that exceed both the WHO Guideline and the NDWQS were to be found in wells with a depth of 36-95 feet. In this depth range, 50 (58.14%) tubewells were above National Drinking Water Quality Standard and 70 (81.39%) tubewells were above the WHO Guideline value (Table 7). At depths of 20-35 feet, 4 tubewells exceeded 10 ppb and 3 tubewells exceeded 50 ppb, whereas, above 95 feet only 3 (12.5%) tubewells exceeded WHO guideline value and non of tubewells exceeded NDWQS value. In one artesian tubewell with depth 630 feet, arsenic concentration was not detected. It therefore seems that the higher arsenic concentrations were generally found at the depths range of 36-95 feet. Regarding the relationship between arsenic and depth of tubewell, there was weekly negative correlation (r=-0.12, p=0.24) based on 86 water samples, which indicates that deeper tubewells have lesser

arsenic concentration. Generally, the value of arsenic concentration of groundwater decreases as the depth of tubewell increases.

Table 7 Classification of arsenic concentration by depth of tubewell

Well Depth (ft)	0-10		11-50		>50		Total	
	No	%	No	%	No	%	No	%
20-35	2	22.22	1	4.17	3	5.66	6	6.98
36-50	1	11.11	8	33.33	6	11.32	15	17.44
51-65	4	44.44	5	20.83	**27**	**50.94**	36	41.86
66-80	0	0.0	4	16.67	13	24.53	17	19.77
81-95	0	0.0	3	12.5	4	7.55	7	8.14
>95	2	22.22	3	12.5	0	0.0	5	5.81
Total	9	100	24	100	53	100	86	100

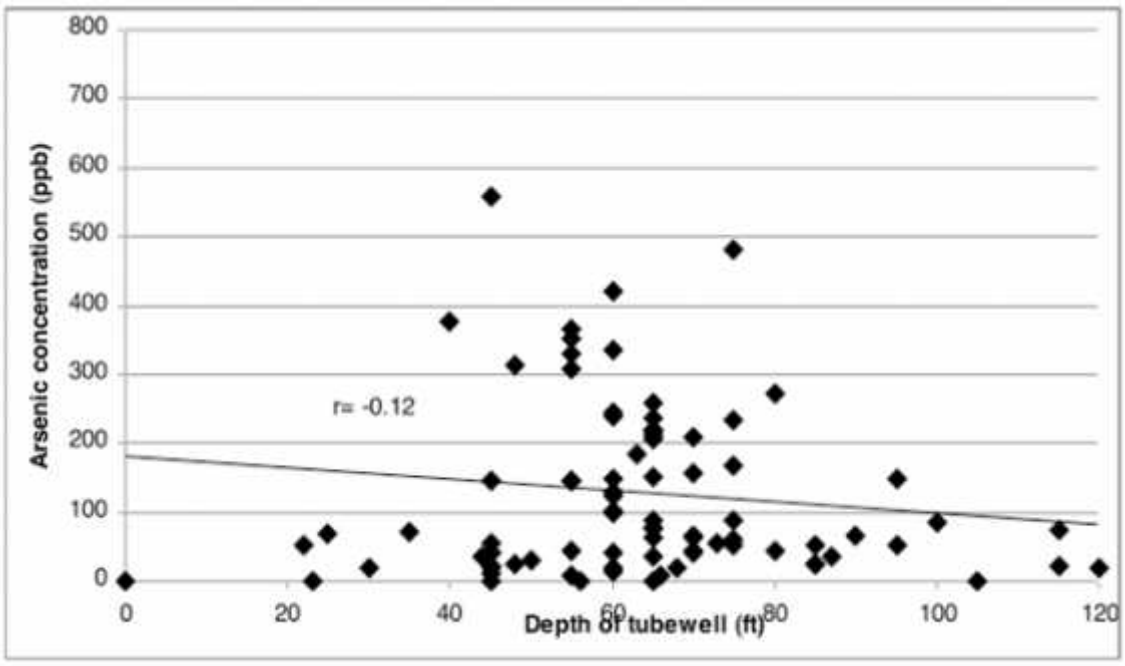

Figure 6 Scattered diagram of depth of tubewells and arsenic concentration

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5.1.4 Clinical manifestations of arsenicosis patient

As shown in table 8, among the 35 female respondents, 12 (34%) female had skin manifestation and out of 55 male respondents, 18 (33%) males observed skin manifestation. Gender difference in skin manifestation was statistically insignificant (χ^2=0.87) at 0.05 levels (Table 8). The reason behind this was unknown but it may be due to hormonal factor or carelessness of male or it may be due to alcohol users which suppress the immune system.

Table 8 Distribution of skin manifestation and gender

Gender	Skin manifestation		Total	Significance (p-value) *
	Yes	No		
Female	12(34)	23(66)	35	0.87
Male	18(33)	37(67)	55	
Total	30	60	90	

* Calculated by Pearson chi-square test

5.1.4.1 Types of arsenicosis among people

Among those 30 patients, table 5.23 shows that the patients were suffer from melanosis in palm, sole, trunk and chest; keratosis in palm and sole. 30% patients were suffered from diffused arsenical melanosis, 20% patients were suffered from diffused arsenical melanosis and keratosis, followed by 16.7% melanosis in palm. The result was shown in table 9.

Table 9 Distribution of arsenicosis by types and sex

Types of dermatological manifestations	Male	Female	Total
Melanosis in palm	2	3	5 (16.7)
Keratosis in sole	1	1	2 (6.7)
Diffused arsenical melanosis	6	3	9 (30.0)
Melanosis in chest	3	0	3 (10.0)
Diffused arsenical melanosis and keratosis	5	1	6 (20.0)
Keratosis in palm	0	1	1 (3.33)
Keratosis in palm and sole	1	3	4 (13.3)
Total	18	12	30

5.1.4.2 Age and sex-wise distribution of arsenicosis cases

The percentage of arsenicosis cases was highest (33.3%, 10 out of 30) in age group 32-41 years followed by 16.8% (5 out of 30) in age group 22-31 years and lowest in age group 62-72 years (table 10). Arsenicosis was found in all age groups but mostly in adult and young age groups. The reason behind this may be due to long exposure on arsenic contaminated water and less population of age above 50 years.

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

Age group	Female	Male	Total
12-21	2	2	4 (13.3)
22-31	2	3	5 (16.8)
32-41	3	7	10 (33.3)
42-51	2	2	4 (13.3)
52-61	2	2	4 (13.3)
62-72	1	2	3 (10.0)
Total	12	18	30

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5.1.4.3 Sex and stage-wise distribution of arsenicosis cases

Arsenicosis is classified in three stages: mild, moderate and severe. Table 11 reveals that all three types of cases were observed during field visit. Since most of its clinical forms was mild cases which was more or less homogeneously distributed among the patients of all age groups. Out of 30 arsenicosis cases, 20 (66.7%) had mild skin manifestation, 7 (23.3%) moderate and 3 (10%) severe.

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

Stages of arsenicosis	Male	Female	Total
Mild	12	8	20 (66.7)
Moderate	3	4	7 (23.3)
Severe	3	0	3 (10.0)
Total	18	12	30

5.1.4.4 Distribution of arsenicosis versus duration of tubewell use

The percentage of arsenicosis cases was higher among respondents having duration of tubewell use >10 years (41%) than ≤10 years (32%). However, there was no statistically significant difference in distribution of arsenicosis cases in such groups of tube well users (p=0.108) (Table 12).

Table 12 Distribution of arsenicosis cases versus duration of tubewell use

Duration of tubewell use	Skin manifestation			Significance (p-value)*
	Yes	No	Total	
≤10 years	18(32)	39(68)	57(100)	0.108
>10 years	12(41)	17(59)	29(100)	
Total	30	56	86	

* Calculated by Pearson chi-square test

5.2 Bacteriological analysis of water

Total eighty-six water samples from tubewell were analyzed for bacteriological study during study period from different places of Nawalparasi District. The membrane filter method was applied for the enumeration of total coliform bacteria and faecal coliform bacteria. Bacteriological analysis by membrane filter was done from influent water (groundwater) and effluent water (filtered water).

5.2.1 Total coliform count

The microbiological analysis of groundwater revealed the presence of total coliforms in 98.84% (85) of total samples i.e. almost all tubewell water was found positive for total coliforms but effluent water from filter shows only 64.28% (figure 7).

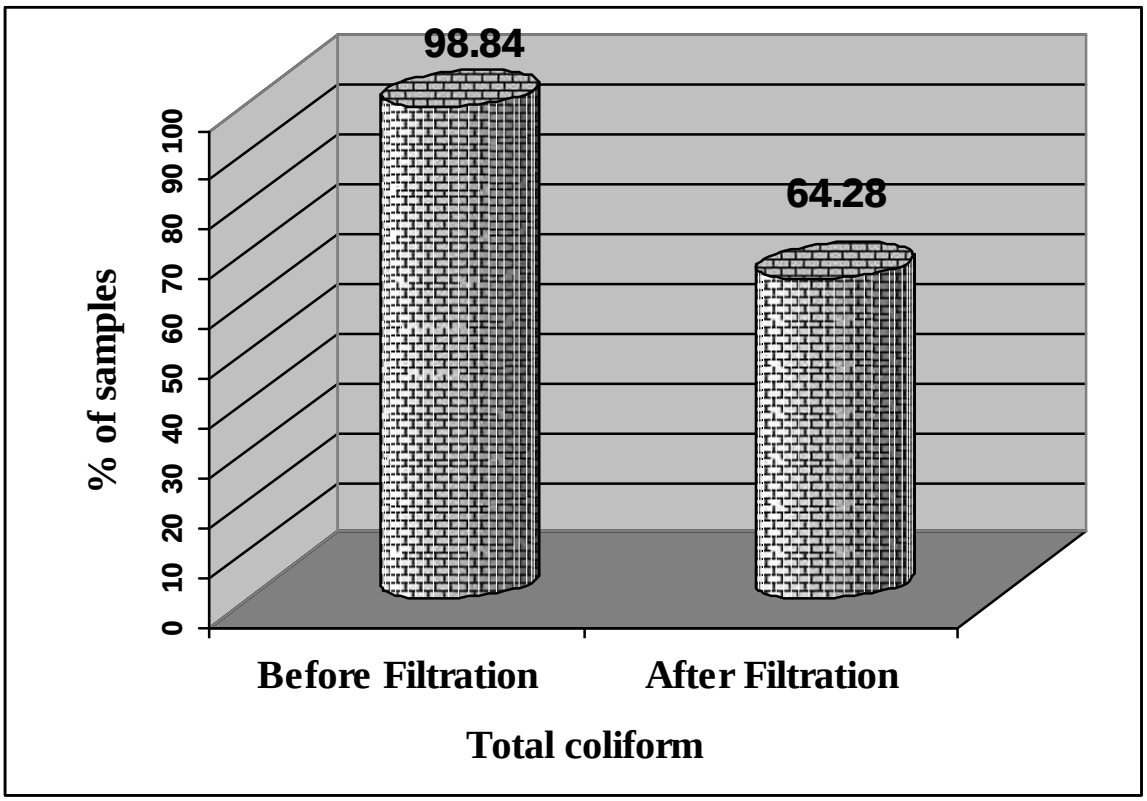

Figure 7 Analysis of water samples for total coliform by membrane filter technique

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5.2.2 Isolation and identification of enteric bacteria from membrane filtration

Altogether nine different kinds of enteric bacteria were isolated from 86 drinking water samples collected from different places of Nawalparasi District. The organisms were subjected to various biochemical tests for their identification. The organisms identified include *Escherichia coli, Klebsiella oxytoca, Klebsiella pneumoniae, Citrobacter* spp*, Enterobacter* spp*, Pseudomonas aeruginosa, Proteus vulgaris, Vibrio cholerae and Salmonella typhi.*

The data pertaining to the source-wise distribution of enteric bacterial isolates of water samples are presented in Table 13. Among the isolates, *E. coli* (37.04%) was found to be the most common in influent water followed by *P. aeruginosa* (16.05%), *Citrobacter* spp. (16.05%), *K. pneumoniae* (10.50%) and so on. Similarly in effluent water also, *E. coli* (39.21%) was found the most common followed by *P. aeruginosa* (19.61%), *K. oxytoca* (13.72%) and *Citrobacter* spp (13.72%).

Table 13 Source-wise distribution of enteric bacterial isolates from water samples

S. N.	Source	*E. coli*	*K. oxytoca*	*K. pneumoniae*	*Pseudomonas aeruginosa*	*Citrobacter* spp.	*Enterobacter* spp.	*Proteus vulgaris*	*Salmonella typhi*	*Vibrio Cholerae*	*Total*
1	Influent water	60 (37.04)*	16 (9.88)	17 (10.50)	26 (16.05)	26 (16.05)	9 (5.56)	4 (2.47)	3 (1.85)	1 (0.62)	162
2	Effluent water	20 (39.21)	7 (13.72)	6 (11.76)	10 (19.61)	7 (13.72)	1 (1.96)	0 (0.00)	0 (0.00)	0 (0.00)	51
Total		80 (38.27)	23 (11.00)	23 (11.00)	36 (17.22)	33 (15.79)	10 (4.78)	4 (1.91)	3 (1.41)	1 (0.47)	213

* Percentage of isolated bacteria

5.2.3 Isolation and identification of arsenic tolerant bacteria from PCA

Altogether thirteen arsenic tolerant bacteria were isolated from plate count agar incorporated with arsenic (V) >50 ppm. As shown in Table 14, the most common isolate was unidentified NP1 (Bluish green) (19.51%) followed by *Pseudomonas* spp. (16.38%), *Bacillus cereus* (11.15%) and so on.

Table 14 Types of bacteria isolated from arsenic contaminated groundwater

Types of bacteria	Total	Percentage
E. coli	19	6.62
Pseudomonas spp.	47	16.38
Klebsiella spp.	26	9.06
Staphylococcus spp	20	6.97
Acinetobacter spp.	14	4.88
Bacillus cereus	32	11.15
Bacillus subtilis	23	8.01
Flavobacterium spp.	6	2.09
Micrococcus spp.	18	6.27
NP1 (Bluish green)	56	19.51
NP2 (Yellow)	8	2.79
NP3 (Reddish brown)	7	2.44
NP4 (Creamy white)	11	3.83
Total	287	100

5.2.4 Colony count in plate count agar in different concentration of arsenic (V)

Table 15 shows that the colony count in PCA decreased as concentration of arsenic (V) increased from 0 ppm to 200 ppm. All 86 samples showed colony count ≥ 300 cfu/ml (TNTC) in 0 ppm arsenic (V). However, in 200 ppm all samples showed colony count below 300 cfu/ml. Similarly, the mean colony count of samples (having colony count

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below 300 cfu/ml) was highest in 10 ppm (216 cfu/ml) and lowest in 200 ppm (70 cfu/ml). Table 15 Colony count in PCA in different concentration of arsenic (V).

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

Arsenic concentration (ppm)	Number of sample	Mean colony count in PCA (cfu/ml)
0	86	TNTC*
10	83	TNTC
	3	216
25	70	TNTC
	16	213
50	16	TNTC
	70	210
100	9	TNTC
	77	142
200	86	70

* TNTC (Too numerous too count) = ≥ 300 cfu/ml

5.2.5 Minimum inhibitory concentration of arsenic tolerant bacteria

Table 16 shows that, among 13 isolates, the highest MIC value of As (III) was obtained for *E. coli* (mean=15 ppm) followed by NP1 (Bluish green) (mean=10.71ppm). Similarly the highest MIC value of As (V) was obtained for *Pseudomonas* spp. (mean=560 ppm) followed by *E. coli* (mean=360ppm).

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

Types of bacteria	N	Types of arsenic	Mean	Max	Min
E. coli	5	As(V)	360	600	200
		As(III)	**15**	25	6.25
Pseudomonas spp.	5	As(V)	**560**	800	200
		As(III)	8.125	12.5	3.125
Klebsiella spp.	5	As(V)	160	400	50
		As(III)	2.81	6.25	1.56
Staphylococcus spp	5	As(V)	160	400	50
		As(III)	2.186	3.125	1.56

Acinetobacter spp.	5	As(V)	180	400	25
		As(III)	2.81	6.25	1.56
Bacillus cereus	5	As(V)	280	400	200
		As(III)	6.25	12.5	3.125
Bacillus subtilis	5	As(V)	220	400	100
		As(III)	4.375	6.25	3.125
Flavobacterium spp.	4	As(V)	**93.75**	200	25
		As(III)	2.34	3.125	1.56
Micrococcus spp.	5	As(V)	210	400	50
		As(III)	3.124	6.25	1.56
NP1 (Bluish green)	7	As(V)	314.28	600	200
		As(III)	10.71	25	6.25
NP2 (Yellow)	5	As(V)	180	400	100
		As(III)	2.186	3.125	1.56
NP3 (Reddish brown)	4	As(V)	175	400	50
		As(III)	3.51	6.25	1.56
NP4 (Creamy white)	4	As(V)	150	200	10
		As(III)	**1.95**	3.125	1.56

5.3 Plasmid analysis

5.3.1 Profiles of plasmids from arsenic tolerant *E. coli*

Plasmids were extracted from 8 arsenic tolerant *E. coli* and one arsenic sensitive *E. coli* and run in 0.8% agarose gel which showed 8 types of plasmid profiles of size ranging from 3.1 to 16.21 kb. Two isolates showed single band, two showed double bands. Similarly one isolates contained three bands and one isolates showed five bands. The most repetitive band was 14.17 kb (in four isolates) followed by 6.16 kb (two isolates) and 8.5 kb (two isolates). A difference was found in size between arsenic tolerant and arsenic sensitive *E. coli* (Lane 13). In arsenic sensitive *E. coli*, one type of band size 4.36 kb was isolated which differs from arsenic tolerant *E. coli*. The results are shown in table 17 and photograph 5.

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

No	Lane	No. of bands		Approx. Size (Kb.)
N2	5	1	1st	14.17
N4	7	2	1st	14.17
			2nd	6.16
N6	9	3	1st	**16.21**
			2nd	4.25
			3rd	3.1
N7	10	2	1st	14.17
			2nd	8.5
N8	11	5	1st	10.1
			2nd	8.5
			3rd	6.16
			4th	5.2
			5th	3.3
N9	12	1	1st	14.17
S	13	1	1st	4.32

CHAPTER-VI

6 DISCUSSION AND CONCLUSION

6.1 Discussion

In the present study 86 drinking water samples from different sites were analyzed to assess the existing status of physical, chemical and bacteriological quality of drinking water sources of Nawalparasi district. In addition, isolation and identification of arsenic tolerant bacteria; MIC of bacteria in two forms As (III) and As (V); plasmid profiling of arsenic tolerant and sensitive *E. coli* was also carried out.

6.1.1 Physico-chemical parameters of water

These samples were analyzed for physicochemical parameters such as temperature, pH and arsenic. No standards or guidelines values regarding the temperature have been set. However, the test results of temperature showed the remarkable variation ranging from the minimum temperature (17°C) in the month of December/January in the tubewell water collected from Baikanthapur to maximum temperature (33°C) was recorded in the month of June from Shivanagar (Ramgram municipality). This result revealed that water bodies undergo temperature variation along the normal climatic fluctuations and seasonal variations. High temperature enhances the growth of microorganisms and may increase taste, odour, colour and corrosion problem.

The test results of pH values showed 88.37% of water samples were within the permissible limit of WHO standard, whereas 11.63% showed their pH values below the permissible limit and none of samples crossed the upper limit of guideline value. Maharjan (1998) had shown similar result with 88.6% within the permissible limit and 11.4% below the permissible. A study conducted jointly by JICA/ENPHO (2005) showed that out of total 134 samples tested in pre-monsoon, the pH value for one-third of the water samples (34.3%) was found to be below WHO guideline value, 65.7% samples within WHO guideline value and none of the sample exceeded the maximum WHO

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permissible limit. In drinking water, pH less than 7.0 may cause corrosion of metal pipes in the distribution system and higher that 8.0 adversely affect the disinfection process. Chlorination may be markedly less effective in increasing pH values.

Arsenic poisoning in the groundwater of Terai in Nepal is becoming a new challenge for nation's water supply sector. The prevalence of arsenicosis was high in whole country. According to Tuinhof and Nanni (2003), 25,000 water analyses of arsenic had been carried out and results indicate that 69% of groundwater's sampled had arsenic concentrations less than 10 ppb, while 31% exceeded 10 ppb and 8% exceeded 50 ppb. A study conducted jointly by NASC/UNICEF (2007), among the 338,949 tubewells tested, approximately 2.8% show values above the NDWQS, while approximately 10.15% of tubewells contain 11-50 ppb of arsenic concentration. Thus, in total, approximately 13% of tubewells are above WHO guideline (10ppb). The percentage of all the tubewells exceeding 50 ppb varies from 0.9% of the wells in Saptari to 11.7% in Nawalparasi. Similarly, the percentage of tubewells exceeding the WHO guideline ranges from 5.4% in Saptari to 23.8% in Nawalparasi. This study also revealed that majority of wells exceeding WHO lie in Kunwar (Ramgram municipality) and Gohini (Sunwal) sites. 53 (61.63%) tubewells was found above National Drinking Water Quality Standard and 89.53% tubewell was found above WHO guidelines. 30.16% filter (19 filtered water) shows above NDWQS whereas 68.25% filter (43 filtered water) shows above WHO guidelines, in 23 households there was no filter. Paired t-test analysis of arsenic concentration (before and after filtration) shows that there was significant difference ($p<0.05$). Out of 38 samples of Ramgram municipality, 53.7% tubewells were above NDWQS.

6.1.2 Age of installation

Concerning the age of installation of contaminated tubewells, it was found 16 (30.19%) tubewells exceed arsenic concentration above NDWQS in tubewell age between 6-10 years followed by 14 (26.42%), 12 (22.64%), 10 (18.87%), 1 (1.88%) tubewells above NDWQS in tubewell age 0-5 years, 16-30 years, 11-15 years and 31-50 years

respectively. The tubewells were of different ages from recently installed to 40 years old. Among 86 tubewells, 57 (66.27%) tubewells were constructed within the last 10 years and 29 (33.73%) tubewells constructed in the last 10-40 years. It was found moderately positive correlation (r=0.23, p=0.83) between the age of installation of tubewells and arsenic concentration. ENPHO/NRCS (2003) conducted similar tests and found that levels of arsenic in water were positively correlated (r=0.079) to the age of tubewells. According to NASC/UNICEF (2005), approximately about 98.9% of the arsenic tested tube wells vary from 0 to more than 100 years old (dug well). In the eight study districts, approximately 74% of the tube wells (335,449) were constructed within the last 10 years and approximately 25% of the tubewells were constructed in the last 10-50 years. The analysis of tube wells in all the districts does not, however, show any significant correlation between arsenic concentration levels and age.

6.1.3 Depth of tubewell

Arsenic concentration was found above both the WHO Guideline and the NDWQS in wells with a depth of 36-95 feet. In this depth range, 50 (58.14%) tubewells were above National Drinking Water Quality Standard and 70 (81.39%) tubewells were above the WHO Guideline value. At depths of 20-35 feet, 4 tubewells exceeded 10 ppb and 3 tubewells exceeded 50 ppb, whereas, above 95 feet only 3 (12.5%) tubewells exceeded WHO guideline value and non of tubewells exceeded NDWQS value. In one artesian tubewell with depth of 630 feet, arsenic concentration was undetectable. It therefore seems that the higher arsenic concentrations were generally found at the depths range of 36-95 feet. There was weekly negative correlation (r= -0.12, p=0.24) between arsenic concentrations level and depth of the tubewell. Generally, it was found that the value of arsenic concentration of groundwater decreases as the depth of tubewell increases. Similar results were obtained by ENPHO/NRCS (2003), NASC/UNICEF (2005) but found very low negative correlation (-0.05) in NASC/ENPHO (2003).

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6.1.4 Health effects

Among total 90 enrollee's dermatological examinations, 35 (38.89%) were female and 55 (61.11%) were male. 30 (33.33%) of these patients among total enrollees had arsenical skin manifestations. Gender difference in skin manifestations was statistically insignificant (p=0.87) at 0.05 levels. This result was different from the findings of Maharjan (2006) 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.

Regarding the demographic feature of the affected cases, majority of them (33.3%) was in reproductive age group (31-42 years) followed by (16.8%) in 22-31 years. The finding is similar to study reported by ENPHO (2002) in Nawalparasi district where majority of arsenicosis was found in reproductive age group (15-49 years). Maharjan (2004) reported similar results that higher prevalence in age group 30-39 and 40-40 years in a community study and Rahman *et al.,* (2006) from Bangladesh, in age group 30-44 years.

Arsenicosis cases were identified by observing skin manifestation on their body. Among the cases, the most common manifestation was diffused arsenical melanosis (30%) followed by diffused arsenical melanosis and keratosis (20%). As in many preceding studies by Mazumder *et al.,* (1998) in West Bengal and Ahmed *et al.,* (1999) in Bangladesh, the current study revealed that manifestations in skin, such as melanosis and keratosis, were common among the arsenicosis patients. In Nepal, the study conducted by Maharjan *et al.,* (2006) found the most common manifestations was melanosis (95.7%) followed by leukomelanosis (57%) and keratosis (55.9%).

Regarding the extent of arsenicosis, most cases were found at the mild (66.7%) and moderate stages (23.3%). Only 3 (10%) arsenicosis patients had severe stage of arsenicosis, leaving majority of patient's mostly mild and moderate dermatological symptoms as detected by Maharjan *et al*., (2006). In Bangladesh, complications, such as non-pitting pedal oedema, gangrene, and cancer, has been reported in different studies

conducted by Ahmad *et al.*, (1999) and Ahmad *et al.,* (2001), but, in Nepal, no such complications have yet been reported. It may be due to lack of equipment for lab diagnosis and trained health professionals.

6.1.5 Microbial quality of water

The majority of diseases in developing countries are infectious in nature caused by bacteria, viruses and other microbes, which are shed in human feces and pollute water supplies which people use for drinking and washing purposes. The various studies related to drinking water quality also showed that the water sources from the rural areas of developed countries were found to be contaminated with coliforms. The fact was clarified in the studies done by Anwar *et al.* (1999), Venus (2005) and Zhang *et al.* (1997).

Last two decades, people are using water from groundwater instead of open dug wells and river water because of regular phenomenon of water borne epidemics. Well protected groundwater is safer in terms of microbiological quality than water from open dug well and ponds. However, it is found that groundwater is prone to toxic chemical including arsenic. Besides this improperly disposed sewage near water sources contaminate groundwater by pathogenic and non-pathogenic bacteria. Therefore, drinking water quality assessment has always been crucial with reference to public health importance. A research on microbiological analysis of water conducted by Bajracharya (2007) revealed that out of 114 samples, only 26.32% tap water and 2.63% tubewell water were total coliform count negative. Source wise distribution of coliform count clearly showed that tubewell (97.37%), tap (73.68%) and stone spout (100%) crossed the WHO guideline value. Similarly Sharma (2005) found *Kanchan*^{TM} Arsenic filters were found to be effective in removing total coliform. Four of five filters were able to remove over 95% total coliforms. Removal efficiency of the filter remained at 0 % until the eighteenth day and, rise to the value of 96 % on the twenty-seventh day of the experimental period. The reason for this low concentration of total coliform might be due to the remains of Piyush

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(0.5% chlorine solution) during the installation of filter.

In the present study, the microbiological analysis of water revealed the presence of total coliforms in 98.84% of total influent water samples from tubewell i.e. the majority of water samples taken were found positive for total coliforms and crossed the WHO guideline. Only 1.16% tubewell samples were total coliform negative. Similarly out of 86 households, only 60 households contain bio-sand filter. Most filters from Gohini and Nadawa sites were not in use. Among the 60 filters present, 49 (81.7%) effluent water from bio-sand filter revealed the presence of total coliform and crossed the WHO guideline value. Only 18.3% effluent water was found total coliform negative. Biological processes in the KAF can give up to 86% efficiency on bacterial removal. However, the bacterial removal efficiency may be low (only 50-60%) during the period immediately after filter installation. It normally takes a period of one to two weeks for a biological layer to develop to maturity in a new filter. The removal efficiency increases with the growth of biological layer. In this research, the efficiency of bacterial removal was found low. This may be due to lack of cleaning filter from long time. Over the long period of use, particles and dirt will be collected on the top of the fine sand layer and arsenic as well as bacterial removal may decreased. These filtered materials tend to clog the filter reducing the filtration rate. Depending on the quality of the influent water (e.g. turbidity, iron concentration, usage, and seasons (e.g. monsoon), the filter may need to be cleaned once every month to once every 6 months (ENPHO, 2005).

6.1.6 Enteric bacteria isolated from membrane filtration

Using total coliform enumeration, enriched alkaline peptone and Selenite F broth, nine different kinds of enteric bacteria were isolated from the arsenic contaminated tubewell samples. Among them, *E. coli* (37.04%) was found to be the most common followed by *P. aeruginosa* (16.05%), *Citrobacter* spp. (16.05%), *K. pneumoniae* (10.50%), *K. oxytoca* (9.88%), *Enterobacter* spp. (5.56%), *P. vulgaris* (2.47%), *S. typhi* (1.85%) and *V. cholerae* (0.62%) in influent water. Whereas in effluent water from 60 bio-sand filters, E. coli (39.21%) was found predominant followed by *P. aeruginosa* (19.61%), *K.*

oxytoca (13.72%), *Citrobacter* spp. (13.72%), *K. pneumoniae* (11.76%) and *Enterobacter* spp. (1.96%).

Maharjan (1998) also isolated *Enterobacter* spp. as the predominant bacteria followed by *E. coli, Citrobacter* spp., *Salmonella* spp. and others. Similarly Prasai (2002) isolated 238 enteric bacteria from different sources. Percentage recovery of *E. coli* (26.4%) was found to be high followed by *Enterobacter* spp. (25.6%), *Citrobacter* spp (22.6%), *Pseudomonas* spp. (6.3%), *Klebsiella* spp. (5.4%), *Shigella* spp. (3.78%), *S. typhi* (3.3%), *P. vulgaris* (2.9%), *Serratia* spp. (2.53%) and *Vibrio cholerae* (0.84%). Bajracharya (2007) also isolated enteric bacteria like *Citrobacter* spp. (26.22%), *E. coli (25%), Enterobacter* spp. (20.73%), *Shigella* spp. (8.54%), *P. vulgaris* (7.93%) etc.

6.1.7 Arsenic tolerant bacteria isolated from PCA

Among thirteen arsenic tolerant bacteria isolated from plate count agar incorporated with arsenic (V) >50 ppm, unidentified NP1 (Bluish green) (19.51%) was predominant one followed by *Pseudomonas* spp. (16.38%), *B. cereus* (11.15%) and so on. Tuladhar (2006) obtained nine arsenic tolerant bacteria isolated which are identified as three *Bacillus* spp., one *Micrococcus* spp., and two *Pseudomonas* spp. The rest three of the arsenic tolerant bacteria are unidentified.

6.1.8 Colony count in PCA in different concentration of As (V)

For the isolation of bacteria, samples were enumerated in PCA. It was found that the colony count in PCA decreased as concentration of arsenic (V) increased from 0 ppm to 200 ppm. All 86 samples showed colony count ≥ 300 cfu/ml (TNTC) in 0 ppm arsenic (V), however, in 200 ppm all samples showed colony count below 300 cfu/ml. Similarly, the mean colony count of samples (having colony count below 300 cfu/ml) was highest from the samples grown at 10 ppm (216 cfu/ml) and lowest in 200 ppm (70 cfu/ml).

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6.1.9 Minimum inhibitory concentration of arsenic tolerant bacteria

Among 13 isolates, the highest MIC value of As (III) was obtained for *E. coli* (mean=15 ppm) followed by NP1 (Bluish green) (mean=10.71 ppm). Similarly the highest MIC value of As (V) was obtained for *Pseudomonas* spp. (mean=560 ppm) followed by *E. coli* (mean=360 ppm). According to Tuladhar (2006), most of the bacteria from isolate have MIC higher than 1000 ppm and some of the arsenic tolerant bacteria like R_1-32 Curl, RJW-18 and R_3-5 (*Bacillus* spp.) having MIC equal to 300 ppm, 350 ppm and 300 ppm respectively.

6.1.10 Plasmids of arsenic tolerant and sensitive *E. coli*

Plasmids were selected extracted from 8 arsenic tolerant *E. coli* and one arsenic sensitive *E. coli* and run in agarose gel which showed 8 types of plasmid profiles of size ranging from 3.1 to 16.21 kb. Two isolates showed single band, two showed double bands. Similarly one isolates contained three bands and one isolate showed five bands. The most repetitive band was 14.17 kb (in four isolates) followed by 6.16 kb (two isolates) and 8.5 kb (two isolates). It was found difference in band size between arsenic tolerant and arsenic sensitive *E. coli*. In arsenic sensitive *E. coli,* one type of band size 4.36 kb was isolated which differs from arsenic tolerant *E. coli.* Multiple band sizes may be due to types of plasmid (linear, supercoiled and nicked). Supercoiled plasmid moves faster in gel than linear, as a result band size may vary. In some lanes, smearing was observed which may be due to either presence of genomic or plasmid DNA that was smeared as a result of preparation.

6.2 Conclusion

The present study disclosed the physiochemical and bacteriological contamination of groundwater in Nawalparasi District. The major source of water for drinking and cooking in selected sites is tubewell. Arsenic contamination was high in most of samples collected from tubewell which exceeds the drinking limit of WHO and NDWQS. There is overwhelming evidence of arsenicosis in this district especially in Thulo Kunwar, Gohini because of consuming elevated levels of arsenic contaminated water. To mitigate arsenic

concentration from drinking water, Kanchan arsenic filter was distributed. Coliform contamination was also high in tubewell as well as in effluent water from filter. Such circumstances are responsible for spreading water borne epidemics. The waterborne diseases are closely related with the conditions of living and environmental sanitation in the community. So, both acute and severe diseases can be effectively controlled by appropriate water management and safe disposal of sewage and excreta. Arsenic concentration was found higher in depth between 36-95 feet which indicates deep tubewell (i.e. artesian tubewell) contain low or no arsenic concentration. Thirteen arsenic tolerant isolates were isolated, among them unidentified (NP1, bluish green) colony showed highest MIC value. Plasmid profiles of *E. coli* showed the most repetitive band was 14.17 kb (in four isolates) followed by 6.16 kb (two isolates) and 8.5 kb (two isolates) but there was found variation in size between arsenic tolerant *E. coli* and arsenic sensitive *E. coli.*

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CHAPTER-VII

7 SUMMARY AND RECOMMENDATIONS

7.1 Summary

1. A total of eighty six samples of tubewell water were collected from working areas of Filters for Families. All water samples were analyzed for physicochemical and microbiological parameters to assess the drinking water quality. Samples were collected from seven sites of Nawalparasi District containing arsenic concentration above WHO guidelines value.

2. The physicochemical analysis of water reflects the following results. The temperature ranges from 17°C to 33°C in different sites. The pH of 11.63% of water samples were found to lie below WHO guideline value.

3. Among seven different places, most of samples (44.19%) were collected from Ramgram Municipality and among them most samples (53.7%) were found above National Drinking Water Quality Standard (50 ppb).

4. Among total 86 water samples, 61.63% tubewell was found above National Drinking Water Quality Standard and 89.53% tubewell was found above WHO guidelines in influent water. In effluent water from filter, 30.16% filter (19 filtered water) shows above NDWQS whereas 68.25% filter (43 filtered water samples) shows above WHO guidelines. In 23 households there was no filter. Paired T-test analysis of arsenic concentration shows that there was significant difference before and after filtration ($p<0.05$).

5. The tubewells were of different ages from recently installed to 40 years old. 16 (30.19%) tubewells exceed arsenic concentration above NDWQS in tubewell age

between 6-10 years. For the relationship of arsenic with tubewell age, moderately positive correlation was obtained between arsenic concentration and age of tubewells (r=0.23, p=0.83).

6. In the depth range 36-95 feet, 50 (58.14%) tubewells were above NDWQS and 70 (81.39%) tubewells were above the WHO Guideline value. Regarding the relationship between arsenic and depth of tubewell, there was weekly negative correlation (r=-0.12, p=0.24), which indicates that deeper tubewells have lesser arsenic concentration.

7. Among the 35 female respondents, 12 (34%) female had skin manifestation and 18 (33%) out of 55 males observed skin manifestation. Gender difference in skin manifestation was statistically insignificant (χ^2=0.87) at 0.05 levels. This data shows that male was most predominant on arsenicosis than female.

8. People were suffer from melanosis in palm, sole, trunk and chest; keratosis in palm and sole. 30% patients were suffered from diffused arsenical melanosis, 20% patients were suffered from diffused arsenical melanosis and keratosis, followed by 16.7% melanosis in palm.

9. Arsenicosis was found in all age groups but mostly in adult and young age groups. The percentage of arsenicosis cases was highest (33.3%, 10 out of 30) in age group 32-41 years followed by 16.8% (5 out of 30) in age group 22-31 years and lowest in age group 62-72 years.

10. Among total arsenicosis patients, 20 (66.7%) had mild skin manifestation, 7 (23.3%) moderate and 3 (10%) severe.

11. Arsenicosis cases was higher among respondents having duration of tubewell use >10 years (41%) than ≤10 years (32%). However, there was no statistically significant

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difference in distribution of arsenicosis cases in such groups of tube well users (p=0.108).

12. The bacteriological analysis of water samples revealed the presence of total coliform in 98.84% of total samples but 64.28% of effluent water also found positive to total coliform.

13. Nine different kinds of enteric bacteria were isolated from the total contaminated samples. *E. coli* was mostly the predominant in influent (37.04%) and effluent water (39.21%).

14. The most predominant arsenic tolerant bacteria obtained in Plate Count Agar incorporated with As (V) above 50 ppm was unidentified NP1 (Bluish green) (19.51%) followed by *Pseudomonas* spp. (16.38%), *Bacillus cereus* (11.15%) and so on.

15. All 86 samples showed colony count ≥ 300 cfu/ml (TNTC) in 0 ppm arsenic (V), however, in 200 ppm all samples showed colony count below 300 cfu/ml. Similarly, the mean colony count of samples (having colony count below 300 cfu/ml) was highest in 10 ppm (216 cfu/ml) and lowest in 200 ppm (70 cfu/ml).

16. Among 13 isolates, the highest MIC value of As (III) was obtained for *E. coli* (mean=15 ppm) followed by NP1 (Bluish green) (mean=10.71ppm). Similarly the highest MIC value of As (V) was obtained for *Pseudomonas* spp. (mean=560 ppm) followed by *E. coli* (mean=360 ppm).

17. Eight types of plasmid profiles of band size ranging from 3.1 to 16.21 kb. Two isolates showed single band, two showed double bands. Similarly one isolates contained three bands and one isolates showed five bands. The most repetitive band size of arsenic tolerant was 14.17 kb (in four isolates) followed by 6.16 kb (two

isolates) and 8.5 kb (two isolates). In arsenic sensitive *E. coli* with band size 4.36 kb was isolated which differs from arsenic tolerant *E. coli.*

7.2 Recommendations

Based on overall understanding about the gravity of the arsenic poisoning situation in Nawalparasi District of Nepal substantiated by the field survey and laboratory findings, it would be pertinent and timely to forward the following recommendations to the competent and concerned authorities:

- The isolated tolerant organisms can be used for the study of their potential in bioaccumulation and bioremediation of heavy metal contamination, as well as transformation and mobilization of arsenic in groundwater. So further study will be useful in developing a combined microbiological–chemical process for treating arsenic-contaminated groundwater.
- Similar studies can be done in other VDCs of Nawalparasi District to cover whole of this district and show the whole scenario.
- In place like Gohini and Kunwar, where efficiency of bio-sand filter (KAF) is low due to high arsenic concentration, it should be replaced by another suitable filter like SONO filter which has more efficiency on both arsenic and pathogen removal.
- Tubewell that has arsenic concentration between WHO guideline and National Drinking Water Quality Standard, or 11-50 ppb, must be the immediate focus of concerned agencies by regular monitoring of these wells in order to determine whether arsenic is increasing in the wells at a specific place.
- Deep artesian tubewell is the effective alternative source to other temporary arsenic removal techniques, as there is an absence of arsenic concentration in deep tubewell.
- Further molecular work such as restriction enzyme digestion, ligation and transformation, polymerase chain reaction (PCR) etc. can be done from purified DNA.

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CHAPTER-VIII

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APPENDIX-I

Household Questionnaire

Serial no. : ___ District: Nawalparasi VDC: _____ Tole: _____
Date of interview: _____ Ward no: ____

General demographic information

1. Information of Respondents

1.1 Name ……
1.2 Sex: a. Male b. Female
1.3 Age …….
1.4 Occupation
a. Farmer b. Labor c. Service d. Business e. Student f. Teacher g. Housewife
1.5 Education
a. Literate b. Illiterate

Technical evaluation

2. At present where do you collect your drinking water?
a) Artesian Tubewell b) Dug wells c) Shallow tubewell d) Tap water
f) River water

3. Age of tube well (installation in yr): …….. Depth of tube well (ft): ……

4. Concentration of Arsenic in tube well if known (µg/l): …….

5. Use of tubewell a) Drinking, cooking and washing b) Irrigation
c) All of above d) Not in use

6. Do you know about arsenic, if yes, where have you heard about arsenic contamination?
a) NGO/INGO b) Radio/Television c) Government d) Health worker/Doctor

7. Do you know the technique of water purification to remove arsenic?
a) Yes b) No
If Yes, how? …….

8. What are the available safe water options to prevent exposure to Arsenic?
a) Use dug tube wells
b) Using filter for removing Arsenic
c) Using tap water
d) Deep water aquifers
e) Filter surface water
f) Rain water harvesting

d) All of the above
e) None of the above

Health related information

9. Do you have any health problem by drinking water containing Arsenic?
i) Yes ii) No
If Yes, What are they?
a) Keratosis on palm
b) Keratosis on sole
c) Melanosis in palm
d) Melanosis in sole
e) Melanosis in chest
f) Diffused Arsenical Melanosis
g) Diffused Arsenical Keratosis

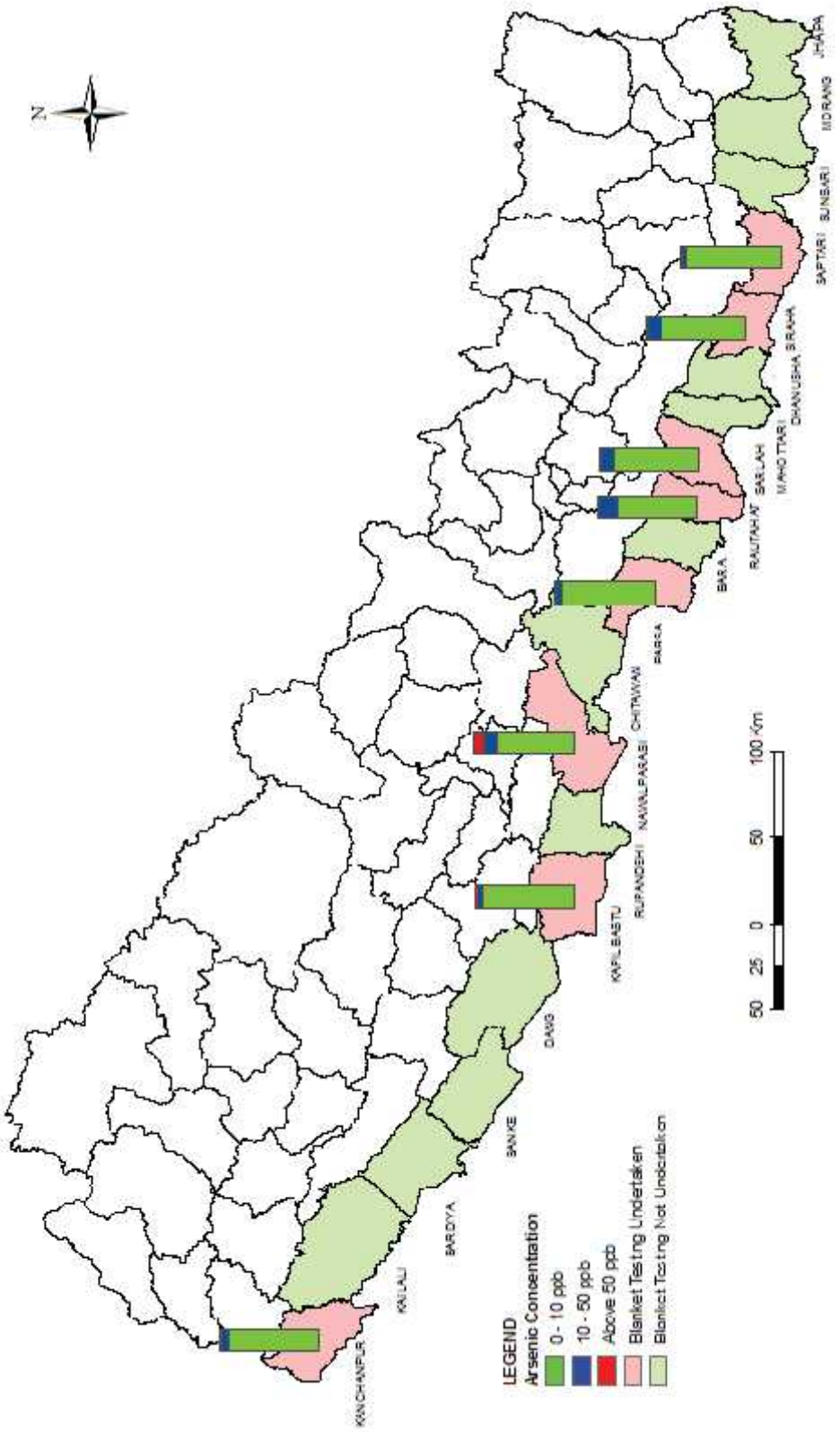
List the name below beside the respondents (if they have symptoms of arsenicosis:

S.N.	Name	Sex	Age	Symptoms
1				
2				
3				

10. Do you have other health problems other than above symptoms? a) Yes b) No
If Yes, What are the problems?
i. Respiratory ii. Cough iii Urinary iv. Weakness v. Eyes
vi. Others: ……

APPENDIX-II

ARSENIC CONCENTRATIONS IN EIGHT DISTRICTS



Source: NASC/UNICEF, 2005

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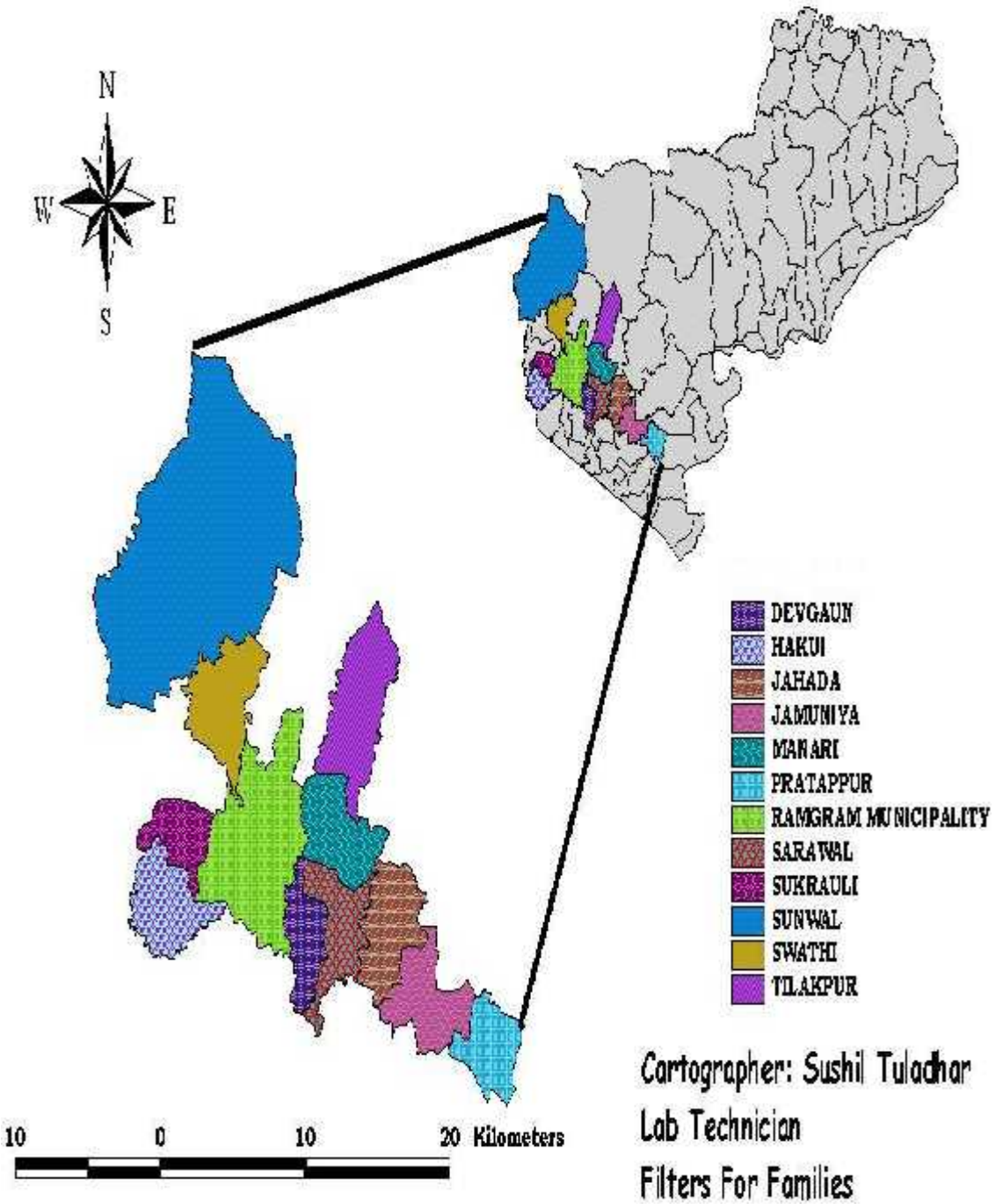
"Working VDC of Filters For Families in Nawalparasi District"



NAWALPARASI DISTRICT
Tanahu
Chitwan
Nawalparasi
Rupandehi
Parasi
Bhairahawa
Legend
Boundaries
Districts
VDC
Major Roads
Highway
Feeder Road
District Road
Other Road
Other VDCs
Municipalities
Water bodies
Map Location
Map Created
June 15, 2006
Km
Office for the Coordination of Humanitarian Affairs (OCHA)
United Nations, Nepal

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APPENDIX-III

LIST OF EQUIPMENT AND MATERIALS USED DURING THE STUDY

A. MATERIALS

Equipments

Hot air oven	Universal (India)
Microwave oven	LG
Incubator	Memmert (Germany)
Autoclave	
Refrigerator	Sanyo (Japan)
Microscope	Olympus (Japan)
Centrifuge	Remi (India), Eppendorf (Germany)
Weighing Machine	Scaltec instruments (Germany)
Water bath shaker	Grant, OLS 200 (UK)
UV transilluminator	Alpha Innotech Corporation (Germany)
Thermal printer	Alpha Innotech Corporation (Germany)
Electrophoresis apparatus	International Biotechnologies Inc, USA
Voltguard	Fisher Scientific, USA
AC automatic voltage	
Regulator	Matsunaga , Japan
Spectrophotometer	Alpha Innotech Corporation (Germany)
pH meter	HANNA Company
Ice box/ Ice packets	

B. MICROBIOLOGICAL MEDIA

Culture media

MacConkey agar	Hi-media Laboratory
Nutrient agar	Hi-media Laboratory
Nutrient broth	Hi-media Laboratory
M-endo agar	Hi-media Laboratory
Plate count agar	Hi-media Laboratory
Peptone	Qualigens Fine Chemicals, India
Bacillus differential agar	Hi-media Laboratory
Luria Berteni agar / broth	

Biochemical media

O/F medium	Hi-media Laboratory
Nitrate reduction medium	Hi-media Laboratory
TSI medium	Hi-media Laboratory
MRVP broth	Hi-media Laboratory
Simmon's citrate agar	Hi-media Laboratory

Starch powder	S.D. fine chemicals Ltd.
Sulphide-indole motility media	Hi-media Laboratory
Urease broth base	Hi-media Laboratory

C. CHEMICALS AND REAGENTS

3%Hydrogen peroxide	Barritt's reagent	Barium chloride
Crystal violet	Absolute (95%) alcohol	Kovac's reagent
Gram's iodine	Sulphuric acid	Calcium chloride
Tween 80	Tris base	Glycine
Agarose	Sodium chloride	Tris buffer
Sodium hydroxide	Potassium acetate	Glacial acetic acid
Ethidium bromide	SDS	Glucose
Glycerol	Bromophenol blue	Sucrose
Iodine	Magnesium chloride	Arabinose
Mannitol	Ethylene diamine tetra acetate (EDTA)	

Staining reagents

Acetic acid	Alcohol	Bromophenol blue
Calcium chloride	Crystal violet	Glycerol
Glycine	Iodine	Magnesium chloride
Safranine		

Tests reagents

α-napthol	3% hydrogen peroxide	Ethyl alcohol
Hydrochloric acid	Mercuric chloride	Methyl red
Potassium hydroxide	Sodium chloride	Sulphanilic acid
Tetra methyl paraphenylene dihydrochloride		

D. MISCELLANEOUS

Conical flasks, Cotton, Distilled water, Droppers, Forceps, Glass slides and cover slips, Immersion oil, Inoculating loop, Inoculating wire, Lysol, Measuring cylinder, Erlenmeyer flask, Petri dishes, Pipettes, Plastic containers, Spatula, Test tubes, Glass rods, Membrane filter

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APPENDIX-IV

COMPOSITION AND PREPARATION OF DIFFERENT CULTURE MEDIA

I. CULTURE MEDIA

1. Alkaline peptone water

Ingredients	Grams/litre
Peptone	10
Sodium chloride	10
Final pH (at 25°C)	8.8±0.2

Preparation: The peptone and sodium chloride was dissolved in water and sterilized by autoclaving at 121°C for 15 minutes.

2. Bacillus differentiation agar (Hi-media)

Ingredients	Grams/litre
Yeast autolysate	0.2
Mannitol	5.0
Monohydrogen ammonium phosphate	1.0
Potassium chloride	0.2
Magnesium sulphate	0.2
Bromocresol purple	0.0075
Agar	15.40
Final pH (at 25°C)	7.2±0.2

Use: For differentiation between *Bacillus cereus* and *B. subtilis* based on mannitol fermentation.

3. MacConkey Agar (MA) (Himedia chemicals, India)

(With sodium taurocholate, with salt and crystal violet)

Ingredients	Gm/liter
Peptone	20.0
Lactose	10.0
Sodium taurocholate	5.0
Crystal violet	0.015
Sodium chloride	5.0
Neutral red	0.04
Agar	20.0
Final pH (at 25°C)	7.4±0.2

Procedure: 55 grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121°C (15 lbs pressure) for 15 minutes.

4. M-endo agar (Himedia chemicals, India)

Ingredients	Grams/litre
Casein enzymatic hydrolysate	3.7
Peptic digest of animal tissue	3.7
Tryptose	7.5
Yeast extract	1.2
Lactose	9.4
Dipotassium phosphate	3.3
Monopotassium phosphate	1.0
NaCl	3.7
Sodium deoxycholate	0.1
Sodium lauryl sulphate	0.05
Basic fuschin	0.8
Sodium sulphate	1.6
Agar	15
Final pH (at 25°C)	7.2±0.2

Procedure: 51 grams M-endo agar was suspended in 980 ml distilled water. It was boiled to dissolve the medium completely but not autoclaved. It was cooled to 45°C and 20 ml of 95% ethanol was aseptically added. It was mixed and dispensed 4 ml amounts into 60 mm Petri plates. In large plates, sufficient medium was used to give a 1.5 mm depth. Plates do not expose to direct sunlight.

5. Nutrient Broth (Hi-media chemicals, India)

Ingredients	(Grams/litre)
Peptic digest of animal tissue	5.00
Beef extract	1.50
Yeast extract	1.50
Sodium chloride	5.00
Final pH (at 25°C)	7.4 ± 0.2

Sterilized by autoclaving at 15 lbs pressure (121°C for 15 minutes).

Procedure: 13 grams of the medium was dissolved in 1000 ml distilled water and autoclaved at 121°C for 15 minutes.

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6. Nutrient Agar (Himedia chemicals, India)

Ingredients	(Grams/litre)
Peptic digest of animal tissue	5.00
Beef extract	1.50
Yeast extract	1.50
Sodium chloride	5.00
Final pH (at 25°C)	7.4 ± 0.2

Procedure: 37 grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121°C (15 lbs pressure) for 15 minutes.

7. Plate count agar (Himedia chemicals, India)

Ingredients	grams/litre
Tryptone	5
Yeast extract	2.5
Dextrose	1
Agar	9
Final pH (at 25°C)	7.4 ± 0.2

Procedure: 23.5 grams of the medium was dissolved in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121°C (15 lbs pressure) for 15 minutes.

8. TCBS agar

Ingredients	grams/litre
Yeast extract	5
Peptone	10
Sodium thiosulphate	10
Sodium citrate	10
OX bile	8
Sucrose	20
Sodium chloride	10
Ferric citrate	1
Bromothymol blue	0.04
Agar	15
Final pH (at 25°C)	8.6±0.2

Procedure: 89 grams of media was dissolved in 1000ml of distilled water, heat to boiling to dissolve the media completely. This medium does not need to be autoclaved.

9. Salmonella Shigella (SS) agar

Ingredients	grams/litre
Beef extract	5
Peptone	5
Lactose	10
Bile salts	8.5
Sodium citrate	10
Sodium thiosulphate	8.5
Ferric citrate	10
Brilliant green	0.00033
Neutral red	0.025
Agar	15
Final pH (at 25°C)	7.4 ± 0.2

Preparation: 63 grams of media was dissolved in 1000ml of distilled water and heated with frequent agitation to dissolve the media completely. The media was not autoclaved and overheating was avoided.

10. Selenite F broth

Ingredients	grams/litre
Tryptone	5
Lactose	4
Sodium phosphate	10
Sodium acid selenite	4
Final pH (at 25°C)	7.4 ± 0.2

Procedure: 23 grams of media was dissolved in 1000ml of distilled water and heated to dissolve the media and then sterilized in a boiling water bath for 10 minutes. The media was not autoclaved and excessive heating was avoided.

II. Biochemical media

1. Simmons Citrate Agar

Ingredients	Grams/litre
Monoammonium phosphate	1.0
Dipotassium phosphate	1.0
Sodium chloride	5.0
Sodium citrate	2.0
Magnesium sulphate	0.2
Bromothymol blue	0.08
Agar	15
Final pH at (25°C)	6.8 ± 0.2

Procedure: 24.28 grams was suspended in 1000 ml distilled water. It was heated to dissolve the medium completely. It was distributed in tubes and sterilized by

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autoclaving at 15 lbs pressure at 121°C for 15 minutes. The medium in tubes were solidified in slanted position.

2. Glucose Phosphate Broth (MR-VP broth)

Ingredients	Grams/litre
Buffered peptone	7.0
Dextrose	5.0
Dipotassium phosphate	5.0
Final pH at (25°C)	6.8 ± 0.2

Procedure: 17 grams was dissolved in 100 ml distilled water. It was distributed in tubes in 10 ml amounts and sterilized by autoclaving at 15 lbs pressure for 15 minutes.

3. Hugh- Leifsons Agar Medium (O/F medium)

Ingredients	Grams/litre
Tryptone	2.0
Sodium chloride	5.0
Dipotassium phosphate	0.3
Bromothymol blue	0.08
Agar	2.0
Final pH (at 25°C)	7.4 ± 0.2

4. Nitrate Broth

Ingredients	Grams/litre
Beef extract	3.0
Peptone	5.0
Potassium nitrate	1.0
Sodium thiosulphate	0.025
Final pH (at 25°C)	7.3 ± 0.2

Procedure: 9 gm was dissolved in 1000 ml distilled water. It was then distributed in test tubes and sterilized by autoclaving at 15 lbs pressure at 121°C for 15 minutes.

5. Sulphide-Indole-Motility Medium

Ingredients	Grams/litre
Beef extract	3.0
Peptone	30.0
Peptonized iron	0.2
Agar	3.0
Sodium thiosulphate	0.025
Final pH (at 25°C)	7.3 ± 0.2

Procedure: 36 grams of the medium was suspended in 1000 ml distilled water and dissolved completely. Then it was distributed in tubes to a depth of about 3 inches

and sterilized by autoclaving at 15 lbs pressure at 121°C for 15 minutes. The medium was allowed to solidify in a vertical position.

6. Urease agar base

Ingredients	Grams/litre
Peptone from meat	1.0
Dextrose	1.0
Sodium chloride	5.0
Monopotassium phosphate	0.8
Dipotassium phosphate	1.2
Phenol red	0.012
Agar	12.0
Final pH (at 25°C)	7.3 ± 0.2

Procedure: 21 grams urea agar base was suspended in 1000ml of distilled water. It was boiled to dissolve completely and sterilized by autoclaving. It was cooled down to 55°C and aseptically introduced urea extra pure crystal solution and mixed well. It was distributed in sterile test tubes and allowed to solidify in slanted position.

7. TSI agar

Ingredients	Grams/litre
Peptic digest of animal tissue	10.00
Casein enzymatic hydrolysate	10.00
Yeast extract	3.00
Beef extract	3.00
Lactose	10.00
Sucrose	10.0
Dextrose	1.0
NaCl	5.0
Ferrous sulphate	0.2
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12.0
Final pH (at 25°C)	7.4 ± 0.2

The TSI agar is used to determine the ability of an organism to utilize specific carbohydrate incorporated in the medium (glucose, sucrose and lactose in concentrations of 0.1%, 1.0% and 1.0% respectively), with or without the production of gas (indicated by cracks in the media as well as an air gap at the bottom of the tube) along with determination of possible hydrogen sulfide production (detected by production of black color in the medium). A pH indicator (phenol red) included in the medium can detect acid production from fermentation of these carbohydrates and it gives yellow reaction at acidic pH, and red reaction to indicate an alkaline surrounding.

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Procedure: The test organism was streaked and stabbed on the surface of TSI and incubated at 37 °C for 24 hours .Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in slant and butt indicates sucrose or lactose fermentation. The results are interpreted as follows:

a. **Yellow (Acid)/ Yellow (Acid), Gas, H_2S** → Lactose/ Sucrose fermenter, H_2S producer.
b. **Red (Alkaline) / Yellow (Acid), No Gas, No H_2S** → Only Glucose, not lactose/Sucrose fermenter, not aerogenic, No H_2S production.
c. **Red (Alkaline) / No Change** → Glucose, Lactose and Sucrose non-fermenter.
d. **Yellow (Acid)/ No Change** → Glucose- oxidizer**.**
e. **No Change / No Change** →Non-fermenter.

8. Carbohydrate fermentation medium

Ingredients	Grams/litre
Peptone	10 gm
Sodium chloride	5 gm
Phenol red	0.018 gm
Carbohydrates	
Glucose- 1%	
Sucrose- 1%	
Mannose- 1%	
Mannitol- 1%	
Galactose- 1%	
Arabinose-1%	
Fructose- 1%	
Final pH (at 25 °C)	7.2 ± 0.2

Procedure: Except sugars all the ingredients were weighed separately and dissolved in 1000 ml distilled water. It was dispensed in 100 ml amounts and was sterilized in the autoclave for 15 minutes at 15 lbs pressure at 121°C to the 100 ml of sterile medium aseptically sterile sugars were added. Each flask was shaked to mix thoroughly and 15 ml amounts were dispensed into sterile test tubes aspetically.

9) Hydrolysis Agar Medias

a) Casein Agar Medium

Ingredients	grams/litre
Nutrient agar	2.8
Casein	1.0
Final pH (at 25°C)	7.2 ± 0.2

b) Gelatin Agar Medium

Ingredients	grams/litre
Nutrient agar	2.8
Gelatin	1.0

Final pH (at 25°C)	7.2 ± 0.2

c) Starch Agar Medium

Ingredients	
Nutrient agar	2.8
Starch	1.0
Final pH (at 25°C)	7.2 ± 0.2

d) Tween-20 Agar Medium

Ingredients	
Nutrient agar	2.8
Tween-20	1.0
$CaCl_2$	0.05
Final pH (at 25°C)	6.8 ± 0.2

Procedure: The above ingredients were measured and suspended in 100 ml distilled water after boiling to dissolve the medium completely it was sterilized by autoclaving then it was poured in petri plate and tested.

10. Chemicals of plasmid DNA analysis

a. Tris- acetate EDTA buffer (50X) (Electrophoresis buffer)

Tris base	242g
Glacial acetic acid	57.1ml
0.5 M EDTA	100 ml (p^H 8.0)
Working solution 1X	

b. 6X gel loading buffer (100 ml)

Bromophenol blue	0.25%
Sucrose in water	40% (w/v)

c. Luria- Bertani Broth/ Agar

Tryptone	10g
Yeast extracts	5g
Nacl	10g
Distilled water	1000 ml
*[Agar	15g (for agar plates)]

* 15 grams agar added in the case of preparation of LB agar

Procedure: All the ingredients were added to a clean 2 L flask that has been rinsed with deionized or distilled water.1 l deionized or distilled water was added to the flask. 0.5 ml of 4-M NaOH was added. The dry ingredients were stirred to dissolve; preferably using a magnetic stir bar. The preparation was autoclaved for 20 minutes at 121°C

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APPENDIX-V

COMPOSITION OF STAINING AND TEST REAGENTS

1. Crystal Violet

Solution A:

Crystal violet	2 gm
95% ethanol	20 ml

Solution B:

Ammonium oxalate	0.8 gm
Distilled water	30 ml

Preparation: Crystal violet was dissolved in ethyl alcohol and ammonium oxalate in distilled water. Then solutions A and B were mixed.

2. 95% Ethyl Alcohol

Preparation: 5ml of distilled water was added to 95 ml of absolute alcohol to make 95% ethyl alcohol solution, or

Acetone-Alcohol Decoloriser

Acetone	500 ml
Ethanol (Absolute)	75 ml
Distilled water	25 ml

Preparation: To 25 ml distilled water, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then immediately, 500 ml acetone was added to the bottle and mixed well.

3. Catalase reagent

Catalase Reagent (3% H_2O_2)

Hydrogen peroxide	3 ml
Distilled water	97 ml

Preparation: To 97 ml of distilled water, 3 ml of hydrogen peroxide was added and mixed well.

4. Grams Iodine

Potassium iodide	20.0 g
Iodine	10.0 g
Distilled water	1000 ml

Preparation: To 250 ml of distilled water, 20 gm of potassium iodide was dissolved. Then 10 gm of iodine was mixed to it until it was dissolved completely. Finally the volume was made 1 litre by adding distilled water.

5. Kovac's Reagent

Isoamyl or amyl alcohol	150 ml
P-dimethylaminobenzaldehyde	10 gm
Conc. Sulphuric acid	50 ml

Preparation: P-dimethylaminobenzaldehyde was dissolved in amyl alcohol and acid was slowly added. It was stored at 4°C.

6. Malachite Green

Malachite green	0.5 gm
Distilled water	100 ml

Preparation: 0.5 gm malachite green was dissolved in 100 ml distilled and it was mixed until the dye was fully dissolved. It was stored at room temperature.

7. Mercuric Chloride Solution

Mercuric chloride	15 gm
Conc. HCl	20 ml
Distilled water	100 ml

8. Methyl Red Reagent

Methyl red	0.1 gm
Ethanol	300 ml
Distilled water	200 ml

Preparation: Methyl red was dissolved in ethyl alcohol and dissolved water was added.

9. Nitrate reduction test reagent

Solution A:

Sulphanilic acid	8 gm
Acetic acid (5N)	1000 ml

Solution B:

α- napthylamine	5 gm
Acetic acid (5N)	1000 ml

10. Oxidase Strips

Oxidase reagent (impregnated in Whatman's No. 1 filter paper)

Tetramethyl *p*-phenylene diamine dihydrochloride (TPD)	1 gm
Distilled water	100 ml

Preparation: This reagent solution was made by dissolving 1 gm of TPD in 100 ml distilled water. To that solution strips of Whatman's No. 1 filter paper were soaked and

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drained for about 30 seconds. Then these strips were freeze dried and stored in a dark bottle tightly sealed with a screw cap.

11. Safranin

Safranin (2.5% solution in 95% ethyl alcohol)	10 ml
Distilled water	1000 ml

Preparation: In a clean piece of paper, 10 gm of safranin was weighed and transferred to a clean bottle. Then 1 litre distilled water was added to the bottle and mixed well until safranin dissolved completely.

12. McFarland tube (No 0.5)

0.5 ml of 0.048 M $BaCl_2$ (1.17%w/v $BaCl_2.H_20$) was added to 99.5 ml of 0.18 M H_2SO_4 1% w/v) was added with constant stirring. The standard was distributed into screw-capped tubes of the same size as those used to prepare the inoculums. The tubes were sealed tightly and stored protecting from light at room temperature.

13. Preparation of arsenic stock solutions

a) 4% sodium arsenate solution

Molecular weight of arsenate salt ($Na_2HAsO_4.7H_2O$) = 311.92
Atomic weight of arsenic (As) = 74.93
Therefore,
To get 74.92 gram of As (V), 311.92 gram of arsenate salt is added.
To get 1 gram of As, 311.92/73.92 = 4.16 gram of salt is needed.
Hence 4.16 gram of salt in 100ml distilled water constitutes 4% As (V) solution.
Or, 4.16×4= 16.64 gram of salt in 100ml distilled water constitutes 4% As (V) solution.
And, 1 gm in 10^6 ml = 1 ppm
1 gm in 1 ml = 10^6 ppm
4 gm in 100 ml=$4\times10^6/100 = 4\times10^4$ ppm
So, for 4×10^4 ppm, 100ml solution is added.
For 1 ppm, $100/4\times10^4$ ml = 2.5µl is needed.
Therefore, to get 100ppm, 2.5×100= 250 µl is needed.

b) 2% sodium arsenite solution

Molecular weight of arsenite salt ($NaAsO_2$) = 129.92
Therefore, to get 1 gm of As (III), 129.92/74.92= 1.73 gram of arsenite salt is needed.
Hence, 1.73×2=3.46 gm of salt in 100ml distilled water constitutes 2% As (III) solution
1 gm in 1 ml = 10^6 ppm
2 gm in 100 ml=$2\times10^6/100 = 2\times10^4$ ppm
So, for 2×10^4 ppm, 100ml solution is added.
For 1 ppm, $100/2\times10^4$ ml =5µl is needed.
Therefore, to get 100ppm, 5×100= 500 µl is needed. So on.

APPENDIX-VI

1. STAINING METHODS:

a. Gram-staining Procedure

Gram staining is the first and foremost step in the presumptive identification of the bacteria. For gram staining a thin smear of fresh culture was made in a clean, wax free glass slide and then it was heat fixed. First devised by Hans Christian Gram during the late 19th century, the Gram-stain can be used effectively to divide most bacterial species into two large groups: those that take up the basic dye, crystal violet (Gram-positive) and those that allow the crystal dye to was out easily with the decolorizer alcohol or acetone (Gram-negative). The following steps are involved in Gram-stain:

1. A thin film of the material to be examined was prepared and dried.
2. The material on the slide was heat fixed and allowed to cool before staining.
3. The slide was flooded with crystal violet stain and allowed to remain without drying for 1 min.
4. The slide was rinsed with tap water, shaking off excess.
5. The slide was then flooded with Gram's iodine for 30 seconds and again washed out with distilled water. The slide was rinsed with tap water, shaking off excess.
6. The slide was flooded with alcohol acetone decolorizer for 10 seconds or 95% ethyl alcohol for 10-20 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer. Thicker smear requires more aggressive decolorizing.
7. The slide was flooded with counter stain (safranin) for 1 min. and washed off with tap water.
8. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 1000X.

b. Spore Staining

A thin film of culture was made on a clean slide. The smear was covered with malachite green and steamed over boiling water for five minutes. Additional stain was added when stain boils off. After slide had cooled sufficiently, it was rinsed with water for 30 seconds. It was counter stained with safranin for about 20 seconds. Then it was rinsed with water and blot dried. The slide was examined under oil immersion.

2. CULTURE CHARACTERISTICS:

The culture characteristics of the organism were studied under following conditions:

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Biochemical characteristics

i) Catalase test

This test was done to demonstrate the presence of catalase and enzymes that catalyses the release of oxygen from hydrogen peroxides. The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria, the main exception being *Streptococcus* spp.

Reduced flavoprotein reacts directly with gaseous oxygen to form hydrogen peroxide, which is an oxidative end product of aerobic breakdown of sugars.

$FPH_2 + O_2$ --------- $FP + H_2O_2$

Hydrogen peroxides thus formed is to the organisms resulting in their death. The enzyme Catalase decomposes hydrogen peroxide into water and oxygen,

$2H_2O_2$---------- $2H_2O + O_2$

Procedure: To examine the catalase activity of the organism, a speck of the culture to be tested was picked from nutrient agar (pure culture) plate with a clean sterile glass rod and placed on a clean glass slide. A drop of 3% H_2O_2 was added over the organisms on a slide. Positive test was indicated by the production of gas bubbled from the surface of the solid culture material.

ii) Oxidase test

This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electrons between electron donors. In the presence of redox dye Tetramethyl-*p*-phenylene diamine dihydrochloride, the cytochrome oxidase oxidizes it into a deep purple colored end product indophenol which is detected in the test. Organism capable of growing in the presence of oxygen i.e. aerobic or facultative anaerobic organisms is mostly oxidase positive and at the same time they produce the enzyme catalase. Obligate anaerobic organism lack oxidase activity since they are unable to live in the presence of atmospheric oxygen and do not posses a cytochrome oxidase system. The test is used for screening species of *Neisseria, Alcaligenes, Aeromonas, Vibrio, Campylobacter* and *Pseudomonas* which give positive reactions and for excluding the Enterobacteriaceae, all species of which give negative reactions.

Procedure: To examine the oxidase activity of the organism in the present study Kovac's reagent coated strives (Whatsmann no 1 filter paper in pigmented with 1% tetra methyl paraphenylene diamine dihydrochloride) was lead in a clean dry glass slide and moistened with distilled water. The colony to be tested was picked with a clean sterile glass rod and smear over the moist area. The positive reaction was indicated by an intense deep purple blue.

iii) Nitrate reduction test

This test was used to differentiate members of the Enteriobacteriaceae that produce the enzyme nitrate reductase, from Gram negative bacteria that do not produce the enzyme. Development of red colour was proof of the presence of nitrate when 1 drops of

sulphanilic acid reagent and one drop of alpha-napthylamine reagent. If no red colour was produced, a very small amount (knife point) of zinc dust powder was added to observe red colouration.

iv) H_2S production test

This test was done to determine whether hydrogen sulphide (H_2S) was liberated due to enzymatic action on sulphur bearing amino acid which is confirmed by a visible black colour produced in the media.

H_2S gas was produced either through the reduction of an organic sulphur source such as thiosulphate or the dilution of organic sulphur supplied by the R_1-SH functional group of the amino acid cysteine, which is present in peptone. Lead acetate, the salt of the metal is an indicator of H_2S production. H_2S is a colourless gas which upon contact with lead acetate produces the sulphide of a black precipitation, indicated by a visible black colour reaction.

$$Pb\ (C_2H_3O_2)_2\ .3H_2O + H_2S \text{ -------- } PbS + H_2S + CH_3COO$$

Procedure**:** H_2S production was tested by using triple sugar iron agar (TSI) and sulphide indole motility agar (SIM). The test organism was stabbed in the TSI and SIM medium and incubated at 30°C for 18 to 72 hrs. The blackening of the medium along the line of inoculation or throughout entire but indicated the positive test for H_2S production.

v) Motility test

Motility test of the isolated organisms were carried out in the SIM medium where the organisms was stabbed and incubated at 30°C for 15 to 72 hrs. The motility was confirmed by the turbidity of the medium.

vi) Citrate Utilization test

This test was performed to determine the ability of an organism to utilize citrate as sole source of carbon and energy for growth and an ammonium salt as sole of nitrogen. Ammonium salt was broken down to ammonia resulting in salt alkalinity that cause change in colour of indicator used in the medium.

Procedure**:** For this test organism was streaked on the slant of Simmon's citrate agar medium and incubated at 30°C for 18 to 24 hrs. Positive test was confirmed from the growth of an intense blue colour on the slant.

vi) Indole test

This test was used to determine the ability of an organism to decompose the amino acid tryptophan to indole.

The principle behind this test is that tryptophanase, complete system of enzymes that mediate the production of indole, catalyzes the degradation of tryptophan. The enzymes

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tryptophanase in the course of degradation catalyzes the reduction deamination reaction of tryptophan by attacking it in its side's chain and leaving aromatic ring intact in the form of indole. Indole accumulated in the medium is then tested for by a colorimetric reaction with p-dimethyl-amino benzaldehyde.

Procedure**:** In present study, the medium was inoculated and incubated at 28°C for 18 to 72 hrs. After incubation a few drops of Kovacs reagent was added. A red ring at the surface of the medium indicated positive test.

v) Methyl red test

This test was done to detect the ability of organisms to produce and maintain stable end product from fermentation.

Methyl red positive organisms produce a high yield of acid: lactic, succinic, acetic and formic. Formic acid breakdown is the pathway for hydrogen and carbondioxide production which result in a low terminal pH, overcoming the phosphate buffering system maintaining acid environment in the medium (pH 4.2 or less) i.e.

$CO_2 + H_2$---------- HCOOH

The methyl red negative organisms continue to further metabolize the initial fermentation product (acidic metabolic by-product) by carboxylation producing neutral acetyl methyl carbin (acetoin), which results in a high terminal pH that decrease the medium acidity raising toward neutrality pH (6.0) or greater.

Procedure**:** In the present study, methyl red test medium was inoculated with the test organisms and incubated at 28°C for 18 to 72 hrs. Methyl red test indicator was added to see the change colour. The positive test was confirmed by colour produced in the medium.

vi) Voges-Proskauer (Acetoin production) test

Many microorganisms ferment carbohydrates with the production of acetyl methyl carbin (acetoin) or its reduction product 2,3- butylenes glycol (2,3 butanediol). The substances can be tested by a colorimetric reaction between dactyl (formed during the test by oxidation of acetion or 2,3 butanediol) and a guanidine group under alkaline conditions. This test is usually done in conjugation with the methyl red test since the production of acetion or 2,3-butanediol usually results in insufficient acid accumulating during fermentation to give a methyl red positive reaction.

Procedure**:** The Voges-Proskauer (VP) test medium was inoculated with test organisms and incubated at 28°C for 18 to 72 hrs. Barrit's reagent was added to the incubated suspension. The positive reaction was indicated by the development of pink colour in the medium.

vii) Urease test

This test demonstrates the urease activity present in certain bacteria which decomposes urea, releasing ammonia and carbon dioxide. Ammonia thus produced changes the color of indicator (phenol red) incorporated in the medium.

Procedure**:** The test organism was cultured in a medium which contains urea and the indicator phenol red. The inoculated medium was incubated at 37°C overnight. If the stain was urease producing, the enzyme will break down the urea (by hydrolysis) to give ammonia and carbondioxide. With the release of ammonia, the medium becomes alkaline as shown by a change in colour of the indicator to red pink.

viii) Coagulase test

This test is used specifically to differentiate species within the genus *Staphylococcus*: *S. aureus* (usually positive) from *S. saprophyticus, S epidermidis* (negative). A positive coagulase test is usually the final diagnostic criterion for the identification of *S. aureus.* Free coagulase and bound coagulase are the two types of coagulase possessed by this organism; most strains possess both free and bound coagulase.

a) Slide Coagulase Test

Bound coagulase (clumping factor) is detected by slide test. The bound coagulase is bound to the bacterial cell wall and reacts directly with fibrinogen. This results in alteration of fibrinogen so that it precipitates on the staphylococcal cell, causing the cells to clump when a bacterial suspension is mixed with plasma.

Procedure**:** For slide coagulase test, a drop of physiological saline was placed on three places of a slide, and then a colony of the test organism was emulsified in two of the drops to make thick suspensions. Later a drop of plasma was added to one of the suspensions and mixed gently. Then a clumping was observed within 10 seconds for the positive coagulase test. No plasma was added in second suspension. This was used for the differentiation of any granular appearance of the organism from true coagulase clumping. The third drop of saline was used for a known strain of coagulase positive staphylococci.

b) Tube Coagulase Test

This test is carried out to detect production of free coagulase. Plasma contains coagulase reacting factor (CRF) which activates free coagulase. The activated coagulase acts upon prothrombin thus converting it to thrombin. Thrombin converts fibrinogen into fibrin which is detected as a firm gel (clot) in the tube test. Tube test is performed when negative or doubtful results are obtained in slide coagulase test.

Procedure**:** In the tube coagulase test, plasma was diluted 1:10 in physiological saline. Four small tubes were taken, one for test organism, one for positive control, one for negative control, and one to observe self clotting of plasma. Then 0.5 ml of the diluted

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plasma was pipetted into each tube and 0.5 ml of test organism, 0.5 ml of positive control (*S. aureus* culture), and 0.5 ml negative control (*S. epidermidis* culture) was added to three tubes, to the fourth tube, 0.5 ml sterile broth was added. After mixing gently, all tubes were incubated at 37°C on a water bath for 6 hours and observed for gel formation in every 30 minutes. The clotting is observed by gently tilting the tube for positive coagulase test.

3. SUGAR UTILIZATION TEST

This test was done on filter sterilized sugar of different types on the basal medium with 0.018% phenol red base. During consumption of the specific sugar source liberate acid which help to drop the pH of the medium and phenol red appears as yellow. During the test, distinct conversion of red to yellow colour was regarded as positive.

Acid production from different sugar containing media was the diagnostic test for the species determination. For diagnostic purpose 8 different sugars were used were mannitol, maltose, fructose, glucose, sucrose, galactose, and arabinose. Phenol red base of concentrations 0.018% was prepared and separately added 1% sugar of each. Utilizing of sugar was confirmed by conversion of colour of phenol red to yellow.

4. HYDROLYSIS TEST

a) Casein hydrolysis

Solidified skim milk agar plates were divided into 6-sectors with each being streaked with the isolate. Observation was made after 7 days of 28°C, for the clear zone around the colony. For confirmation, mercuric chloride solution was flooded on the plate.

b) Gelatin hydrolysis

Each of the solidified gelatin agar plate was divided into 6-sectors and isolates were streaked with on it. The plates were incubated at 30°C for 7 days. Hydrolysis was confirmed by flooding the plates with mercuric chloride solution.

c) Starch hydrolysis

Solidified starch agar plates were inoculated with the isolates and incubated for 7 days at 30°C. Then iodine solution was flooded onto the plates to ascertain the clear zone of hydrolysis around the colonies.

APPENDIX-VII

Table: Distinguishing reactions of the commoner and pathogenic Enterobacteriaceae

Species	Test/ substrate											
	lac	mot	gas	ind	VP	cit	PDA	ure	lys	H_2S	inos	ONPG
E. coli	+	+	+	+	-	-	-	-	+	-	-	+
Shigella groups A, B, C	-	-	-	±	-	-	-	-	-	-	-	-
Sh. sonnei	-	-	-	-	-	-	-	-	-	-	-	+
Salmonella (most serotypes)	-	+	+	-	-	+	-	-	+	+	±	-
S. typhi	-	+	-	-	-	-	-	-	+	+	-	-
S. paratyphi A	-	+	+	-	-	-	-	-	-	-	-	-
C. freundii	±	+	+	-	-	+	-	±	-	±	-	+
C. koseri	±	+	+	+	-	+	-	±	-	-	-	+
K. pneumoniae	+	-	++	-	+	+	-	+	+	-	+	+
K. oxytoca	+	-	++	+	+	+	-	+	+	-	+	+
E. aerogenes	+	+	++	-	+	+	-	-	+	-	+	+
E. cloacae	+	+	+	-	+	+	-	±	-	-	-	+
Hafnia alvei	-	+	+	-	+	-	-	-	+	-	-	+
Serratia marcescens[b]	-	+	±	-	+	+	-	-	+	-	±	+
P.mirabilis	-	+	+	-	±	±	+	++	-	+	-	-
P. vulgaris	-	+	+	+	-	-	+	++	-	+	-	-
M. morganii	-	+	+	+	-	-	+	++	-	±	-	-
Providencia rettgeri	-	+	-	+	-	+	+	++	-	-	+	-
P. stuartii	-	+	-	+	-	+	+	±	-	-	+	-
P. alcalifaciens	-	+	+	+	-	+	+	-	-	-	-	-
Yersinia enterocolitica[c]	-	-	-	±	-	-	-	±	-	-	±	+
Y. pestis	-	-	-	-	-	-	-	-	-	-	-	±
Y. pseudotuberculosis	-	-	-	-	-	-	-	+	-	-	-	±

[a] lac, inos, fermentation of lactose, inositol; mot, motility; gas, gas from glucose; ind, indole production; VP, Voges-Proskauer; cit, Citrate utilization (Simmons'); PDA, phenylalanine deaminase; ure, urease; lys, lysine decarboxylase; H_2S, H_2S produced in TSI agar; ONPG, metabolism of *o*-nitrophenyl-β-D-galactopyranoside.

[b] Some strains of Serratia marcescens may produce a red pigment

[c] *Yersinia* are motile at 22°C.{Key: +, ≥85% of strains positive; -, ≥ 85% of strains negative; 16-84% of strains are positive after 24-48 hour at 36°C}(Source: Collee *et al.,* 1996)**.**

APPENDIX-VIII

a) MIC of As (III)

1. For each bacterium, a set of 12 screw-capped test tubes containing 1 ml sterile nutrient broth was added to each of them with micropipette.
2. The test tubes were labeled as positive growth control, negative control and numbers 1 to 10.
3. Then 200 ppm As (III) salt solution (i.e. 1ml of 2% stock solution) was added aseptically to tube 1 containing 1 ml nutrient broth and it was mixed properly. Then 1 ml mixture of nutrient broth and As (III) was transferred aseptically to 2^{nd} tube.
4. Similarly the second tube was mixed properly and 1ml of its content was transferred to 3^{rd} tube. This process of homogenization followed by transfer was carried upto 10^{th} tube. This resulted serial double dilution of the salt solution in decreasing concentration. Finally after homogenization 1 ml of content from the 10^{th} tube was discarded.
5. Finally one loopful of organisms was transferred in each tube and positive control from stock culture prepared in nutrient broth. In negative control, only nutrient broth and As (III) were added and in positive control only nutrient broth and one loopful organism was added.
6. All the tubes were incubated at 37°C for 24 hours and observed turbidity by comparing with positive and negative controls.

b) MIC of As (V)

i. 13 sterile test tubes were labeled as 1, 2, 3 …. 13 and 1 ml sterile nutrient broth was added to each of them with a micropipette.

ii. Using a micropipette, 0.5 ml, 0.7 ml, 0.9 ml, 1.1 ml, 1.3ml, 1.5 ml, 1.7 ml, 1.9 ml, 2.1 ml, 2.3 ml and 2.5 ml As (V) salt solution from 4% stock solution were transferred to tube no. 2, 3, 4, 5, and so on up to 13 respectively. This resulted 200 ppm, 280 ppm, 320 ppm, 440 ppm, 520 ppm, 600 ppm, 680 ppm, 760 ppm, 840 ppm, 920 ppm and 1000 ppm As (v) concentration in successive test tubes. In tube no. 13, 1 ml salt solution was added.

One loopful of fresh 0.5 McFarland matched pure culture in nutrient broth was transferred aseptically to all the tubes except tube no. 13. Tube no. 1 and 13 acted as growth positive and negative controls, respectively. All tubes were incubated at 37°C for 24 hours and MIC value was recorded as that lowest concentration of the salt solution which did not show visible turbidity.

APPENDIX- IX

PROTOCOLS

A. Extraction of plasmid DNA

i) 10 arsenic tolerant bacteria and one arsenic sensitive *E. coli* were selected.
ii) 10 ml overnight culture was prepared of each in Luria Bertani Broth at 35°C.
iii) 5 ml overnight culture of plasmid carrying bacteria was transferred to a clean 1.5 ml microfuge tube.
iv) The microfuge tube was centrifuged at 10,000 rpm for 2 minutes and the supernatant obtained was poured off immediately.
v) Again 1.5 ml of the culture was transferred to the same microfuge tube with pellet and centrifuged at 10,000 rpm. The supernatant was poured off as in step (iii).
vi) The pelleted bacterial cell was resuspended in 400 µl of solution G1 with RNase and mixed well. No cell clumps should be visible.
vii) 450 µl of G2 solution was added and mixed gently by inversion. The reaction was not allowed to proceed for more than 5 min.
viii) 500 µl of solution G3 was added and mixed by inversion. Then it was spin at 13,000 rpm for 10 minutes at 4°C. A white pellet was formed.
ix) The spin column was kept in a 2 ml collection tube. Then the supernatant was passed through the spin column, 400 µl each time, spin at 10,000 rpm for 60 seconds. After each spin, the elute was discarded before proceeding next spin.
x) Now, it was washed with 500 µl wash buffer I and centrifuged for 60 seconds. The elute was discarded and the same 2 ml collection tube was used before proceeding with next spin.
xi) The column was washed with 750 µl of wash buffer II and centrifuged at 10,000 rpm for 60 seconds. The elute was discarded and the same 2 ml collection tube was used before proceeding with next spin.
xii) A final spin was done at 10,000 rpm for 3 min which ensure removal of alcohol from column.
xiii) In fresh 1.5 ml microfuge tube, DNA was eluted with 50 µl elution buffer twice by spinning for 60 sec at 10,000rpm. Elution buffer was added to the center of the column.
xiv) The obtained plasmid was kept in freeze for further processes.

B. Agarose gel electrophoresis:

i Preparation of agarose gel (0.8%)

1. 0.5g of agarose was weighed out into a 250mL conical flask. 100mL of 1X TAE was added and swirled to mix.
2. The preparation was micro-waved for about 1 minute to dissolve the agarose.
3. It was left to cool on the bench for 5 minutes down to about 60°C.
4. 5µL of ethidium bromide was added (10mg/mL) and swirled to mix.

5. The gel was poured slowly into the tank. Any bubbles were pushed away to the side using a disposable tip. The comb was inserted and double checked that it is correctly positioned.
6. It was left to set for at least 30 minutes, preferably 1 hour, with the lid on if possible.
7. 1X TAE buffer was poured into the gel tank to submerge the gel to 2–5mm depth. This is the running buffer.

ii. Preparation of sample

15 µL of each sample was transferred to a fresh microfuge tube. 3 µL of 6X gel loading buffer was added into each tube and left the tip in the tube. Similarly 15 µL supercoiled DNA markers were prepared.

iii. Loading of sample in the wells

The first well was loaded with 15 µL marker. The samples (15 µL) were loaded in the adjacent wells.

iv. Electrophoresis

1. The gel tank was closed, the power-source was switched on and the gel was run at 70V for3 hours.
2. Current flowing was checked.

v. Monitoring

1. The progress was monitored on the gel by reference to the marker dye.
2. The gel was stopped when the bromophenol blue had run $3/4^{th}$ the length of the gel.
3. The power was switched off.

vi. Photo-documentation (visualization)

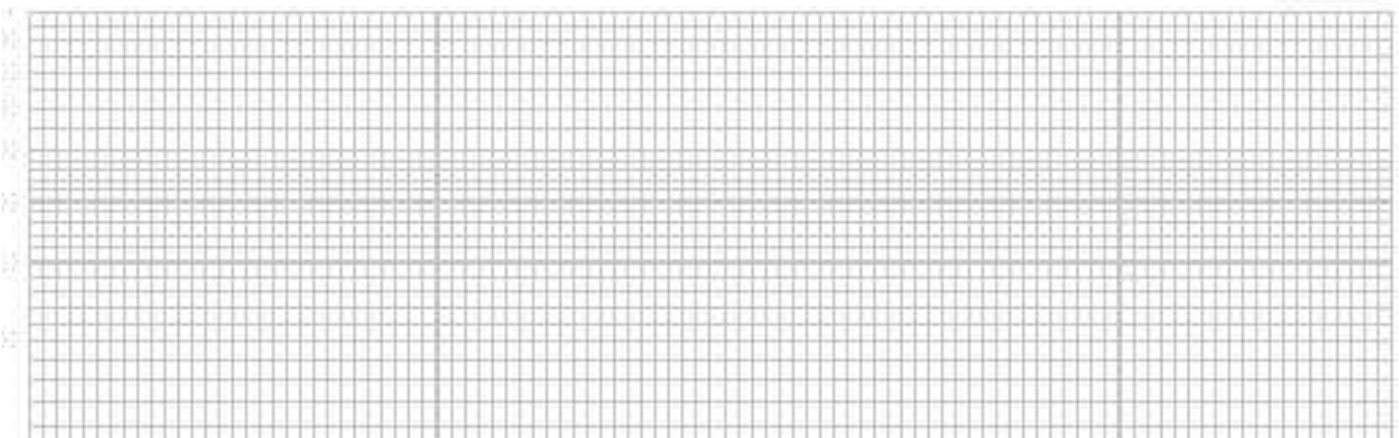
1. The gel was carried (in its holder if possible) to the room to look at on the UV trans-illuminator.
2. The gel was illuminated at 300nm UV and the picture was saved for analysis.

vii. Decontamination of ethidium bromide

Sufficient water was added to reduce the concentration of EtBr to < 0.5 µg/ml. One volume of 0.5 M $KMnO_4$, one of volume of 2.5 N HCl was mixed and it was allowed to stand for several hours at room temperature.1 volume of 2.5N NaOH was mixed and the solution was discarded

APPENDIX XI

Standard curve of known DNA bands Vs distance



APPENDIX-XIII

Method of application as described in Wagtech international arsenic test kit leaflet

Stage 1: Loading bung device

a. Hydrogen sulphide removal filter was inserted into the bottom of the bung device.
b. Black arsenic filter slide was opened.
c. One filter paper was taken from black labeled pot, forceps and/or gloves were used to insert into position ensuring the filter paper was evenly covering the hole. Then firmly slide was closed, taking care to locate the pins in the slide correctly.
d. The black filter slide (arsenic test) was inserted into the bottom slot of the Bung device.
e. Again red arsenic filter (removal) slide was opened.
f. One filter paper was taken from red labeled pot, forceps and/or gloves was used to insert into position (filter paper insertion was repeated as mentioned in step c).
g. The red filter slide (removal) was inserted into the top slot of the bung device.

Stage 2: Sample preparation

a. The flask was filled to the 50 ml line with sample water and placed on a firm surface.
b. Loaded bung device was picked up and checked that filter slides are fully pushed into Bung device.
c. A1 powder sachet was poured into the flask.
d. With one hand, A2 tablet was hold with forceps or gloved hand over the neck of the flask. With the other hand, the loaded Bung device was hold directly above. Immediately after dropping the A2 tablet into the flask, the bung device was pushed into the neck of the flask, sealed it tightly. Reaction would begin immediately.
e. The flask was kept for 20 minutes minimum for the complete reaction and arsenic detection.

Stage 3: Arsenic measurement

a. Black Filter Slide was removed from Bung device.

b. The Black Filter Slide was placed behind the Color Chart and the test result was matched with the corresponding color.
c. If test result reads above 300 µg/l (ppb), dilution tube was used with de-ionized water and converted accordingly. Then stage 1 was repeated and test was conducted again.
d. Finally, both filter slide holders was opened and used tests papers was disposed into the disposal bag provided in the Ask Arsenic kit. Then the flask was washed clean water using the cleaning brush provided and prepare for the next test.

Note: Black Filter Slide will collect the Arsenic Gas

Red Filter Slide removes excess Arsenic Gas