DETECTION OF COLIFORM BACTERIA IN DRINKING WATER SUPPLY OF SHIMLA CITY

Project Report submitted in partial fulfillment of the requirement

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Under the Supervision of

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By

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To

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

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CERTIFICATE

This is to certify that the work titled (**Detection of Coliform Bacteria in Drinking Water Supply of Shimla city**" has been submitted by **Akansha Rajput** and **Radhika Batta** in partial fulfillment for the award of degree of **B.Tech Biotechnology** from Jaypee University of Information Technology, Solan has been carried out under my supervision. This work has not been submitted partially or wholly at any other university or institute for the award of this or any degree or diploma.

Signature of Supervisor:

Name of Supervisor: Dr. Sudhir Kumar

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Date:-

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Radhika Batta

Akansha Rajput

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AIM OF THE STUDY

1. To analyze the drinking water for both physical and biological parameters.
2. To quantitate and validate the presence of coliform bacteria in drinking water.
3. To suggest the department concerning water supply for its treatment.

ABSTRACT

Shimla is a famous tourist destination of the state hosting millions of tourists (both domestic and international) every year. The civic infrastructure requirements including the water demand of the town accelerated manifold being the capital city and the concentration of large scale tourist infrastructure. Providing potable drinking water supply to its inhabitants and the floating population remains a major challenge for the irrigation and Public Health department and the Municipal Corporation which are charged with the responsibility of supplying water to the town.

Water is supplied to Shimla town from various sources like Dhalli Catchment Area, Cherot Nallah, Jagroti Nallah, Chair Nallah, Gumma Khad and Ashwani Khad. Our study focuses on the analysis of the water quality at two important sources of water supplying water to the Shimla town. We have collected sample from Ashwani Khad and Cherot Nallah in summer and winter seasons respectively. In this study, we detected and validated the presence of coliform bacteria. These strains were characterized using morphological and biochemical methods. We detected the presence of *Salmonella typhi* in the Ashwani Khad sample in both seasons. We also detected *E.coli* in Cherot Nallah water. Quality of water was further asserted by doing the physical characterization of water. The prime purpose of our study is to quantitate the coliform bacteria as a potential indicator of presence of human pathogens in the drinking water. We also intend to find out the potential of water for human consumption and to suggest the authorities for improving the water treatment process.

Radhika Batta

Akansha Rajput

Chapter 1 INTRODUCTION

Shimla the queen of hills is located at an altitude of 2130 m above mean sea level. Owing to its scenic beauty and heritage, Shimla is a famous tourist destination of the state hosting millions of tourists (both domestic and international) every year. The civic infrastructure requirements including the water demand of the town accelerated manifold being the capital city and the concentration of large scale tourist infrastructure. Providing safe drinking water to its inhabitants and the floating population remains a major challenge for the irrigation and Public Health department and the Municipal Corporation which are responsible for supplying water to the town. The I&PH takes into consideration the bulk supply and treatment of water while the SMC is concerned with distribution and pumping, metering and billing of potable water to domestic and commercial connections.

Water is supplied at varying elevations from various sources like Dhalli Catchment Area, Cherot Nallah, Jagroti Nallah, Chair Nallah, Gumma Khad and Ashwani Khad. The total installed capacity is 47.54 mld. The IP&H department installed four water treatment plants at different places namely Gumma Khad, Ashwani Khad, Cherot Nallah and Dhalli catchment area (Centre for Science and Environment 2010).

Our study focuses on the analysis of the water quality at two important sources of water supplying water to the Shimla town. We have collected sample from Ashwani Khad and Cherot Nallah in summer and winter seasons respectively. The selection of our site is based on the fact that cases of water borne disease have been reported among the population supplied with water from the above mentioned areas. There have been reported cases of jaundice and diarrhea in 2007, 2010 and 2013, respectively. In the light of the fact, the study was conducted in order to validate water samples fit or unfit for human consumption.

Our study aimed at conducting analysis of water to detect the presence of coliform bacteria as an indicator organism for the presence of disease causing human pathogens. We tested for coliform bacterial count in the samples collected in summer and winter respectively. Therefore, we checked for the seasonal variation in the level of contamination. Water samples from Ashwani

Khad area have been found contaminated by fecal matter in 2007 and 2010, respectively. 17 samples were sent to the National Institute of Communicable Diseases, New Delhi. They reported the water to be contaminated by fecal matter (Centre for Science and Environment 2010). It is important to study the water quality of this hill town since water-related diseases, including diarrhea, are a major cause of death amongst young children world-wide. Each year they kill more children than HIV/AIDS. In India too child mortality due to HIV/AIDS counts for 0.7 % while 20% die from diarrheal diseases.

Chapter 2 REVIEW OF LITERATURE

The chemical, physical and bacterial characteristics of water determine its efficacy and utilization. While the prime objective of a water supply agency is to provide hygienically safe-water to its consumers, it has been observed that many people do not have access to clean and safe drinking water and many die because of waterborne bacterial infections. Inadequate drinking water supply and poor sanitation are the major cause of morbidity and mortality all around the world. According to World Health Organization (WHO), 80% of human diseases in developing countries are waterborne. Enteric pathogens are disease-causing microorganisms, predominantly of fecal origin and are major causative agents of waterborne disease. It is estimated that 1.1 billion people globally drink unsafe water and the vast majority of diarrhea disease in the world (88%) is attributable to unsafe water, sanitation and hygiene.

Among the various sources of drinking water are surface and underground sources. However, both surface and ground water sources could become contaminated by biological and chemical pollutants arising from point and non point sources. Sources of water contamination can be old water distribution networks, leakage in pipeline, bad sanitary condition and improper management of waste disposal. In India, it is reported that about 70% of the available water is polluted. The chief source of pollution is identified as sewage constituting 84 to 92 percent of the waste water. Water quality is the degree of portability which is determined by the amount and kinds of suspended and dissolved substances in water.

2.1 Total Coliform Bacteria can be detected by performing Total Coliform Bacteria count and the group includes thermotolerant coliforms and bacteria of fecal origin, as well as some bacteria that are isolated from environmental sources. Therefore, the presence of total coliform may or may not indicate fecal contamination which grows on a lactose rich media and at an ambient temperature of 35 or 37 °C. They can be detected by the production of acid and gas upon lactose fermentation (National Health and Medical Research Council 2003).

2.2 Fecal Coliform or Thermotolerant Bacteria are present in the gut and feces of warm blooded animals. They are considered the best indicators of animal and human waste. Fecal coliforms are the member of family *Enterobacteriae* including *Esherichia coli, Citrobacter, Enterobacter* and *Klebsiella* species. They are gram negative, aerobic or facultatively anaerobic, non spore forming rod shaped bacteria. They are oxidase negative and nitrate reducing bacteria. Fecal coliforms can also be used as indicator of pathogenic micro-organisms because they are present in large number and are technically easier to detect and quantitate. The members of this group can cause mild to severe diseases. (Sulehria et al. 2011; Tortorello M. 2003).

2.3 Study on Bacteriological Parameter of Drinking Water

Analysis of Bacteriological parameter helps in determination of bacteriological contamination in drinking water which is as important as study of Physico-chemical contamination. Bacteriological contamination causes the water borne disease which may pose a risk to human health and to environment. We took the sample from various sites in Shimla and performed MPN test to detect the presence of coliform bacteria. MPN test is the most probable number test. This number is the statistical estimate of the mean number of coliform present in the sample. The precision of this method is low and depends upon the number of tubes used for analysis. The time required for obtaining result is higher than the membrane filter technique. Yet it is more popularly used as it is easy to implement and inexpensive. (Rompre et al.2002).

McEgan et al. (2013) conducted the Coliforms, *Escherichia coli*, and various physicochemical water characteristization as indicators of microbial water quality. The relationship between the presence and/or concentration of *Salmonella* and biological, physical, or chemical indicators in Central Florida surface water samples over 12 consecutive months was explored. Samples were taken monthly for 12 months from 18 locations throughout Central Florida. Aerobic plate counts and most probable numbers (MPN) for *Salmonella*, *E. coli*, and coliforms were performed.

Sulehria et. al (2011) conducted the MPN test for fecal coliform. Thirty samples (ninety in total) were collected from each site i.e., main reservoir, distribution line and consumer taps. All samples revealed the presence of coliform bacteria. The counts were higher than the standards

established by World Health Organization (WHO). The bacterial counts were higher in consumer taps (3.99-4.39 log cfu/100 ml), followed by distribution line (3.65-4.25 log cfu/100 ml) and main reservoir (3-3.96 log cfu/100 ml). The pH was found within the limits of WHO standard (6.5-8.5), however, there was no sign of residual chlorine in any sample of drinking water. Therefore, they conclude that the quality of drinking water in Mughalpura is not up to WHO standards.

MPN can be performed by a technician with basic microbiological training. However, it is also relatively inexpensive, as it requires unsophisticated laboratory equipment.

Another method which could be used for detection of coliform bacteria is Membrane filtration method. The Membrane Filter (MF) Technique was introduced in the late 1950s as an alternative to the Most Probable Number (MPN) procedure for microbiological analysis of water samples. The concentration of larger samples on a membrane filter is a key benefit of the technique over the MPN procedure as well as over Pour Plate and Spread Plate techniques.

Messer et al. (2005) performed the variation of the standard two step membrane filter technique for the detection of *Enterococci* in fresh and marine recreational water. They modified the original mE medium by reducing the triphenyltetrazolium chloride from 0.15 to 0.02 g/liter and adding 0.75 g of indoxyl b-D-glucoside per liter. The mEI medium, detects *Enterococci* in 24 h comparable to 48h detected by the original mE medium. Colonies from mEI medium were examined for false-positive and false-negative occurrences. mEI medium had a false-positive rate of 6.0% and a false-negative rate of 6.5%. Inter laboratory testing of the MF method with mEI medium was also performed. The results of the same showed that the relative reproducibility standard deviations among laboratories ranged from 2.2% for marine water to 18.9% for freshwater.

Other advantages of membrane filtration method are that it reduces preparation time, allows isolation and enumeration of discrete colonies of bacteria, and provides presence or absence information within 24 hours. This method is thus used for monitoring coliform bacteria in drinking, waste, and surface water and also for the microbial monitoring in pharmaceuticals, cosmetics, and food and beverage industries. The disadvantage of MF is its inability to recover

stressed or injured coliforms. Bacteria can undergo various chemical and physical stresses involved in drinking water treatment, including disinfection, exposure to chlorine.

Another method which is used for the detection of coliform bacteria is molecular methods. Molecular methods mainly comprises of Polymerase Chain Reaction (PCR) method. The PCR method is used for the detection and identification of microorganisms in foods, soils, sediments and waters. The use of PCR method for the detection of coliform in drinking water is rather recent. The disadvantage of PCR-based analysis is that it cannot provide information on the physiological status of targeted cells. Another limitation of PCR analysis of environmental samples is the inhibition of the enzymatic reaction. Humic substances are known as polymerization enzyme inhibitors and colloid matter has a high affinity for DNA. The presence of these elements in a water sample can therefore considerably decrease the amplification yield of PCR applied to the detection of greatly diluted bacteria (Rompre et al. 2002).

Tantawiwat *et al.*, (2005) developed Multiplex PCR for the Detection of total coliform bacteria for *E. coli* and *Clostridium perfringens* in drinking water. The multiplex PCR amplification of lacZ, uidA and plc genes led to the simultaneous rapid detection of total coliform bacteria, *E. coli* and *Clostridium perfringens* in drinking water. An enrichment step was included so that only cultivable pathogens can be detected. The specificities and sensitivities of the three primer sets used were separately studied with the optimized multiplex.

Riyaz-ul-hassan *et al.*, (2004) detected *Salmonella* in milk samples through the means of Polymerase Chain Reaction. They designed four pairs of oligonucleotide primers to amplify different fragments of this important pathological marker. The protocols were standardized with serotype *S. typhimurium*. However, these primers were found to generate specific amplicons with all the serotypes of *Salmonella* tested. The PCR protocols were found to be highly specific as no amplifications, specific or non-specific, were found when reactions were run using non-*Salmonella* DNA as template.

Moreno *et al., (*2002), evaluated PCR and FISH techniques for detecting *Arcobacter* and *Campylobacter* strains in river water and waste water samples. Both 16S and 23S rRNA sequence data were used to design specific primers and oligonucleotide probes for PCR and

FISH analysis, respectively. In order to assess the suitability of the methods, the assays were performed on naturally and artificially contaminated samples and compared with the isolation of cells on selective media. The results show both, the great prevalence of *Arcobacter* in surface and wastewater, and the inadequacy of available cultural methods for its detection. So according to the study's results; both rRNA based techniques have the potential to be used as quick and sensitive methods for detection of the pathogens *Arcobacter* and *Campylobacter* in the water sample.

2.4 Study on Physico-chemical Parameter of Drinking Water

Physico-Chemical analysis determines the physical and chemical condition of the drinking water. Following are some of the studies done by researchers on Physico-chemical analysis of drinking water:

Shittu *et al.*, (2008) conducted the experimental study on physico-chemical analysis of well water, stream water and river water used for drinking and swimming purposes in Abeokuta, Nigeria. The results obtained were compared with WHO and EPA standards for drinking and recreational water. With the exception of Sokori stream and a well water that did not comply with Turbidity and Mg^{2+} standards respectively, all others were within the standards set for pH, Color, Total solids, Total dissolved solids, acidity, total hardness, Ca^{2+} hardness, chloride and Iron.

Abdul et al. (2011) conducted the test to determine the quality of municipal tap water of Bholakpur area in Hyderabad, India and to further identify the cause of diarrheal illness. The study consists of the determination of physico-chemical properties, trace metals, heavy metals, rare earth elements and microbiological quality of drinking water. The water samples were analyzed for 27 elements (Li, Be, B, Na, Mg, Al, Si, K, Ca, V, Cr, Mn, Fe, Ni, Co, Cu, Zn, As, Se, Rb, Sr, Mo, Ag, Cd, Sb, Ba and Pb) using inductively coupled plasma-mass spectrometry (ICP-MS). The microbiological quality of water was tested using standard plate count, membrane filtration technique, thermo tolerant coliform (TTC), and most probable number (MPN) method

Chapter3 MATERIALS AND METHOD

3.1 Materials- water samples, glasswares, chemicals (Mac Conkey agar, EMB agar, triple sugar iron, LB broth, hydroxylamine, 1, 10 –phenanthroline, sodium acetate, MR-VP media, XLD agar)

3.2 Methods

3.2.1 Sampling- Water samples were collected from the area of Ashwani Khad (Fig 1) in Shimla city on 15/08/2013, 29/09/2013 and 12/01/2014 respectively. This was done in order to check for the seasonal variation in the coliform count. We also collected water from Chirot Nallah on 12/01/2014(Fig 2). Water from JUIT Campus, Khumbar Nala was also taken as a sample. Refer to table no. 1

Table no.1: List of the samples we analyzed in our project.

Sample no.	Water Samples
1	Main Reservoir(Phase I) of Ashwani Khad
2	Pumping Station(Phase II) of Ashwani Khad
3	Distribution Line(Phase III) of Ashwani Khad
4	Consumer Tap(Phase IV) of Ashwani Khad
5	Main Reservoir(Phase I) of Cherot Nallah
6	Pumping Station(Phase II) of Cherot Nallah

7	Distribution Line(Phase III) of Cherot Nallah
8	Consumer Tap(Phase IV) of Cherot Nallah

ASHWANI KHAD

MAIN RESERVOIR(PHASE1)

PUMPING STATION(PHASE 2)

DISRIBUTION LINE(PHASE 3)

CONSUMER TAP(PHASE 4)

Fig 1: The various phases of water treatment at Ashwani Khad area in Shimla

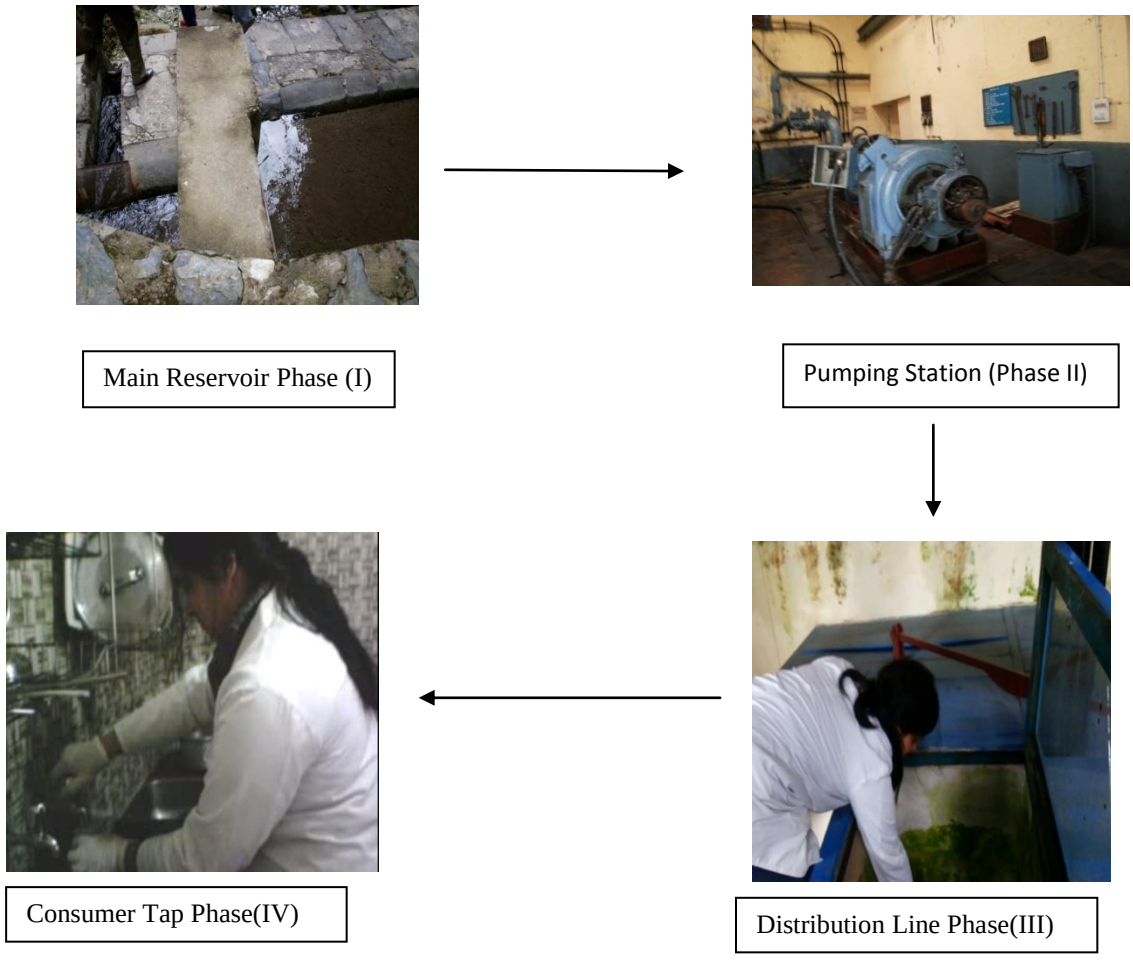


Fig 2: The various phases of water treatment at Cherot Nallah area in Shimla

3.2.2 Methods of Detection of Coliform Bacteria

(Approved by US Environment Protection Agency EPA)

- Multiple Tube Fermentation Method or Most Probable Number Method
- Membrane Filter Technique
- Enzymatic Methods
- Molecular Methods

3.2.2.1 Multiple Tube Fermentation Method

The Multiple-Tube Technique is a two staged test that begins with a **presumptive test** using lactose fermentation tubes inoculated with the water sample to determine if acid and gas fermentation of lactose occurs indicating the presence of coliforms. Three different volumes of sample water are used to inoculate lactose fermentation tubes in replication. If gas and acid fermentation of lactose has occurred in any of the tubes after incubation, coliforms are presumed to be present in the water sample (Fig 3). If the tubes show an orange coloration it indicates the presence of lactose fermenting bacteria. The orange coloration is due to the decrease of pH caused due to acid production (Fig 13). If the tube shows a yellow coloration it indicates the presence of non lactose fermenting bacteria. The second stage of this method uses **Eosin-Methylene Blue agar** as a **confirmatory** method to identify the positive lactose fermentation tubes as fecal coliforms. The tube showing positive presumptive test can then be streaked onto EMB agar. The result of MTF is expressed in terms of **Most Probable Number (MPN)** (Rompre et al.2002).The MPN value is calculated by the table standardized by WHO (Table no.2)

Table no.2: MPN values per 100ml of sample (when five 10-ml, five 1-ml and five 0.1ml test portions are used)

10ml	1ml	0.1ml	MPN Index/100ml
0	0	0	<2
0	0	1	2
0	1	0	2
0	2	0	4
1	0	0	2
1	0	1	4
1	1	0	4
1	1	1	6
1	2	0	6
2	0	0	4
2	0	1	7
2	1	0	7
2	1	1	9
2	2	0	9
2	3	0	12

3	0	0	8
3	0	1	11
3	1	0	11
3	1	1	14
3	2	0	14
3	2	1	17
4	0	0	13
4	0	1	17
4	1	0	17
4	1	1	21
4	1	2	26
4	2	0	22
4	2	1	26
4	3	0	27
4	3	1	33
4	4	0	34
5	0	0	23
5	0	1	30
5	0	2	40
5	1	0	30
5	1	1	50
5	1	2	60
5	2	0	50
5	2	1	70
5	2	2	90
5	3	0	80
5	3	1	110
5	3	2	140
5	3	3	170
5	4	0	130
5	4	1	170
5	4	2	220
5	4	3	280
5	4	4	350
5	5	0	240
5	5	1	300
5	5	2	500
5	5	3	900
5	5	4	1600
5	5	5	>1600

3.2.2.2 Membrane Filter Technique

This method consists of filtering water sample on a sterile filter with a 0.45 micrometer pore size which retains bacteria, incubating filter on selective medium and enumerating colonies on the filter. The most widely used media for this method is m-Endo-type media. This is selective and differential media that inhibits the growth of gram positive bacteria and enhances the growth of gram negative. Coliform bacteria form red colonies with a metallic sheen on Endo-type medium containing lactose. Mac Conkey agar can also be used as a media. The presence of high number of heterotrophic bacteria has shown a decreased coliform recovery by this method. This method cannot detect stressed or injured coliforms. A significant advantage over MTF method is that through MF examination of larger volumes of water is feasible thus increasing its sensitivity and reliability.

3.2.2.3 Enzymatic Methods

Beta-D-glucuronidase is an enzyme which catalyzes beta-D-glucopyranosiduronic into aglycons and D-glucuronic acid. Activity of this enzyme is limited to *E.coli*. Therefore, this enzyme is used for the detection of *E.coli* in water. Beta-D-galactosidase catalyzes the breakdown of lactose into galactose and glucose. To detect the activity of beta-D-glucuronidase, indoxyl-beta-D-glucuronide (IBDG) is used as chromogenic substrate. p-nitrophenyl-beta-D-galactopyranoside (PNPG) is used to detect beta-D-galactosidase.

3.2.2.4 Molecular Methods

They increase the sensitivity and specificity of the detection of cultivable as well as non cultivable bacteria. The most common method employed for detecting bacteria are the immunological methods and PCR based method.

3.2.2.5 Immunological Methods

Immunological methods are based on the specific recognition between antigen and antibody. ELISA method can be used by utilizing a monoclonal antibody against the enterobacterial common antigen (ECA), a lipopolysaccharide which is linked within the outer membrane of ***Enterobacteriaceae***. Pre-cultivation of the sample in a selective broth for 24 h was carried out to increase the sensitivity of ELISA. It's application for the detection of specific cells from natural contaminated sample is limited as non-targeted microflora may interfere with the sensitivity of ELISA(Rompre et al.2002)

3.2.2.6 Polymerase Chain Reaction Technique

PCR amplification is performed on the nucleic acid content obtained by a cellular lysis followed by a chemical extraction. PCR includes Denaturation of the DNA, annealing primers on the template and extension. Amplification of the DNA requires 20-30 cycles. PCR product is then analyzed by electrophoresis on agarose gel (Rompre et al.2002)

3.2.3 Biomolecular Tests

IMViC Tests

They are indole test, methyl red test, Voges-Proskauer test and Citrate test.

3.2.3.1 Indole Test

This test is performed in a peptone water culture. This test checks for the production of indole from tryptophane. 0.5ml of Kovac's reagent is added. A red coloration indicates a positive result (Fig 7).

3.2.3.2 Methyl Red Test

This test is done to check for the production of acid during fermentation of glucose. Five drops of 0.04% solution of methyl red is added in MR-VP medium. Red coloration indicates a positive result (Ananthanarayan R. & Paniker. C.K. 2009) (Fig 8).

3.2.3.3 Voges-Proskauer Test

This test is done to detect the production of acetyl methylcarbinol from pyruvic acid, an intermediate in the conversion to 2:3 butyl glycol. The test is performed by the addition of alpha-naphthol in ethanol and 0.2ml of 40% KOH to 1ml of MR-VP culture of organism. Pink coloration in 2-5 minutes which further deepens to red-brown color in half an hour, shows a positive result (Ananthanarayan R. & Paniker. C.K. 2009) (Fig 9).

3.2.3.4 Citrate Test

It is based on the ability of the bacterium to utilize citrate as a sole carbon source. It uses a Simmon Citrate agar medium. A positive result is indicated by turbidity and a blue color. Negative result indicates the lack of growth (Ananthanarayan R. & Paniker. C.K. 2009) (Prescott L.M 1993)(Fig 10).

Procedure Performed

Four tubes of NB broth were prepared and colonies from (E1), (E2), (S1) and (S2) were inoculated and incubated for 24hrs. The above mentioned biochemical tests (Table 6) were performed by inoculating the culture from NB broth. Four tubes each of Peptone water, MRVP broth and slants of Simmon Citrate agar was prepared and inoculated with NB broth samples. Incubation of 24 hrs at 37 $^{\circ}$C was provided. After which Indole test (Fig 7), Methyl red (Fig 8), Voges- Proskauer test (Fig 9) and Simmon Citrate test (Fig 10) were performed.

3.2.4 Miscellaneous Tests Performed

- EMB
- Mac Conkey
- XLD
- TSI

3.2.4.1 EMB Test

This test uses the Eosin-methylene blue agar which is a selective and a differential media. It allows the growth of gram-negative bacteria and inhibits the gram-positive. The bacteria that ferment lactose in the medium form colored colonies, while non lactose fermenting forms colorless colonies.

EMB agar contains peptone, lactose, sucrose, eosin Y and methylene blue dyes. The dye methylene blue in the medium inhibits the growth of gram-positive bacteria. Eosin dye responds to changes in the pH, going from colorless to dark purple under acidic conditions. The medium contains lactose and sucrose as energy sources. This medium detects the presence of fecal coliforms on their ability to ferment lactose. Therefore, a lactose fermenting bacteria will acidify the medium, the dye produces a dark purple complex which may or may not be associated with a green sheen. A slow fermenter of lactose will give a brown-pink coloration of growth. Colonies of a non-lactose fermenting bacteria appears translucent or pink (Fig 4) (Fig 13)

3.2.4.2 Mac Conkey Test

This test uses the Mac Conkey agar which is used for the isolation and differentiation of gram-negative enteric bacilli. It consists of lactose as a carbon source. Enzymatic Digest of Gelatin, Enzymatic Digest of Casein and Enzymatic Digest of Animal Tissue are the nitrogen and vitamin sources. During lactose fermentation pH drops around the colony causing a color change in the pH indicator, Neutral Red and bile precipitation. Bile Salts mixture and Crystal Violet are the selective agents, inhibiting Gram-positive bacteria and allowing only Gram-negative to grow (Fig 11).Pink colored colonies show the presence of fecal coliforms (Ananthanarayan R. & Paniker. C.K. 2009) (Fig 5) (Fig 14)

3.2.4.3Xylose-Lysine Deoxycholate (XLD) Test

This test uses the XLD agar which is a selective growth media used in isolation of *Salmonella typhi* and other *Salmonella* species. Degradation of xylose, lactose and sucrose to acid causes phenol red indicator to change its color to yellow. It utilizes sodium deoxycholate as the selective agent and therefore it is inhibitory to gram-positive microorganisms (Forbes B.A 2002)

3.2.4.4Triple Sugar Iron (TSI) Test

This test utilizes the TSI medium which is a composite medium. It consists of glucose, sucrose and lactose as the carbon source. Slants are prepared of the above media. After inoculation, if the

slant remains red and the butt becomes yellow, all the sugars are fermented by the bacteria. The presence of bubbles in the butt area indicates gas production and blackening of the medium shows formation of hydrogen sulphide. Blackening of the media is the characteristic feature of *Salmonella (*Table 5*)*(Ananthanarayan R. & Paniker.C.K. 2009) (Prescott L.M 1993). We prepared the TSI slants and streaked colonies of non-lactose fermenting (S1) and non-lactose fermenting (S2) from main reservoir and orange colored (E1) and (E2) colonies of Pumping Station (Fig 6). After 24 hrs incubation a color change was observed.

3.2.5 Strain Identification using Biochemical Strip

KB002 is a test system that can be used for identification of gram negative rods. HiAssorted Biochemical test kit can be used for screening pathogenic organisms from urine, enteric specimens and other relevant clinical samples. It can also be used for validating known laboratory strains. (Fig 12)

Each HiAssorted Biochemical test kit is a standardized colorimetric identification system utilizing seven conventional Biochemical tests and five carbohydrate utilization test. The tests are based on the principal of pH change and substrate utilization. On incubation organisms undergo metabolic changes which are indicated by a color change in the media that can be either interpreted visually or after addition of reagent. We performed this test using Orange and Dark orange colored MPN vials of Main Reservoir samples (Ashwani Khad) (Table 7).

KB009 is a comprehensive test system that can be used to study the biochemical profile of a wide variety of organisms. It can also be used for validating known laboratory strains. Each HiCarbohydrate kit is a standardized colorimetric identification system utilizing thirty five carbohydrate utilization test. Tests are based on the principal of pH change and substrate utilization. On incubation organisms undergo metabolic changes those are indicated by spontaneous color change in the medium. We performed this test using the Orange and Dark orange colored MPN vials of Main Reservoir samples of Ashwani Khad. In case of vials showing orange coloration, we got a negative result for raffinose, melibose, inositol, cellobiose.

In case of vial showing dark orange coloration we get a negative result for cellobiose, rabiose, lactose, xylose.

3.2.6 Physico-chemical Analysis for the Drinking Water Quality

Parameters for drinking water quality typically fall under two categories - Chemical and Physical. Chemical parameters causes chronic health risks through built up of heavy metals whereas physical parameters affect the aesthetics and taste of drinking water. Drinking water is analyzed for various parameters such as turbidity, pH, nitrate, fluoride, chloride, residual free chlorine and other ions.

The pH of water is extremely important. The fluctuations in optimum pH ranges may lead to an increase or decrease in the toxicity of poisons in water bodies. The pH is measure of the intensity of acidity or alkalinity and the concentration of hydrogen ion in water. pH has no direct adverse effects on health, however, higher values of pH accelerate the scale formation in water heating apparatus and also reduce germicidal potential of chloride. High pH induces the formation of tri-halomethanes which are toxic (Kumar *et al*., 2010) (Table 8). Another important Physico-chemical parameter is turbidity of water which is actually the expression of optical property in which the light is scattered by the particles present in the water. Change in water color from transparent to light-yellowish, reddish or grayish indicates the increase in turbidity. High turbidity in water also interferes with chlorine disinfection process and provides a growth medium to pathogenic microbes. Similarly, when the fluoride concentration in water increases more than 1.5 mg/L, it induces a teeth disease in calcification stage of children. The pathological condition becomes evident from yellow to brown patches on teeth. When fluoride concentration exceeds the level 8 mg/l, it may cause endemic, cumulative fluorosis with resultant skeletal damage in both children and adult. People become old in short age. Their joints are almost finished at the age of 30-40 (Meena *et al.,* 2011). Major source for fluoride contamination is the industrial effluent run off and geological source. Similarly, groundwater contains nitrate contamination due to leaching with the percolating water. Runoff from agricultural fields contributes major in nitrate pollution. Excessive concentrations of nitrates cause blood disorders. Nitrate after reduction to nitrite in body deplete oxygen in blood resulting in methemoglobinemia or what is commonly known as "blue baby syndrome" disease **(**Mathur *et al*., 2012**).** Some recent studies have shown that nitrates in drinking water besides causing methemoglobinemia can result

in various other clinical manifestations like recurrent stomatitis, recurrent respiratory tract infections (RRTI) etc**.** Another important parameter is the presence of iron in drinking water. Iron in water is predominantly present as Fe^{3+}. It is necessary to reduce Fe^{3+} to Fe^{2+}. This is accompanied by addition of reducing agent hydroxyl amine. Fe^{2+} is quantitatively complexed by 1, 10 –phenanthroline in the pH range from three to nine. Sodium acetate is used as buffer to maintain pH 3.5. Detection of iron-phen complex is performed by spectrometer at 508nm. Iron compounds can have more serious effects on health than the element itself. Water soluble compounds like iron chloride can cause lethal effects upon a concentration of 200mg, and are toxic for adults upon the dose of 10-50g (Fig 16). Heavy metals are metals with high molecular weights that are of concern because they are generally toxic to animal life and human health if naturally occurring concentrations are exceeded. Heavy metal toxicities are relatively uncommon. However, failure to recognize and treat heavy metal toxicities can result in significant morbidity and mortality. Dehydration is common. Encephalopathy is a leading cause of mortality in patients with both acute and chronic heavy metal toxicity. For example at lower doses, copper ions can cause symptoms typical of food poisoning (headache, nausea, vomiting and diarrhea) (Abdul et al.2012)(Table 9). Thus, physico-chemical analysis is important to determine quality of drinking water as well as in water management studies.

Chapter 4 Results and Discussion

4.1 Analysis of water using MPN method

We performed MPN analysis using Ashwani Khad(Table 3) sample taken on 15/08/2013 and 12/01/2014. The same analysis was carried out using samples of Cherot Nallah(Table 4) taken on 12/01/2014.

Table 3- Seasonal analysis of water samples from Ashwani Khad

Water(Ashwani Khad)	**MPN(July-Oct)**	**MPN(JAN)**	**Permissible limit**	**Human consumption**
Main Reservoir	1800cfu/100ml	1600cfu/100ml	6cfu/100ml	UNFIT
Pumping Station	34cfu/100ml	200cfu/100ml	6cfu/100ml	UNFIT
Distribution Line	2cfu/100ml	4cfu/100ml	6cfu/100ml	FIT
Consumer Tap	0cfu/100ml	2cfu/100ml	6cfu/100ml	FIT

As it can be seen, MPN value increases in summer season in comparison to winter season. Therefore, we can infer that the coliform count is more in the summer season leading to water contamination. This could be further verified by the reported cases of water born diseases which

are more in summers as compared to that in winters. The MPN test when conducted in Mughalpura, Lahore they found high cfu count in consumer tap water (3.99-4.39 log cfu/100 ml), followed by distribution line (3.65-4.25 log cfu/100 ml) and main reservoir (3-3.96 log cfu/100 ml). (Sulehria et.al 2011)

Table 4- Analysis of water samples from Cherot Nallah taken on 12/01/2014

Water(Cherot Nallah)	MPN	Permissible limit	Human Consumption
Main Reservoir	350cfu/100ml	6cfu/100ml	UNFIT
Pumping Station	350cfu/100ml	6cfu/100ml	UNFIT
Distribution Line	2cfu/100ml	6cfu/100ml	FIT
Consumer Tap	0cfu/100ml	6cfu/100ml	FIT

As it can be, MPN value decreases from Main Reservoir to Consumer Tap phase. The MPN value of Consumer Tap being 0cfu/100ml which is lower than the permissible limit making the water fit for human consumption. Thus we can infer that the water treatment procedure is properly implemented owing to the decreased contamination of this water.

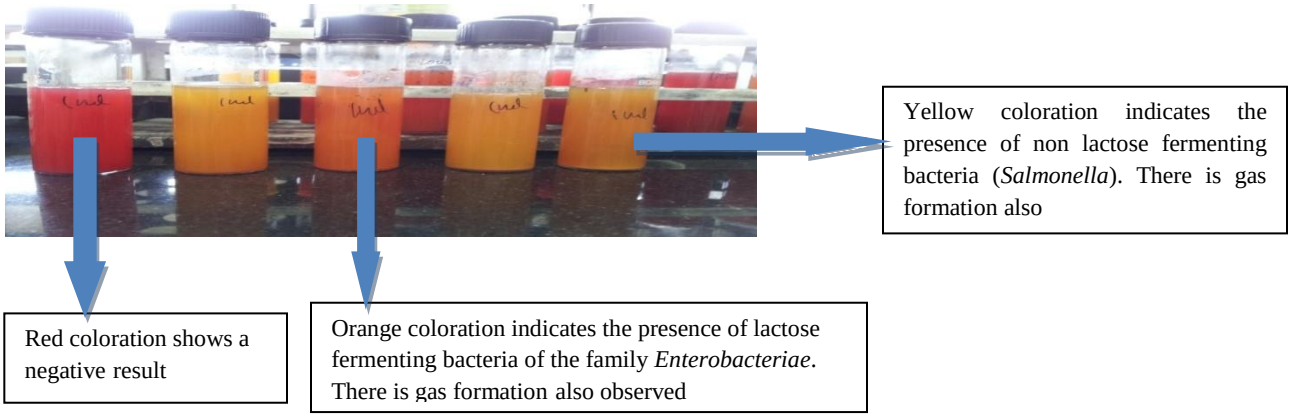
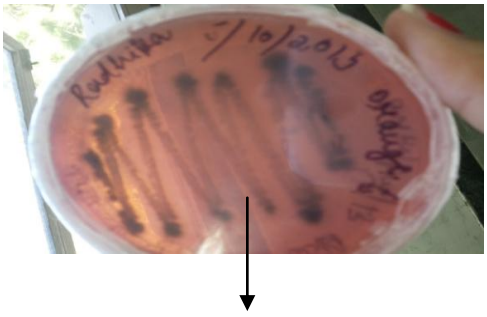


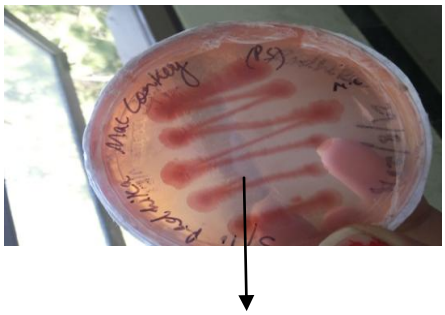
Fig 3: MPN performed for 1ml of Main Reservoir Sample of Ashwani Khad area.

4.2 Characterization of Bacteria

Fig 4- Spreading the water samples showing yellow and orange coloration in MPN respectively, Main Reservoir and Pumping Station sample of Ashwani Khad area on EMB Agar.



Main Reservoir MPN orange sample on Mac Conkey



Pumping Station MPN Yellow sample on Mac Conkey

Fig 5- Streaking the colonies taken from EMB plates above, on Mac Conkey Agar

Fig 6- Results of TSI of E1, E2, S1, S2 (left to right) samples

Table 5- Results of TSI slants of water samples taken from ASHWANI KHAD

	E1	**E2**	**S1**	**S2**
Butt and Slant	Brown, Red	Brown, Red	Yellow, Red	Red, Red
Bacterium	*Salmonella typhi*	*Salmonella typhi*	*Klebsiella pneumonia*	*Pseudomonas*
Comments	Typhoid Fever	Typhoid Fever	Pneumonia	GU infections

After 24 hrs of incubation we observed a color change (Fig 6). A brown color was observed in (E1) (E2) with the blackening of Butt. Blackening is due to the formation of H_2S gas indicating the presence of *Salmonella typhi* in the sample. Yellow coloration was observed in (S1) it means glucose fermentation only and peptone is catabolized indicating the presence of *Pseudomonas* and red in (S2) it means no fermentation and peptone is catabolized indicating the presence of *Klebsiella.* At the university of Kentucky TSI test was performed, 48 isolates from the *Vibrio* were inoculated into triple sugar iron agar slants, incubated overnight and tested for oxidase activity. On TSI slants 13 isolate produced and all were oxidase-positive. (Skillern et. al 1983).

Fig 7- The indole tests results of E1, E2, S1, S2

The positive result is indicated by the red layer at the top of the tube after the addition of Kovac's reagent. A negative result is indicated by the lack of color change at the top of the tube after the addition of Kovac's reagent.

Fig 8- The methyl red test results of E1, E2, S1, S2

Positive methyl red test are indicated by the development of red color after the addition of methyl red reagent. A negative methyl red test is indicated by no color change after the addition of methyl red reagent.

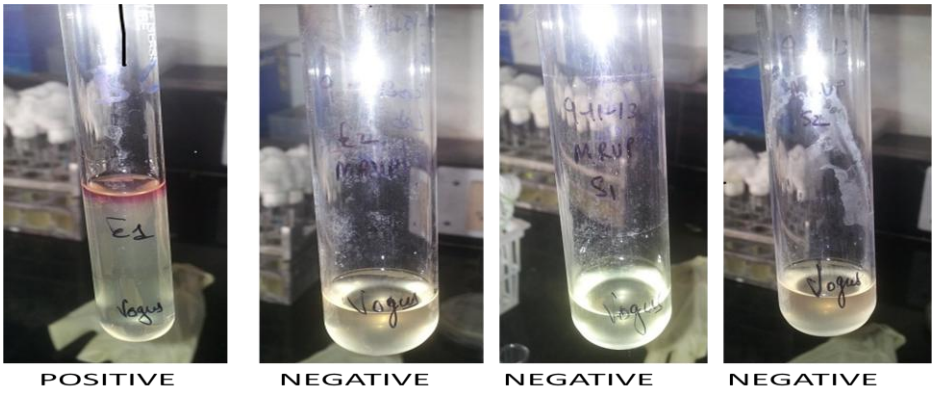


Fig 9- Voges-Proskauer test results of E1, E2, S1, S2

Negative test is indicated by lack of color change after the addition of Barritt's A and Barritt's B reagents. A positive Voges-Proskauer test is indicated by the development of red-brown color after the addition of Barritt's A and Barritt's B reagents.

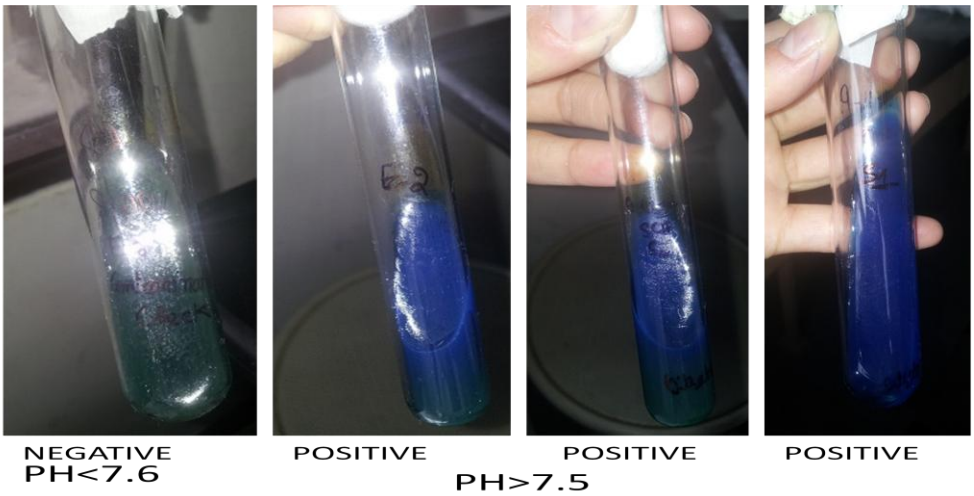


Fig 10- Simmon Citrate test results of E1, E2, S1, S2

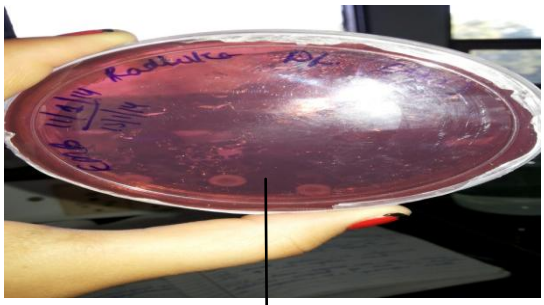
Negative citrate utilization test is indicated by the lack of growth and color change in the tube. A positive citrate result as indicated by growth and a blue color change**.**

Table 6- Results of the biochemical tests done from Main Reservoir and Pumping Station (Ashwani Khad)

Tests Performed	Time Incubated	E1	E2	S1	S2
TSI	24-48 hours	Formation of Hydrogen	Formation of Hydrogen	Yellow color	Red color
Indole	Immediate	Negative	Negative	Negative	Negative
Methyl Red	Immediate	Negative	Positive	Positive	Positive
Voges-Proskauer	20 minutes	Positive	Negative	Negative	Negative
Citrate	24 hours	Negative	Positive	Positive	Positive

As per the pattern observed in the biochemical tests we can infer the presence of *Salmonella* in (E2) ,(S1), (S2) and *Pseudomonas* in (E2). This indicates that the water is potentially unsafe for human consumption by the very high counts of potential pathogens enumerated and identified.

Salmonella is an agent of gastrointestinal illness that belongs to the same microbiological family as *E. coli*, *Enterobacteriaceae*. *Salmonella* are non-spore-forming, facultative anaerobic, Gram-negative bacilli that are 2–5 µm long and 0.8–1.5 µm wide (AWWA, 2006). This pathogen is associated with cases of human gastroenteritis. The absence of *E. coli* during routine verification should be an adequate indication of the sufficient removal and inactivation of *Salmonella. Pseudomonas* is part of a large group of free-living bacteria that are ubiquitous in the environment. This organism is often found in natural waters such as lakes and rivers. It can cause endocarditis, osteomyelitis, pneumonia, urinary tract infections, gastrointestinal infections, and meningitis, and is a leading cause of septicemia.



No green sheen, absence of *E.coli*

Green sheen is visible therefore *E.coli* is detected

Fig 11- Streaking of Main Reservoir and Distribution Line (Ashwani Khad) water samples taken on 12/01/2014.

Fig 12- Strip 1 and 2 of KB002 series of HiAssorted biochemical test kit identifying the strain

Table 7- Results of biochemical tests performed with the help of KB002 strips

TEST	CITRTE UTILISATION	LYSINE	ORNITHINE	UREASE	TDA	NITRATE REDUCTION	H_2S PRODUCTION	GLUCOSE	ADONITOL	LACTOSE	ARABINOSE	SORBITOL
Salmonella cholerasuis	+	+	+	-	-	+	+	+	-	+	+	-
Vibrio cholerae	-	+	+	+	-	-	+	-	+	-	-	-

As per the pattern observed in the test we can infer the presence of *Salmonella cholerasuis* sub species indica in orange sample and *Vibrio cholera* in dark orange sample of Main Resevoir (Ashwani Khad).

Serotype *Choleraesuis* is a host-adapted pathogen that causes swine paratyphoid. It is also highly pathogenic to humans, usually causing septicemic disease with little involvement of the intestinal tract. In comparison with other highly prevalent serotypes of *Salmonella*, such as serotypes *typhi*, *typhimurium*, and *enteritidis*, this organism has received much less attention; therefore, not surprisingly, our knowledge of it is not only incomplete but also significantly lacking. *Vibrio cholerae*, the bacterium that causes cholera, is usually found in food or water contaminated by feces from a person with the infection. It causes severe watery diarrhea, which can lead to dehydration and even death if untreated.

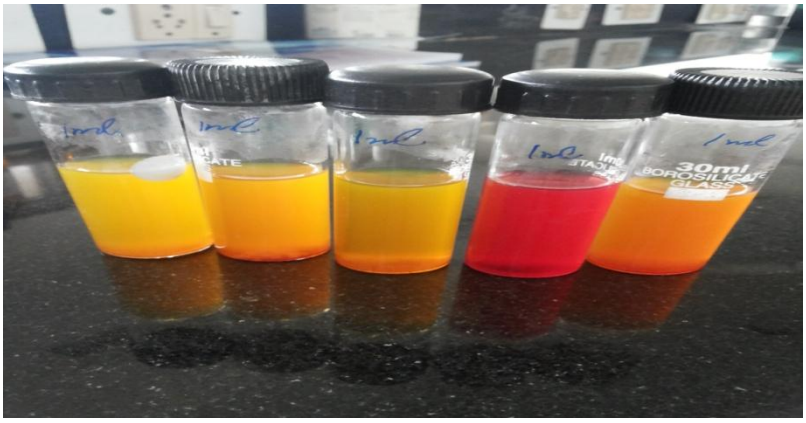


Fig 13- Result of MPN analysis of Consumer Tap Cherot Nallah

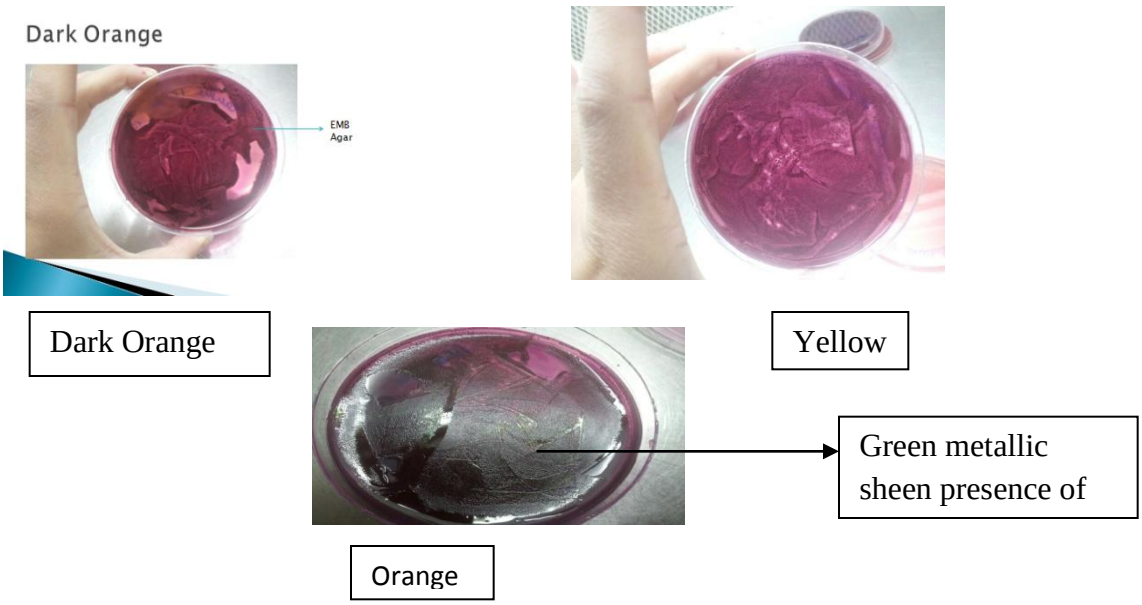


Fig 14- Orange, Dark orange and yellow colored MPN samples of Cherot Nallah Consumer Tap was spread on EMB agar.

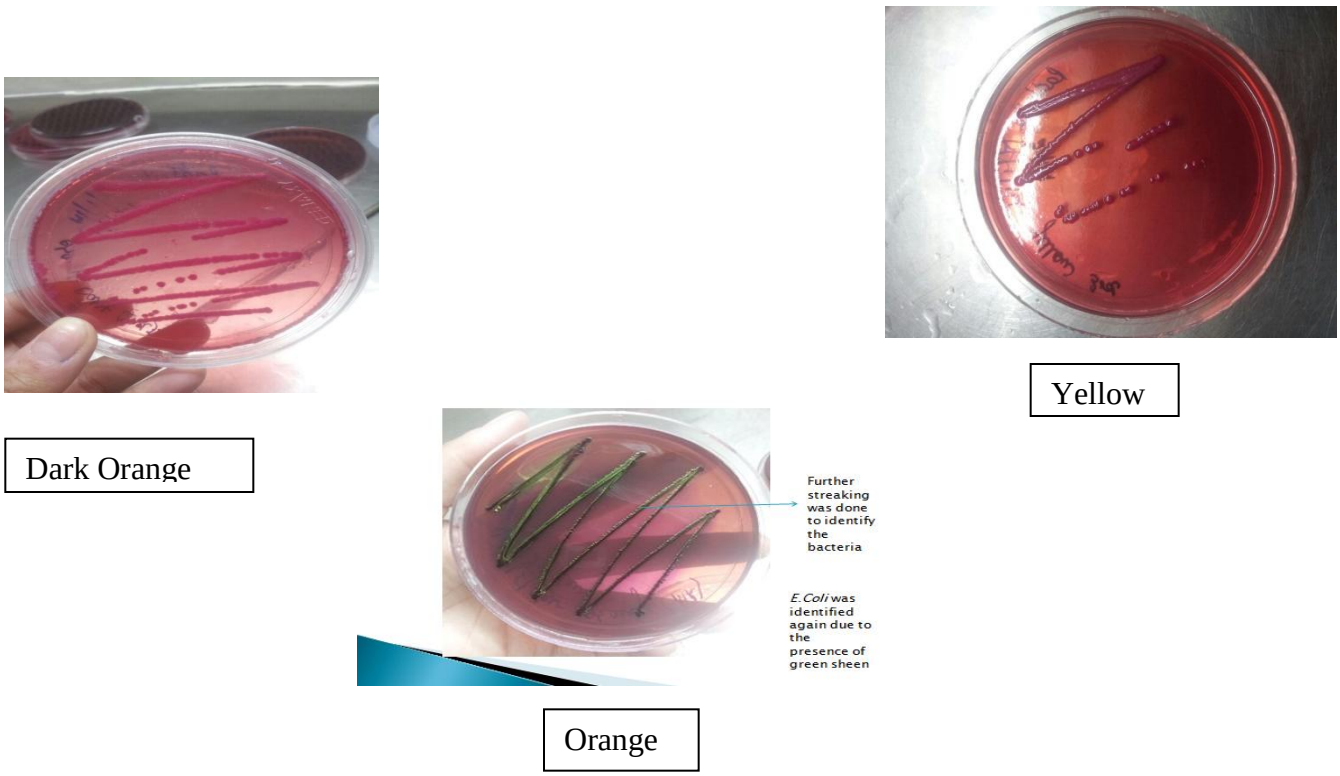


Fig 15- Above (Fig 14) spread colonies were further streaked

On further characterization of Consumer Tap (Cherot Nallah) green sheen indicating the presence of *E.coli* was observed. This indicates that the water is unfit for human consumption as these causes bloody diarrhea. The presence of *E.coli* indicates that the water is contaminated by feces. People can become infected when a contaminated city or town water supply has not been properly treated with chlorine or when people accidentally swallow contaminated water while swimming in a lake, pool, or irrigation canal.

4.3 Physico-chemical properties of water samples

4.3.1 pH analysis

The pH of water samples range from 7.09 to 7.94 with a mean of 7.42. According to WHO for drinking water quality the permissible limit of pH is 6.5 to 8.0. The statistical analysis of physico-chemical properties of drinking water quality are given in Table 8.

Table 8 The statistical analysis of Physico-chemical properties in water samples of Ashwani khad and Chamber Nallah area in Shimla.

Parameters	**pH**
Number of Samples	8
Minimum	7.09
Maximum	7.94
Arithmetic Mean	7.42
Median	7.44
Mode	7.42
WHO Permissible limits	6.5-8.0

For an effective chlorination the pH range should be 6.5 to 8.0(WHO 2004). The pH measured falls within this range therefore the disinfection procedure is being properly implemented and the water is fit for human consumption

4.3.2 Trace, heavy and rare earth elements analysis using AS

The concentrations of Chromium (Cr), Nickel(Ni),Cobalt(Co), Copper(Cu), Zinc(Zn), Aluminum(Al) in drinking water samples are presented in Table 9.

The concentration of Cr in water samples was found to be 0.090 mg/L within desirable limit of Cr specified as 0.05 mg/L for drinking water (WHO 2004) .Cr is not acutely toxic to humans. Cr^{+6} is more toxic than Cr^{+3} because of its high rate of adsorption through intestinal tracts. The concentration of Ni in the water samples ranges from 0.023 to 0.136 mg/L. The desirable limit for Ni is 0.2mg/L for drinking water. Ni compounds induce nasal, laryngeal and lung cancer (Lessard et al. 1978). Co concentration in the water samples was nil. The concentration of Cu in the water samples ranges from 0.027 to 0.070 mg/L. The desirable limit of Cu in the drinking water is 0.05 mg/L (ISI 1983). 12.5% of the samples show high Cu concentration. High doses of Cu can cause stomach and intestinal distress, liver and kidney damage and anaemia (USEPA 1999). Acute Cu toxicity causes headache, nausea, vomiting, gastrointestinal irritation (Stenhammar 1999). The concentration of Zn in drinking water samples ranges from 0.025 to 0.092mg/L. The desirable limit of Zn in drinking water is specified as 3 mg/L. All the water samples are within the desirable limits of WHO. High concentration of Zn can be toxic, plays a very important role in the physiological processes of many organisms (Pillai 1983). Al concentration in water samples ranges from 0.29 to 0.36 mg/L. However the desirable limit of Al in drinking water is 0.2mg/L. 90% of the samples show high Al concentration. Al has been considered to be a causative agent for various neurological disorders including the Alzheimer's disease (Gardner and Gunn 1995).

Table 9 Concentration of trace and heavy metals in drinking water supply of Shimla area (Ashwani Khad and Cherot Nallah)

Elements	Maximum	Minimum	Average	WHO(2004)
Chromium(Cr)	0.090	0.090	0.00	0.05
Nickel(Ni)	0.136	0.023	0.05	0.02
Cobalt(Co)	0	0	0	N.A
Copper(Cu)	0.070	0.027	0.051	0.05
Zinc(Zn)	0.092	0.025	0.047	3
Aluminium(Al)	0.39	0.29	0.33	0.2

N.A not available, concentration mg/L

The concentration of Al, Cu, and Ni exceeds the permissible limit of drinking water quality. However none of the water samples analyzed for Cr, Co and Zn exceeds the limit permitted by WHO. The gastrointestinal infections witnessed in the past could be a reason of copper contamination in water. Therefore the water is not recommended as fit for human consumption.

Chapter 5 Conclusion

Water is an essential natural resource for sustaining life. Contamination in drinking water results in increase in mortality rate and economic burden due to health losses in the society. Therefore, it is important to maintain the drinking water quality to protect the environment and to maintain public health. In the present study, water samples were collected from Ashwani Khad and Cherot Nallah areas of Shimla city. Analysis of Physico-chemical parameters were found to be within the permissible limits but the Bacteriological parameters of Ashwani Khad were found to be above the permissible limit prescribed by the WHO. Enteric bacteria were detected in all water samples. We found *Salmonella typhi* and *Pseudomonas* in the Ashwni Khad sample during the winter season. *Vibrio cholera* and *Salmonella cholerasuis* were found in the water of same source during the summer season. *E.coli* was detected in the water from Cherot Nallah. Thus consumption of this water produces a higher risk to the consumers.

Majority of the people living in Shimla depend on direct water supplies therefore this study suggests that government should take necessary action to provide hygienically safe drinking water in order to protect the environment and to safeguard public health. Regular monitoring and maintenance of drinking water supplies should be done. Proper drinking water treatment filtration plant must be set. Treatment procedure should be properly implemented. This may result in a number of substantial benefits to individual and to the society.

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