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Ministry of Higher Education
Prince Sattam Bin Abdulaziz University
College of Applied Medical Sciences
Department of Medical Laboratories Sciences



**Assessment of Coliform Pathogenic Bacteria in Tank Drinking and Tap Water in Different Areas in
Al-kharj and Its Villages Affiliate, Kingdom of Saudi Arabia**

Graduation project

In

Medical Laboratory Department

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WHO	World Health Organization
<i>E. coli</i>	<i>Escherichia coli</i>
PCR	polymerase chain reaction
<i>P.aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
HIV	Human immunodeficiency Virus
AIDS	Acquired immunodeficiency syndrome
ETEC	Enterotoxigenic
EHEC	Enterohemorrhagic
EIEC	Enteroinvasive serotypes
<i>S. dysenteriae</i>	<i>Shigella dysenteriae</i>
MAC	MacConkey's Agar
EMB	Eosin-methylene blue
XLD	Xylose lysine deoxycholate
CFU	Colony-forming units

Abstract

Water is one of the most abundant and essential commodities of man occupying about 70% of the earth's surface and 60% of the human body therefore it should be continuously protected against microbial infections. Tank drinking water and tap water from different water resources such as wells and tanks should be free from contamination with waterborne pathogens including bacteria. In this study, samples of both drinking and tap water were collected from different regions of Al-Kharj and its villages affiliate (Al- kharj, Al-hayathem ,Al-dalam, Al- rfaya, Al-hawta), Kingdom of Saudi Arabia. This study aim to determine the presence of coliform pathogenic bacteria in tap water and tank drinking water.

Methods: Both water samples were tested for the presence of coliform pathogenic bacteria and this was determined by centrifugation technique. In addition, bacterial isolates were identified by phoenix 100 BD company automated identification and sensitivity machine. Finally, bacterial count dilution technique was used to count the bacterial content in the water samples used in our study.

Results: It was found that there is no bacterial contamination in tank drinking water. Otherwise, there's no contamination with coliforms bacteria in all samples, but the tap water samples were contaminated with pathogenic bacteria. It was also shown that the bacterial isolates found in tap water including *pseudomonas aerruginosa*, *pseudomonas putida*, *Acinetobacter iwoffii* and *Moraxella species*. In tap water of (Al-kharj, Al- hayathem ,Al-dalam, Al- rfaya, Al-hawta)

Conclusion: Thus water considered to be consumed by humans must maintain good microbial qualities within the acceptable ranges and must undergo effective treatment in order to reduce bacterial count and infection.

Key words: Coliform, Pathogenic bacteria, Tap and Tank drinking water, Al- kharj, KSA

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1.introduction

The World Health Organization(WHO) estimated that up to 80% of all sicknesses and diseases in the world are caused by inadequate sanitation, polluted water or unavailability of water. The Water is the most important resource for humans, it forms 50 to 60% in weight of our body and play an active role in all the vital processes of our body. ^[1]

Water cushions joints, and protects tissues and organs from shock and damage. It acts as a lubricant for joints, mouth and digestive system in saliva, and in nose, throat, eyes, and stomach as part of mucus. It also aids in digestion and absorption of food, as well as in the removal of wastes from the body. ^[2] Every day after tank drinking water or eating watery food, this replenish our metabolic reserve. ^[2-3]

Microbiological pollution in tank drinking water includes bacteria, virus and fungi. These microorganisms can be responsible of serious diseases as typhoid, cholera, and hepatitis. Their presence can be easily detected. Bacterial re-growth is encouraged by the lack of a residual disinfectant and by the possibly great variability of nutrients in water, such the low-mineral water, particularly if it has a high temperature. ^[3, 4]

The quality and safety of tank drinking water remain as an important public health issue. Contamination of tank drinking water has frequently been blamed for the transmission of infectious diseases that have caused serious illnesses with associated mortality worldwide. ^[5,6] Each year, an estimated 1.9 million deaths, primarily of children under 5 years of age, result from unsafe tank drinking water and inadequate sanitation and hygiene. ^[7]

The WHO estimates that improving water, sanitation, and hygiene could prevent approximately 9.1% of the global burden of disease and 6.3% of all deaths.^[8] Groundwater is still and will continue to be the main source of safe and reliable tank drinking water in arid regions like Saudi Arabia, where surface water is scarce, rainfall is irregular and rates of evaporation are very high. Hence, groundwater is a key source for urban and rural supplies and is considered the only source to meet domestic and agricultural needs in towns and villages.^[9]

Tank drinking water should be free from known pathogenic microorganisms and indicator bacteria which is a symptom for fecal contamination of water . Coliforms are the most important indicator bacteria which are considered in the bacteriological examination of water.^[10] Coliform bacteria, members of the Enterobacteriaceae (e.g., species of *Enterobacter*, *Klebsiella*, *Citrobacter*, and *Escherichia*) Determination of coliform as an indicator of water contamination has been explained in 1011 standard (microbiological characteristics of water). The use of *Escherichia coli* (*E. coli*) and other coliform bacteria and those coliforms that are indicator bacteria has been recommended for the daily control of tank drinking water. Coliform bacteria are used for monitoring the bacteriological safety of water supplies on the basis of the realization that the presence of coliform bacteria in water is an indicator of potential human fecal contamination and therefore the possible presence of enteric pathogens.^[11]

In general, this standard focuses on three groups of bacteria including coliform, thermotolerant coliform and *E.coli*.^[12] *E.coli* is used as an indicator of fecal contamination of water. Detection of indicator bacteria is one of the best ways to evaluate the effectiveness of water disinfection methods. .^[12]

The most important indicator bacteria, in terms of their importance, include *E. coli*, coliforms and other thermotolerant coliforms. The presence of these bacteria in the water is an indicator of insufficient disinfection process and also recent and frequent contamination of water with human and animal feces.^[13]

Thermo tolerant coliforms, except *E. coli*, can enter the tank drinking water through water contaminated by industrial wastewaters and under deterioration soil and water.^[14] polymerase chain reaction (PCR) has been recommended as a specific and reliable method for the detection of coliforms in tank drinking water.^[15]

The presence of *E.coli* in water is a strong indication of recent sewage or faecal contamination. Sewage may contain many types of disease causing organisms. *E. coli* comes from human and animal waste. During rainfalls, snow melts, or other types of precipitation, *E.coli* may be washed into creeks, rivers, streams, lakes, or groundwater. When water is used as a source of tank drinking water and the water is not treated or inadequately treated, *E.coli* may end up in the tank drinking water.^[16]

Faecal coliforms and *E.coli* are bacteria whose presence indicates that the water may be contaminated with human or animal wastes. Microbes in these waters can cause short-term effects, such as diarrhea, cramps, nausea, headaches, or other symptoms. They may pose a special health risk for infants, young children, some of the elderly, and people with severely compromised immune systems.
[17-18]

Pet wastes (cats,dogs) can contribute to faecal contamination of surface waters. Runoff from roads, parking lots, and yards can carry animal wastes to streams through storm sewers. Birds can be a significant source of faecal coliform bacteria. Birds (seagulls, geese, swans) can all elevate bacterial counts, especially in freshwater systems (wetland, rivers, lakes and ponds).Some waterborne pathogenic diseases that may coincide with faecal coliform contamination include ear infections, viral and bacterial gastroenteritis, dysentery, typhoid fever and hepatitis A. ^[19-20]

1.1. Coliforms

By definition, coliform bacteria are facultative anaerobes, Gram-negative, non- spore-forming, rod-shaped bacteria that ferment lactose with acid production in 24 to 48 h at 36 °C, and indole-negative. Coliforms belong to the family *Enterobacteriaceae* and include *Escherichia*, *Enterobacter*, *Klebsiella* and *Citrobacter*, *Kluyvera*, *Leclercia* genera, and some members of the genus *Serratia*. These bacteria were classically used as indicators of faecal contamination of waters because they were considered to be inhabitants of the intestinal tracts of homoeothermic animals. ^[21,22]

1.2. Pathogenic *Escherichia coli* Strains

E. coli strains isolated from intestinal diseases have been grouped into at least six different main groups, based on epidemiological evidence, phenotypic traits, clinical features of the disease and specific virulence factors. From these, enterotoxigenic (ETEC, namely O148), enterohemorrhagic (EHEC, namely O157) and enteroinvasive serotypes (EIEC, namely O124) are of outstanding importance and can be transmitted through contaminated water. ^[23,24]

1.2.1. Enterotoxigenic *E. coli* (ETEC) Strains

Enterotoxigenic *E. coli* (ETEC) serotypes can cause infantile gastroenteritis. The number of reports of their occurrence in developed countries is comparatively small, but it is an extremely important cause of diarrhea in the developing world, where there is no adequate clean water and poor sanitation. In developing countries, these strains are the most commonly isolated bacterial enteropathogen in children below 5 years of age, and account for several hundred million cases of diarrhea and several ten of thousand deaths each year. [23-25]

Disease caused by ETEC follows ingestion of contaminated food or water and is characterized by profuse watery diarrhea lasting for several days that often leads to dehydration and malnutrition in young children. [23,25] ETEC also are the most common cause of “travelers’ diarrhea” that affects individuals from industrialized countries travelling to developing regions of the World. [23,24]

1.2.2. Enterohemorrhagic *E. coli* (EHEC) Strains

Reported outbreaks had been associated mainly with the consumption of contaminated foods, such as raw or undercooked ground meat products and raw milk. The primary reservoir of this bacterium has been found to be healthy cattle. [24,26,27]

E. coli serotype O157:H7 causes abdominal pain, bloody diarrhea, and hemolytic uremic syndrome. This bacterium produces Shiga-like toxins. The incubation period is 3– 4 days, and the symptoms occur for 7–10 days. It is estimated that 2–7% of *E. coli* O157:H7 infections result in acute renal failure. [24,26,27]

Although *E. coli* O157:H7 is not usually a concern in treated tank drinking water, outbreaks involving consumption of tank drinking water contaminated with human sewage or cattle feces have been documented. An increasing number of outbreaks are associated with the consumption of fruits and vegetables (sprouts, lettuce, coleslaw, salad) contaminated with feces from domestic or wild animals at some stage during cultivation or handling.

EHEC has also been isolated from bodies of water (ponds, streams), wells and water troughs, and has been found to survive for months in manure and water-trough sediments.^[26,27] Person-to-person contact is an important mode of transmission through the oral-fecal route. An asymptomatic carrier state has been reported, where individuals show no clinical signs of disease but are capable of infecting others.^[26,27]

1.2.3. Enteroinvasive *E. coli* (EIEC) Strains

Enteroinvasive *E. coli* (EIEC) behave in many respects like shigellae. They are capable of invading and multiplying in the intestinal epithelial cells of the distal large bowel in humans. The illness is characterized by abdominal cramps, diarrhea, vomiting, fever, chills, a generalized malaise, and the appearance of blood and mucus in the stools of infected individuals.^[23,24,28] Use of *Escherichia coli* as indicator organism *Escherichia coli* are the predominant member of the facultative anaerobic portion of the human colonic normal flora.^[29] The bacterium's only natural habitat is the large intestine of warm-blooded animals and since *E. coli*, with some exceptions, generally does not survive well outside of the intestinal tract, its presence in environmental samples, food, or water usually indicates recent faecal contamination or poor sanitation practices in food- processing facilities.^[30]

1.3. *Salmonellosis*

1.3.1. The Genus *Salmonella*.

Pathogenicity of Main Serovars the genus *Salmonella*, a member of the family Enterobacteriaceae, include Gram-negative motile straight rods. Cells are oxidase- negative and catalase-positive, produce gas from D-glucose and utilize citrate as a sole carbon source. *Salmonellae* have several endotoxins: antigens O, H and Vi. ^[31,32]

1.3.2. Characterization of the Diseases

Salmonellae pathogenic to humans can cause two types of salmonellosis: (1) typhoid and paratyphoid fever (do not confuse with typhus, a disease caused by a rickettsia, (2) gastroenteritis. ^[31] Low infective doses (less than 1,000 cells) are sufficient to cause clinical symptoms. Salmonellosis of newborns and infants presents diverse clinical symptoms, from a grave typhoid-like illness with septicemia to a mild or asymptomatic infection. In pediatric wards, the infection is usually transmitted by the hands of staff. ^[32]

Food-borne *Salmonella* gastroenteritis are frequently caused by ubiquitous *Salmonella* serovars such as Typhimurium. About 12 h following ingestion of contaminated food, symptoms (diarrhea, vomiting and fever) appear and last 2–5 days. Spontaneous cure usually occurs. *Salmonella* may be associated with all kinds of food.

Prevention of *Salmonella* food-borne infection relies on avoiding contamination (improvement of hygiene), preventing multiplication of *Salmonella* in food (constant storage of food at 4 °C), and use of pasteurization (milk) or sterilization when possible (other foods). Vegetables and fruits may carry *Salmonella* when contaminated with fertilizers of fecal origin, or when washed with polluted water. ^[31]

The incidence of typhoid fever decreases when the level of development of a country increases (i.e., controlled water sewage systems, pasteurization of milk and dairy products). Where these hygienic conditions are missing, the probability of fecal contamination of water and food remains high and also the incidence of typhoid fever. ^[32]

1.4. Shigellosis or Bacillary Dysentery

1.4.1. The Genus *Shigella*

Shigella species are Gram-negative, non-sporeforming, non-motile, straight rod-like members of the family Enterobacteriaceae. Cells ferment sugars without gas production. ^[33,34,35]

1.4.2. Characterization of the Disease

The incubation period is 1–4 days. The disease usually begins with fever, anorexia, fatigue and malaise. Patients display frequent bloody stools of small volume (sometimes grossly purulent) and abdominal cramps. Twelve to 36 hours later, diarrhea progresses to dysentery, blood, mucus and pus appearing in feces that decreases in volume (no more than 30 mL of fluid per kg per day). ^[35–36]

Although the molecular basis of shigellosis is complex, the initial step in pathogenesis is penetration of the colonic mucosa. The resulting focus of *Shigella* infection is characterized by degeneration of the epithelium and by an acute inflammatory colitis in the lamina propria. Ultimately, desquamation and ulceration of the mucosa cause leakage of blood, inflammatory elements, and mucus into the intestinal lumen. Under these conditions the absorption of water by the colon is inhibited and the volume of stool is dependent upon the ileocecal flow. As a result, the patient will pass frequent, scanty, dysenteric stools. ^[37,38]

1.4.3. Ecology of *Shigellae* and the Cycle of Shigellosis

Shigella is typically an inhabitant of the intestinal tract of humans and other primates. ^[33,34,36,39] It is typically spread by fecal-contaminated tank drinking water or food, or by direct contact with an infected person.

In water, *shigellae* can survive for at least six months at room temperature, and this high survival favors transmission through water. Flies have been implicated on the transmission of *Shigella* cells from human feces to foods. The hand is an important vehicle for transmission of shigellosis, since *Shigella dysenteriae* (*S. dysenteriae*) serotype 1 cells survives for up to one hour on a human's skin and a very small inoculum is required to unchain infection and disease. ^[36-40]

Table 1. **The main bacterial diseases transmitted through tank drinking water.** ⁽⁴⁰⁾

Disease	Causal bacterial agent
Cholera	<i>Vibrio cholerae</i> , serovarieties O1 and O139
Gastroenteritis caused by vibrios	Mainly <i>Vibrio parahaemolyticus</i>
Typhoid fever and other serious salmonellosis	<i>Salmonella enterica subsp. enterica serovar Paratyphi</i> <i>Salmonella enterica subsp. enterica serovar Typhi</i> <i>Salmonella enterica subsp. enterica serovar Typhimurium</i>
Bacillary dysentery or shigellosis	<i>Shigella dysenteriae</i> <i>Shigella flexneri</i> <i>Shigella boydii</i> <i>Shigella sonnei</i>
Acute diarrheas and gastroenteritis	<i>Escherichia coli</i> , particularly serotypes such as O148, O157 and O124

2. Aim of the work

This study aim to determine the presence of coliform pathogenic bacteria in tap water and tank drinking water. These samples were collected from different regions of Alkharj and its villages affiliate, Saudi Arabia. All samples were taken from 5 regions of Kharj (i.e. Al-kharj, Al-dalam , Al-hawta, Al-rfaya, Al-hayathem). Samples were examined microbiologically using Centrifugation method, identification of bacteria was also determined. In addition, bacterial count was also calculated in our project.

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3. Material and Methods

In our study, we conducted several steps for microbial content of tank drinking and tap water in Alkharj and its villages affiliate, Saudi Arabia. Both water samples were taken from 5 regions of Kharj.

3.1. Sources of water samples

3.1.1. Procedures

1. Ten samples were collected from different water sources within Alkharj and its villages affiliate.
2. These different water samples were analysed for bacterial contamination especially pathogenic coliform bacteria.
3. Each sample was collected in sterile container sealed with screw cap after disinfection of dispensing point with alcohol swab.
4. Then, samples were kept on ice till analysis take place in the laboratory within three hours.
5. There were five sources of water included in this study: Al-kharj, Al-dalam, Al-hawta, Al-rfaya, Al-hayathem

3.2. Collection of water samples⁽⁴¹⁾

3.2.1. Procedure

1. Remove any external fittings from the tap, such as an antispash nozzle or rubber tube.
2. Clean carefully the outside nozzle of the tap, especially any grease which has collected.
3. Turn the tap on full, and allow the water to run to waste for 1 minute. This allows time for the nozzle of the tap to be flushed and any stagnant water in the service pipe to be discharged.
4. Sterilize the tap using the flame of a blowlamp or gas torch, or by igniting a piece of cotton-wool soaked in methylated spirit and holding it with a pair of tongs close to the nozzle until the whole tap is unbearably hot to the touch.
5. Allow the tap to cool by running the water to waste for a few seconds.
6. Fill the sample bottle from a gentle flow of water, and replace the cap of the bottle.
7. Using a water-proof marker or grease pencil, number the bottle with the sample code number.
8. A total of 10 samples of tap water and tank drinking water were taken from all 5 areas of Alkharj its villages affiliate.
9. All samples were stored placed in sterilized tubes prior to analysis in the laboratory and stored at 25-30 °C.
10. Bottles containing the samples were labeled appropriately and transport it to the laboratory as soon as possible (≤ 6 hours).
11. Samples can be refrigerated in case of delay. Samples were centrifuged for 2 minutes in 3,000 rpm.
12. 5ml from each water sample were inculcate into MacConkey Broth and incubate overnight.

3.3. Detection of coliform

3.3.1. Preparation of MacConkey Broth^(42,43,44)

3.3.1.1. Principle:

It is used for the detection of coliform bacteria in milk and water. MacConkey broth is used for cultivating gram-negative, lactose-fermenting bacilli and as a presumptive test for coliform organisms. It has been used to analyze food, milk and water samples¹⁰⁻¹³ for coliforms.

3.3.1.2. Procedure:

1. Dissolve 40 g of the MacConkey broth medium in one liter of purified water.
2. Mix thoroughly.
3. Use hot plate for 10min to make sure they're homogeneous.
4. Five ml from MacConkey broth were added in all the sterilize tubes.
5. Autoclave at 120C for 15 minutes.

3.3.2. Subculture from broth to MacConkey agar^(45,46)

3.3.2.1. Principle:

MacConkey agar is a selective and differential culture medium for bacteria designed to selectively isolate Gram-negative and enteric bacilli and differentiate them based on lactose fermentation. The original MacConkey medium was used to differentiate strains of *Salmonella typhosa* from members of the coliform group. Formula modifications improved growth of *Shigella* and *Salmonella* strains.

3.3.2.2. **Procedure:**

1. Suspend the 50 gram of MacConkey agar powder in 1 L of purified water and mix thoroughly.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Autoclave at 121°C for 15 minutes.
4. Pour the autoclaved MacConkey agar into sterile plate dishes and leave it to cool.
5. Take an inoculum from MacConkey broth and subculture into MacConkey agar.
6. Incubate the plates overnight at 37°C.
7. Record the result

3.3.3. **Subculture from broth to Eosin-methylene blue media(EMB):**^(47,48)

3.3.3.1. **Principle:**

EMB media is used for isolation, enumeration and differentiation of members of *Enterobacteriaceae*. This medium is recommended for the detection, enumeration and differentiation of members of the coliform group.

3.3.3.2. Procedure:

1. Suspend 37.5 g of the EMB medium in one liter of purified water.
2. Heat with frequent agitation and boil for one minute to completely dissolve the medium.
3. Autoclave at 121°C for 15 minutes.
4. Pour the autoclaved EMB agar into sterile Petri plates and leave it to cool.
5. Take an inoculum from MacConkey broth and subculture into EMB agar.
6. Incubate the plates overnight at 37°C.
7. Record the result

3.3.4. Subculture from broth to Xylose-Lysine deoxycholate (XLD):⁽⁴⁹⁾

3.3.4.1. Principle:

Xylose-Lysine deoxycholate Agar is a selective medium recommended for the isolation and enumeration of coliform bacteria specially *Salmonella* and *shigella* species.

3.3.4.2. Procedure:

1. Suspend 54.8 grams of dehydrated XLD medium in 1000 ml purified/ distilled water.
2. Heat with frequent agitation until the medium boils. Do not autoclave.
3. Transfer immediately to a water bath at 50°C. After cooling, pour into sterile Petri plates. Leave it to cool.
4. Take an inoculum from MacConkey broth and subculture into XLD agar.
5. Incubate the plates overnight at 37°C.
6. Record the result.

3.4. Biochemical tests

3.4.1. Oxidase test⁽⁵⁰⁾

3.4.1.1. Principle:

The Oxidase test is used to identify oxidase positive organisms. It is based on the bacterial production of an enzyme called indophenol oxidase. The oxidase test is a key test to differentiate between the families of Pseudomonadaceae (ox +) and Enterobacteriaceae (ox -).

3.4.1.2. Procedure:

1. Pick a good-sized amount of inoculum (already incubated and grown) from a plate culture and place it on a piece of filter paper FIRST.
2. Add one drop of the reagent (if it is dark blue, it is old and should not be used).
3. TIME the reaction: a positive reaction will occur within 20 seconds. DO NOT READ the reaction after 30 seconds.

3.4.2. Indole test⁽⁵¹⁾

3.4.2.1. Principle:

This test is used to identify *E. coli* (indole positive). This indole testing consists of peptone water which is used for the cultivation of non-fastidious microorganisms. Peptone Water is a minimal growth medium. This non-selective medium has been used as a basal medium for biochemical tests such as carbohydrate fermentation patterns and production of indole.

3.4.2.2. Procedure :

1. Dissolve 20 g of peptone water powder in 1 L of distilled water.
2. Distribute into suitable containers and sterilize in the autoclave at 121⁰C for 15 minutes.
3. Inoculate from MacConkey broth into the peptone water and the tube incubated overnight at 37⁰C.
4. After incubation, a few drops of Kovak's reagent added to the peptone water
5. The result was indicated by formation of red color in the surface layer within 10 minutes.

3.5. Gram staining: ^(52,53)

3.5.1. Procedures:

1. One drop of saline was mixed with a single colony on slide and fixed with gentle heat.
2. Crystal Violet Oxalate was poured on slide for 2-3 minutes.
3. Iodine (mordant) was poured on slide for 2-3 minutes.
4. Alcohol decolorized was poured on slide for 1 minute.
5. Safranin counterstain was poured on slide for 2-3 minutes.
6. In each step, the slide was washed with distilled water.
7. The slide was examined microscopically.
8. Gram positive bacteria were blue or violet while gram negative bacteria were pink or red.

3.6. Bacterial identification

Bacterial identification is confirmed by phoenix 100 BD company automated identification and sensitivity machine in the laboratory of King Khalid Hospital.

3.7. Bacterial count ⁽⁵⁴⁾

3.7.1. Principle:

The three bacterial plate count methods must be serially diluted until having 30-300 colony forming units (CFU) on the plate. Plates with more than 300 CFU are very difficult to count. Plates with less than 30 CFU are not statistically reliable.

3.7.2. Procedure:

Materials:

- 9mL sterile water bottles
- 1mL sterile pipettes with blue pipetting aid
- MacConkey plates (or other plate appropriate for your organism)
- Broth culture of organism .

1. The tricky part of doing serial dilutions is determining the correct dilution to get 30-300 CFU's per plate.
2. Labelled three screw-capped tubes 1:100, 1:10,000 and 1: 1,000,000.
3. Using a sterile 10mL pipette, aliquot 9.9mL of sterile water into each tube.
4. Using a 100µL micropipettor and sterile tip, transfer 0.1mL of your broth culture into the 10-2 tube.

5. Cap the tube.
6. Either mixed it for a few seconds on a vortex mixer or vigorously flicked the tube to adequately disperse the bacteria evenly throughout the tube and break up bacterial clumps. Do NOT shake the test tube.
7. This first tube now has a 1:100 (10⁻²) dilution of the original broth culture. If there are still too many bacteria to count, so further dilution is necessary.
8. Using a new sterile tip, transfer 0.1mL from the first tube (1:100) and add it to tube labeled 1:10,000.
9. Repeat the tube mixing procedure. This second tube now has a 1:10,000 dilution of the original broth culture. If there are probably still too many bacteria in this dilution to count, so the dilution process needs to be repeated once more.
10. Using a new sterile tip, transfer 0.1mL from the second tube (1:10,000) and add it to the tube labeled 1:1,000,000.
11. Repeat the tube mixing procedure .
12. Labelled two plates “0.1mL and 1mL of 1:10,000 dilution. Label the other two plates “0.1mL and 1mL of 1:1,000,000.
13. Quickly mixed or flicked the dilution tube and aliquot the indicated amount from the appropriate tube onto the center of the plate.
14. Disperse it in 2-3 drops around the center of the plate.
15. By using a sterile blue L-shaped spreader, spread the inoculum evenly around the plate.
16. Do not invert the plates until all the liquid has absorbed into the surface of the agar.
17. Incubate the plates for the appropriate time and temperature. This is usually 24 hours at 35-37°C.
18. Calculation: no of colony forming unit (CFU) /Amount of diluted bacterial cells Plated **X**
Dilution factor= CFU/ml



Fig.1: All media used in the study



Fig.2: Pouring of the plates

4. Result

Microbial analysis of water samples in AlKharj and its villages affiliate (Alhawta, Al- hayathem, Al-delm, Al-rfaya, Al-kharj), kingdom of Saudi Arabia revealed that there is no bacterial contamination in tank drinking water.

On the other hand, microbial analysis of tap water sample that obtained from Al-Kharj region revealed that sample was contaminated with *pseudomonas putida*. while, tap water sample from Alhawta region revealed that the sample was contaminated with *Acinetobacter iwoffii*.

Tap water sample obtained from Alhayathem region revealed that the sample was contaminated with *pseudomonas aerruginosa*. In addition, tap water sample obtained from Aldelm region revealed that the sample was contaminated with *pseudomonas* species. Finally, tap water sample obtained from Alrfaya revealed that the sample was contaminated with *Moraxella species*.



Fig.3: MacConkey broth without bacterial cells (negative control)



Fig.4: MacConkey broth showing bacterial growth

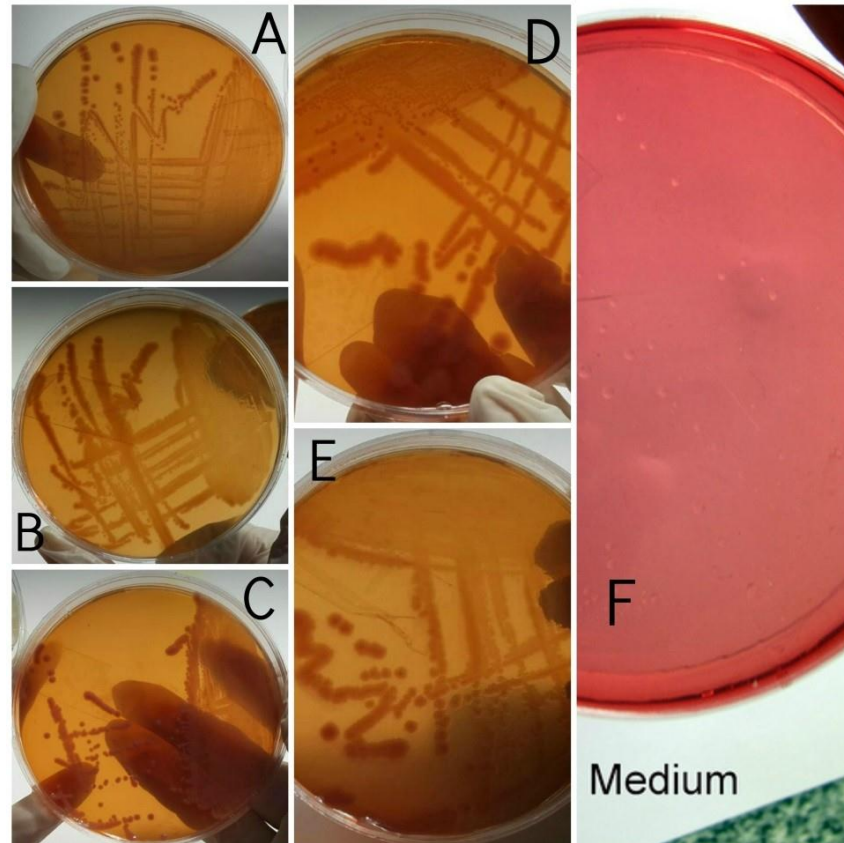


Fig. 5: MacConkey agar plates showing bacterial growth; A: showing *Morexlla* species colony (Alrafaya tap water) B: showing *Pseudomonas species* (Aldelam tap water) C: showing *Acinetobacter iwoffii* colony (Alhawta tap water). D: showing *Pseudomonas putida* colony (Alkharj tap water) E: showing *P. aeruginosa* colony (Alhayathem tap water). F: showing no bacterial growth (ALL TANK DRINKING WATER SAMPLES).

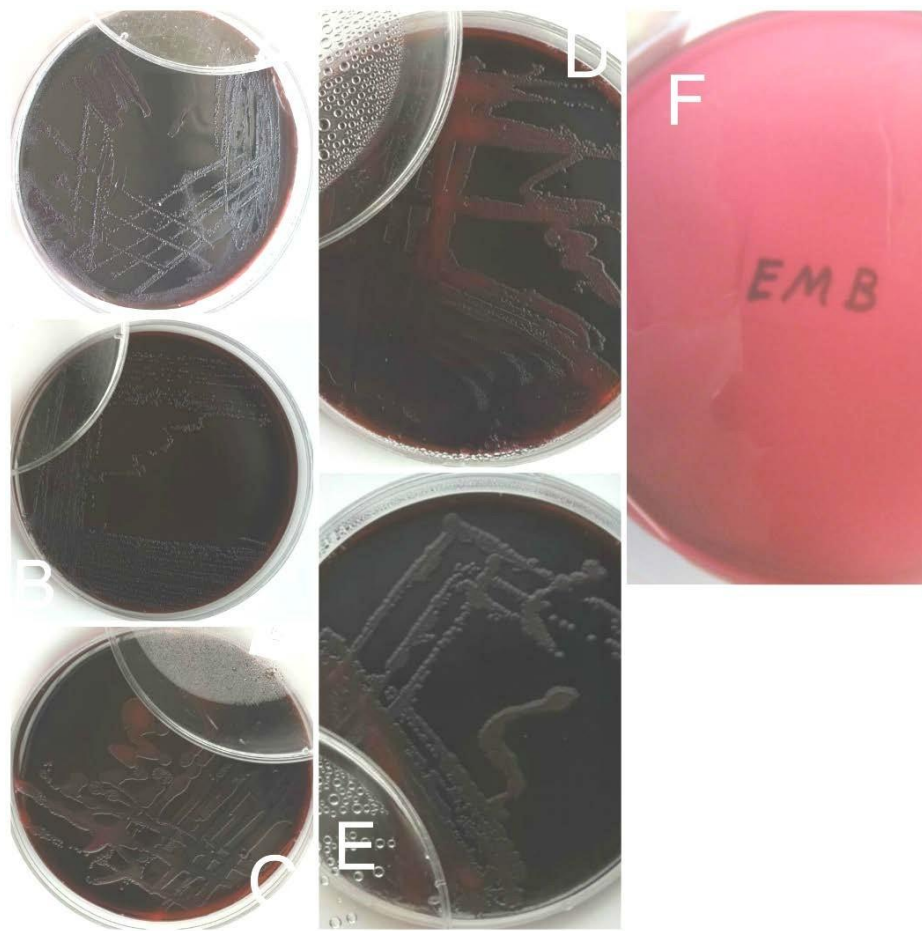


Fig.6: EMB agar plates showing bacterial growth: A: showing *Morexlla* species colony(Alrafaya tap water). B: showing *Acinetobacter iwoffii* colony (Alhawata tap water). C: showing *Pseudomonas putida* colony (Alkharj tap water). D: showing *Pseudomonas* species(Aldelam tap water). E: showing *P.aerruginosa* colony (Alhayathem tap water). F: showing no bacterial growth (ALL TANK DRINKING WATER SAMPLES).

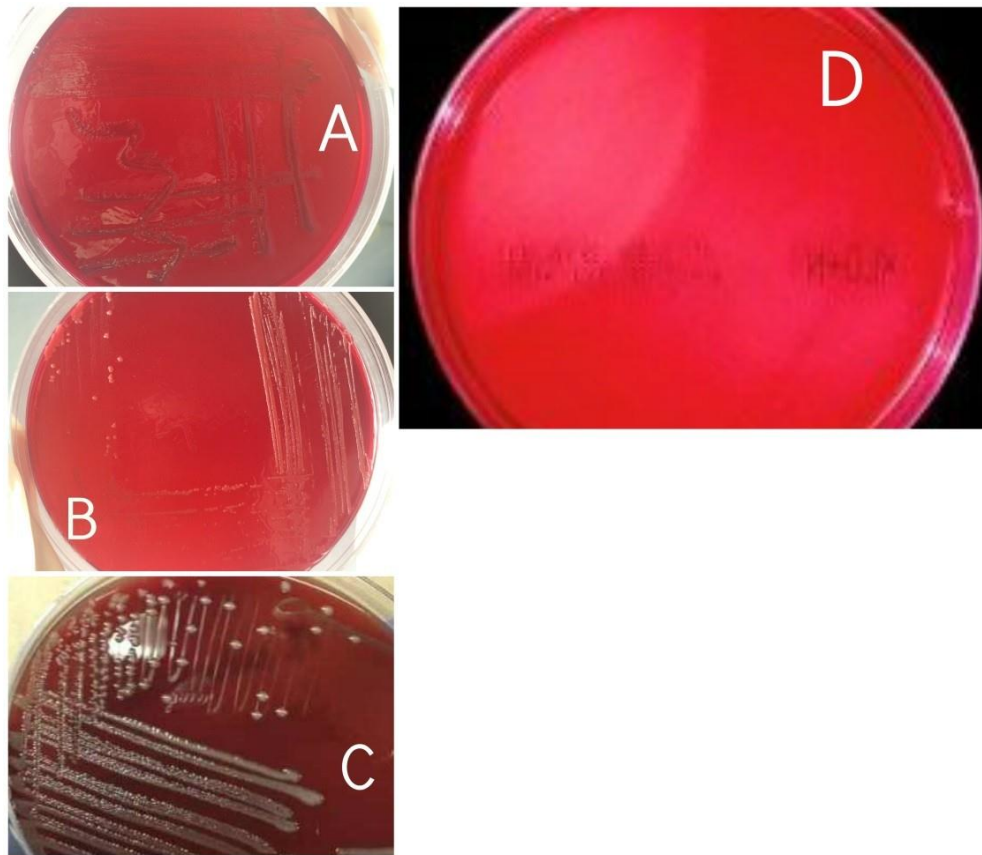


Fig.7: XLD agar plates showing bacterial growth: A: showing *pseudomonas species* colony (Alkharj,Aldelam,Alhayathem tap water).B:showing *Acinetobacter iwoffii* colony (Alhawata tap water). C: showing *Moraxella species* colony (Alrafaya tap water) D:showing no bacterial growth (ALL DRINKING SAMPLES).



Fig.8: Oxidase test: Oxidase test was performed on all tap water samples from all regions, four water samples (Kharj, Hyathym, Delam, rfaya) produced purple color (indicate that oxidase test is positive).



Fig.9: Gram stain test showing presence of *Moraxella* species in tap water sample obtained from Alrafaya region.

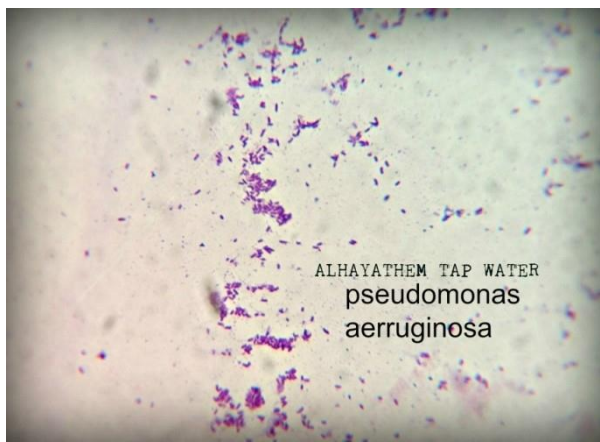


Fig.10: Gram stain test showing presence of *P.aeruginosa* in tap water sample obtained from Alhayathem region.



Fig.11: Gram stain test showing presence of *Pseudomonas* species in tap water sample obtained from Aldelm region.



Fig.12: Gram stain test showing presence of *Pseudomonas putida* in tap water sample obtained from AlKharj region.



Fig.13: Gram stain test showing presence of *Acinetobacter iwoffii* in tap water sample obtained from Alhawta region.



Fig.14: Indole test: Positive test is indicated by a pink ring. Negative indole test - yellow ring. All samples of tap water showing indole negative.

Table 2: Percentages of bacterial content in the water samples collected from five different regions

The region	Organism in Tap water	Confidence value	Tank drinking water
ALKHARJ	<i>pseudomonas putida</i>	90%	NO GROWTH
ALDELM	<i>pseudomonas species</i>	95%	NO GROWTH
ALRFAYA	<i>Moraxella species</i>	99%	NO GROWTH
ALHWT	<i>Acinetobacter iwoffii</i>	99%	NO GROWTH
ALHAYATHEM	<i>pseudomonas aerruginosa</i>	95%	NO GROWTH

Table 3: The number of colony forming unit of bacterial contamination in investigated water collected from five different regions

Bacteria	Number of colony	Tap water(n=5). %	Tank drinking water(n=5). %
Morexlla species	272	2.72×10^7	0
<i>Pseudomonas species</i>	364	3.64×10^7	0
<i>Acinetobacter iwoffi</i>	240	2.40×10^7	0
<i>pseudomonas putida</i>	367	3.67×10^7	0
<i>Pseudomonas aeruginosa</i>	340	3.40×10^7	0

Calculation:

#Colony-forming units (CFU) /Amount Plated X Dilution factor= CFU/ml

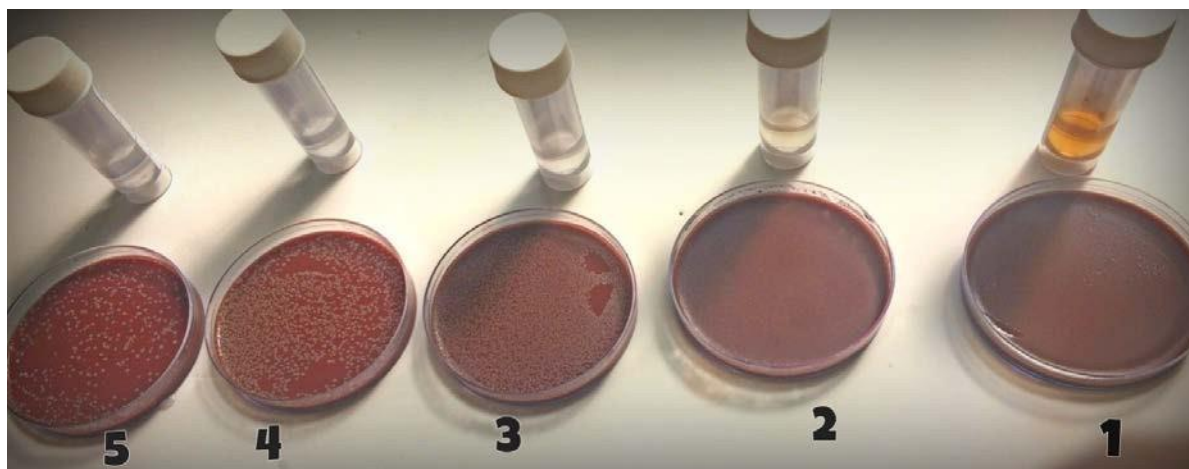


Fig15: Bactrial count test by using serial dilution technique.

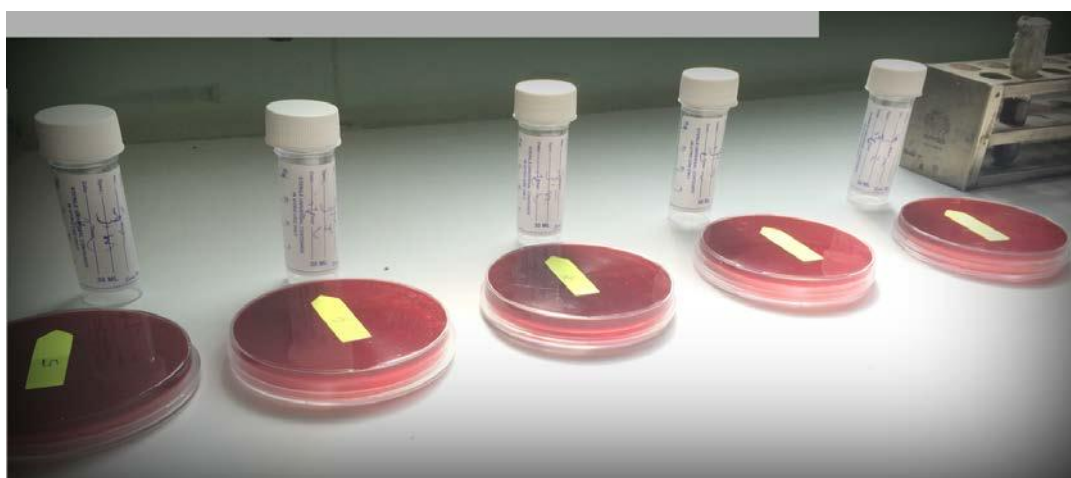


Fig16: Prepreion of bacteria serial dilution.



Fig17: (Inoculate directly from broth)



Fig18: (Inoculate from normal saline)

5. Discussion

Tank drinking water and tap water from different water resources should be free from contamination with waterborne pathogens including bacteria. In this study, samples of both drinking and tap water were collected from different regions of AlKharj and its villages affiliate (Alhawta, alhayathem, aldelm, alrfaya, alkharj), kingdom of Saudi Arabia. In addition presence of coliform pathogenic bacteria was analyzed in all water samples in AlKharj and its villages affiliate.

In our study, microbial analysis revealed that there is no bacterial contamination in tank drinking water. On the other hand, tap water samples showed presence of bacteria. By using bacterial identification technique, AlKharj region showed that tap water sample was contaminated with *pseudomonas putida*. while, tap water sample from Alhawta region revealed that the sample was contaminated with *Acinetobacter iwoffii*. Tap water sample obtained from Alhayathem region revealed that the sample was contaminated with *pseudomonas aerruginosa*. In addition, tap water sample obtained from Aldelm region revealed that the sample was contaminated with *pseudomonas* species. Finally, tap water sample obtained from Alrfaya revealed that the sample was contaminated with *Moraxella species*.

Our study revealed that there is no coliform pathogenic bacteria in all water samples of tank drinking water collected from Al-Kharj. On the other hand, a study of *Hrudey SE, et al*⁽⁵⁵⁾ showed that detection of bacterial indicators in tank drinking water suggests the presence of pathogenic organisms that are the source of water borne diseases.⁽⁵⁵⁾

In this previous study, the results of total coliform count showed that 20% of the samples from wells exceeded the guideline values recommended by national and international standards of tank drinking water.⁽⁵⁶⁾ In a previous large investigation of the quality of water samples from 1062 wells from seven regions in Saudi Arabia, fecal streptococci were detected in 8% of samples.⁽⁵⁷⁾ In another study which evaluated the bacteriological characteristics of tank drinking water in Khamis Mushait Governorate, Southwestern Saudi Arabia, fecal coliform, and fecal streptococci were detected in 87.9% and 57.6% of 33 well water samples.⁽⁵⁸⁾ The presence of coliform may be attributed to contamination of the hoses used by humans, including farmers and livestock owners; and the exposure of these delivery hoses to dust storms.⁽⁵⁹⁾ Previous studies have indicated that dust storms and livestock activity in the vicinity of surface wells increase microbial levels and bacterial input.⁽⁶⁰⁾

In another study reported by WHO⁽⁶¹⁾, 33% of the water samples from tankers had higher total coliform than stipulated by the national and international guideline values. In a previous study in Shebaa area, Southwestern Saudi Arabia, only 2.6% of 39 water samples from tankers was positive for total coliform. The investigators concluded that transportation of desalinated water by water tankers had not significantly contributed to its contamination in their region.⁽⁶²⁾ However, Mihdhdhir reported that 68.8% and 37.5% of samples from tankers in Makkah Al-Mokarama were positive for total coliform and fecal coliform, respectively.⁽⁶³⁾

Our results showed that *P. aeruginosa* was the most common microbial contamination in tap water sample obtained from Alhayathem region. These samples were contaminated due to lack of proper treatment and cleaning. Bari et al.,⁽⁶⁴⁾ showed lower *P. aeruginosa* contamination (4%) from wells.

Geldreich ⁽⁶⁵⁾ found that *P. aeruginosa* was widely distributed in nature and most prevalent opportunistic pathogen isolated from the water samples. *P. aeruginosa* 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 in concentrations of 10/100 mL to >1,000/100 mL. However, it is not often found in tank drinking water. Usually it is found in 2% of samples, or less, and at concentrations up to 2,300 mL(-1) often at 3-4 CFU/mL. Its occurrence in tank drinking water is probably related more to its ability to colonize biofilms in plumbing fixtures (i.e., faucets, showerheads, etc.) than its presence in the distribution system or treated tank drinking water ⁽⁶⁶⁾

Trautmann et al., ⁽⁶⁷⁾ in between 1998 and 2005 showed that 9.7% and 68.1% of randomly taken tap water samples were positive for *P. aeruginosa*. Although much has been written about biofilms in the tank drinking water industry, very little has been reported regarding the role of *P. aeruginosa* in biofilms. Tap water appears to be a significant route of transmission in hospitals, from colonization of plumbing fixtures. Outbreaks have been reported from exposure to *P. aeruginosa* in swimming pools and water slides. Contamination of tap water samples with *P. aeruginosa* in our results showed that this organism was the most common microbial contamination in water sources due to lack of proper treatment and cleaning.

By using bacteriological analysis of water samples from roof tanks, another study revealed a higher levels of bacterial indicators in many samples than the national and international guideline values. ⁽⁶⁸⁾ Abu-Zeid *et al.* found that 26.4% of 201 samples from house tanks showed contamination. The investigators suggested that water contamination obviously occurred during storage in house reservoirs, and was possibly implicated, at least partly, in the increased prevalence of diarrhea among residents in the Shebaa area. ⁽⁶⁹⁾

Similarly, our results indicated that the tap water obtained from Alrafaya region was contaminated with *Morexella* species and tap water obtained from Alhawata region was contaminated with *Acinetobacter iwoffii* colony. This could be a result of biofilm growth in the household tanks.⁽⁷⁰⁾ The use of roof tanks for water storage is a common practice in Saudi Arabia.

In many previous studies, diarrhea was strongly associated with the cleaning of water tanks.⁽⁷¹⁾ A finding of considerable concern in these studies is flooding the sewage in winter and summer. This could be the cause of the infiltration of wastewater which in turn may contribute to microbial contamination of water in the wells and house tanks. To maintain the quality of tank drinking water in roof tanks as received from the source, it would be necessary to implement effective awareness and educational programs. Although most people reported that drinking and tap water transmitted diseases, less than one-third of those interviewed had attended any educational program on the effects of polluted water on health. These awareness programs are supposed to show the importance of keeping house tanks closed, hand washing before handling areas close to the nozzle of the hose, cleaning of tanks on a regular basis, and possibly the addition of small amounts of chlorine into the water stored in roof tanks.

Similarly with our study,⁽⁷²⁾ showed that the genus *Acinetobacter* was detected at high relative abundance ranging from 1.5% to 48% of the total groundwater microbial community. However, culture-based analysis did not recover any antibiotic-resistant bacteria or opportunistic pathogens from these groundwater samples. In addition, opportunistic pathogenic *Enterococcus faecalis* and *Pseudomonas aeruginosa* were isolated from the fruits irrigated with the groundwater from wells.

Although the groundwater was compromised, quantitative microbial risk assessment suggests that the annual risk incurred from accidental consumption of *E. faecalis* on these fruits was within the acceptable limit of 10^{-4} . However, the annual risk arising from *P. aeruginosa* was 9.55×10^{-4} , slightly above the acceptable limit. Our findings highlight that the groundwater quality at this agricultural site in western Saudi Arabia is not pristine and that better agricultural management practices are needed alongside groundwater treatment strategies to improve food safety.

Another result that is in agreement with our data, Ahmed F, et al analyzed different drinking bottled and tap water samples from different locations in and around Taif city. chemical and bacteriological characteristics of these water samples indicated that total coliforms, fecal coliforms, *E. coli*, fecal streptococci, *P. aeruginosa* and heterotrophic plate count (HPC) were detected. In addition, the previous study revealed that no *E. coli* was observed in any samples of tap or bottled water. No fecal streptococci were observed in tap water but 0.97 % only in bottled water. They also found that *Pseudomona* spp. , *Acinetobacter calcoaceticus*, *S. pyogenes*, *K. pneumonia*, *P. aeruginosa* were higher in tap water (14.3%) than bottled water (1.9%).⁽⁷³⁾

At the end, our results could help health authorities consider a proper regular monitoring program and a sustainable continuous assessment of the quality of tap water. In addition, this study highlights the importance of the awareness and educational programs for residents on the effect of polluted water on public health.

6. Conclusion & Recommendations

Tank drinking water is an important source of tank drinking water in the KSA, which has limited resources of fresh water. Continuous monitoring of water quality and effectiveness of the treatment processes, and obeying regulations, are required to ensure that the water quality meets the set standards and to meet the increasing demand for good quality tap or bottled water.

From the obtained results in our study we can conclude that:

1. No coliform bacteria was detected throughout the study period either in tap or tank drinking water samples in AlKharj and its villages affiliate (Alhawta, Al- hayathem, Al-delm, Al-rfaya, Al-kharj), kingdom of Saudi Arabia.
2. Certain regions in and near AlKharj were found to be contaminated with different microbial pathogens
3. *Pseudomonas aerruginosa* was detected only in tap water sample obtained from Alhayathem region.
4. Microbial analysis of tap water sample that obtained from alKharj region revealed that sample was contaminated with *pseudomonas putida*.
5. In addition, tap water sample obtained from Aldelm region revealed that the sample was contaminated with *pseudomonas* species.
6. Tap water sample from Alhawta region revealed that the sample was contaminated with *Acinetobacter iwoffii*.
7. Finally, tap water sample obtained from Alrfaya revealed that the sample was contaminated with *Moraxella species*

From the obtained results in our study we can recommend that:

1. Periodical monitoring of water sources for pollutants (chemical & microbial).
2. Periodical testing of water tankers for their microbial contamination. The risk of microbial contamination in tanks can be reduced by several well-known practices. These include the installation of first flush devices, cleaning gutters, both of which are designed to reduce the build-up of potential contaminants and the use of filtration to remove potential contaminants before use.
3. Enhanced funding is needed to validate newer molecular detection tools, understand the ecology of pathogens in aquatic ecosystems, better predict disease outbreaks, and improve emergency responses. A preventive approach to pathogen pollution should be taken by developing countries in the form of a source water protection program for all major freshwater sources.
4. The identification and control of threats posed by waterborne pathogens will also require effective pathogen detection techniques. The need to develop, evaluate and validate newer molecular tools for pathogen detection such as PCR techniques and DNA microarrays. Rapid advances in fields such as genomics offer the potential to develop improved pathogen detection tools.
5. Encourage infrastructure planning, including technological advances, to ensure that improved treatment and environmental protection measures are not diminished by development or population growth.
6. Programs are needed to assess the effects of aerial emissions on tank drinking water quality.
7. An improved understanding is needed of methods for assessment and risk analysis of the cumulative effects of agricultural, forestry and other land use activities (e.g., ore, oil and gas exploration) as well as pointsource inputs (e.g., municipal and industrial discharges) on surface and ground waters.

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الملخص العربي

المياه هي واحدة من أكثر السلع وفرة وهي من أساسيات الحياة للإنسان وتحتل حوالي 70٪ من سطح الأرض و 60٪ من جسم الإنسان ولذلك يجب أن تكون محمية بشكل مستمر ضد التلوث الميكروبي. مياه الشرب ومياه الصنابير من مختلف المصادر المائية مثل الآبار والخزانات يجب أن تكون خالية من التلوث بمسببات الأمراض التي تنتقل عن طريق المياه بما في ذلك البكتيريا. ولقد أجريت هذه الدراسة على مياه الشرب ومياه الصنابير من مناطق مختلفة من الخرج والقرى التابعة لها (الخرج، الهياثم، الدلم، الرفايع، الحوطة) داخل المملكة العربية السعودية. هذه الدراسة تهدف إلى الكشف عن وجود بكتيريا القولونية المسببة للأمراض في مياه الصنابير وخزانات مياه الشرب.

الطريقة: تم اختبار جميع عينات المياه المجمعة من عدة أماكن للكشف عن وجود البكتيريا القولونية. وتم تحديد وجود البكتيريا عن طريق الترسيب بواسطة تقنية الطرد المركزي. وبالإضافة إلى ذلك، تم التعرف على وجود هذه البكتيريا عن طريق جهاز phoenix 100 BD company automated identification and sensitivity machine. هذا بالإضافة إلى استخدام طريقة العد البكتيري لعد مستعمرات البكتيريا الموجودة في عينات المياه.

النتائج: تبين أنه لا يوجد أي تلوث بكتيري في خزانات مياه الشرب، وبالتحديد ليس هناك تلوث بالبكتيريا القولونية في كل العينات، ولكن كانت مياه الصنابير ملوثة بأنواع من البكتيريا المسببة للأمراض. والبكتيريا التي تم تحديدها هي: *pseudomonas aeruginosa*, *pseudomonas putida*, *Acinetobacter iwoffii* and *Moraxella species*. والتي وجدت بمياه الصنابير في كل من الخرج، الهياثم، الدلم، والحوطة، الرفايع.

الخلاصة: لذلك، المياه التي يستهلكها البشر يجب أن تكون ذات جودة جيدة ويكون وجود الميكروبات فيها داخل نطاق مقبول، وإيضاً يجب أن تخضع لعلاج فعال من أجل خفض عدد البكتيريا والعدوى.

الكلمات الرئيسية: القولونية، البكتيريا المسببة للأمراض، مياه الصنابير وخزانات مياه الشرب، الخرج، المملكة العربية السعودية.

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المياه هي واحدة من أكثر السلع وفرة وهي من أساسيات الحياة للإنسان و تحتل حوالي 70٪ من سطح الأرض و 60٪ من جسم الإنسان ولذلك يجب أن تكون محمية بشكل مستمر ضد التلوث الميكروبي. مياه الشرب ومياه الصنابير من مختلف المصادر المائية مثل الآبار والخزانات يجب أن تكون خالية من التلوث بمسببات الأمراض التي تنتقل عن طريق المياه بما في ذلك البكتيريا. ولقد أجريت هذه الدراسة على مياه الشرب ومياه الصنابير من مناطق مختلفة من الخرج والقرى التابعة لها (الخرج، الهياثم ، الدلم، الرفايح، الحوطة) داخل المملكة العربية السعودية. هذه الدراسة تهدف إلى الكشف عن وجود بكتيريا القولونية المسببة للأمراض في مياه الصنابير وخزانات مياه الشرب.

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الكلمات الرئيسية: القولونية، البكتيريا المسببة للأمراض، مياه الصنابير وخزانات مياه الشرب، الخرج، المملكة العربية السعودية.



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كلية العلوم الطبية التطبيقية قسم
علوم المختبرات الطبية

تقييم البكتيريا القولونية المسببة للأمراض في خزانات مياه الشرب ومياه الصنبور من مناطق مختلفة في الخرج والقرى التابعة لها، بالمملكة

العربية السعودية

مشروع التخرج

في

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إعداد:

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