Isolation of multiple antibiotic resistant bacterial isolates from potable and non-potable drinking water sold in tea-stalls

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN MICROBIOLOGY

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DECLARATION

I hereby declare that the thesis project titled **"Isolation of multiple antibiotic resistant bacterial isolates from potable and non-potable drinking water sold in tea-stalls"** submitted by me has been carried out under the joint supervision and able guidance of Associate Professor Dr. M. Mahboob Hossain , Microbiology Program, BRAC University in partial fulfilment of BS in Microbiology, at BRAC University, Dhaka. It is further declared that the research work presented here is original, has not been submitted anywhere else for any degree or diploma.

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Isolation of multiple antibiotic resistant bacterial isolates from potable and non-potable drinking water sold in tea-stalls

ABSTRACT

Water, another name is life. It is impossible for all living body to survive without water. About 75% of the whole volume of earth is water among which only 1% is drinkable. Worldwide 1.2 billion people suffer from inadequate access to clean water. Day by day the life of human is becoming very busy. Gradually for livelihood people are becoming outbound. In context of Bangladesh, Dhaka is a very busy city. Most of the cases people do not carry enough drinking water with them. They feel comfortable to buy bottled mineral water or drink water from the nearby tea-stall. The purpose of this study was to check how much pure this type of drinking water which is normally found at tea-stall.

Multiple Antibiotic Resistant (MAR) strain is a threat for public health especially in developing countries like Bangladesh. For this reason in this research work MAR isolation from drinking water has been carried out. In this study, water samples for faecal indicators and their sensitivity label was evaluated. Heterotrophic Plate Count (HPC) and antibiotic sensitivity tests were done to identify the MAR strain. In this study seven isolates were found with MAR characteristics.

For the isolation of various bacterial species five types of agar media were used i.e. TCBS for *vibrio spp,* XLD for *Salmonella spp.* and *Shigella spp,* MAC for coliform and enteric pathogen, NA for total viable count, m-FC for faecal coliform and cetrimide for *Pseudomonas spp*.

In the present study *Pragia fontium, Erwinia tracheiphila, Citrobacter rodentium, Yersinia aleksiciae, Tatumella punctata, Enteric Group68, Vibrio sinaloensis, Vibrio fluvialis, Tatumella citre, Tatumella punctata, Yersinia bercovieri, Yersinia mollaretii, Tatumell apunctata, Tatumell acitrea* were observed in different drinking water samples. The findings are redoubtable since frequently suggested antibiotics revealed incompatible with intended pathogens and demand further extensive study with combined drug effect.

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Abbreviations

mm: Millimetre

μm: Micrometer

mg : Milligram

g : Gram

Kg: Kilogram

e.g.: For example

et al.: And others

pH: Negative logarithm of hydrogen ion concentration

CFU : Colony Forming Unit

spp.: Species

% : Percentage

^{o}C: Degree Celsius

TVC: Total viable count

CEC: Coliform and Enteric pathogen

SC: Staphylococci Count

FC: Fungal Count

NA: Nutrient Agar

Mac: MacConkey agar

MSA: Mannitol Salt Agar

EMB: Eosine Methylene Blue Agar

SDA: Sabouraoud Dextrose Agar

XLD: Xylose lysine deoxycholateagar

TCBS: Thiosulfate-citrate-bile salts-sucrose agar

Chapter 1

Introduction

1.1 Introduction

Water is a very essential need for Human to survive. But polluted water can cause of death. So safety of drinking water is mandatory. Water is one of the easiest vehicles for some of the pathogenic organisms and the contaminating water bodies may help in the outbreak of epidemic diseases The pathogenic most frequently transmitted though water are those which cause infection of the intestinal tract, namely typhoid, paratyphoid diarrhoea, dysentery and cholera (Pelezar and Reid, 1978). Human health should therefore be protected by preventing microbial contamination of water that is intended for consumption (Völker, Schreiber, and Kistemann, 2010). In rural communities, untreated surface water from rivers, dams, and streams is directly used for drinking and other domestic purposes (Biyela, Lin, and Bezuidenhout, 2004). These unprotected water sources can be contaminated with microbes through rainfall run-off and agricultural inputs, mixing with sewage effluents and faeces from wild life, which render them unacceptable for human consumption. *Faecal coliforms, Aeromonas* and *Pseudomonas* are used as indicators of faecal contamination in water (Webster, Thompson, Fulton et al., 2004) and the presence of these pathogens may have severe health implications on consumers especially those that are immune compromised (Pavlov, Wet, Grabow, and Ehlers, 2004; Dumontet, Krovacek, Svenson, Pasquale, Baloda, and Figliuolo, 2000).

As people's life are getting busy day by day. Even they do not manage time for healthy eating. Most of the time they have to depends on outside food and also for drinking water. Many people in our country use to drink water from tea stall or they have to buy a mineral water bottle from any shop. Even when people travel from one place to another they may have not to eat but they have to drink water. As we know, water is an essential element of our body that's why we need to drink at least 7-8 litters of water per day. Although potable water is suitable for human consumption, it does not guarantee that so called quality is maintaining or not.

Water is a common medium for microbes. Now a day's water born disease is a threat for our country as well as world. In our county people (including children)are suffering from many water borne diseases like typhoid, paratyphoid diarrhoea, dysentery and cholera. To manage the quality of drinking water is now a challenge to us. The current study has been done to observe the microbial load of non-potable drinking water & potable drinking water which are sold in tea stall. Total six samples are collected from an area name Mohakhali among those samples three

were mineral water bottle (potable) and three were non-potable. The goal of the study was to compare the quality of these two types water which are normally found intea stall, also isolation of multiple antibiotic resistance bacteria from the water sample.

Fig: 1.1: Drinking water

Multidrug resistant water borne microorganisms made the food safety situation more vulnerable in public health (Ali et al., 2011). Diarrheal is the most common water-bone common disease and approximately 30 million people are suffering from water borne illness each year in Bangladesh (FAO, 2012). Microbial toxin or human body's reactions to the microbe cause the disease (Khairuzzamanet al., 2014).

Biological treatment processes in the waste water treatment plants may result in a selective increase of antibiotic-resistant bacteria and therefore increase the occurrence of multidrug-resistant organisms (Zhang, Marrs, Simon, and Xi, 2009) Although microorganisms in drinking water are reduced by chlorination, they may survive the treatment process and enter the distribution system (Faria, Vaz-Moreira, Serapicos, Nunes, and Manaia, 2009). Moreover, the presence of antibiotic resistance in microorganisms has been previously reported (Mulamattathil, Esterhuysen, and Pretorius, 2000; Lin and Biyela, 2005; Kinge and Mbewe, 2010). Six selected sample's microbial quality was analysed from tea stalls. After observing microbial growth and biochemical tests, the test results were put into ABIS online software which has shown four most probable bacteria name. According to this result further analysis were done.

1.2. Objectives of the study

1.2.1 General objective:

Water borne illness is common phenomenon in Bangladesh even more or less every country's common problem where general hygienic precautions are not followed. In this study water were collected which are analysed in street side tea stall & mineral bottle water. Mainly one area was selected to analyse the water quality of local & mineral drinking water. Some random water from street-side was analysed for microbial load determination. ABIS (Analysis of Bacterial Identification Software) online software reliability also observed by input the biochemical test result.

❖ **Specific objectives:**

- To analyse the microbial load of water which is sold in tea stall but not in potable form & the potable mineral water.
- Isolate and identify the pathogenic microorganisms from water;
- Identification of multiple antibiotic resistance microbes.
- Statistical analysis between potable & non potable water microbial load.

Chapter 2

Materials and Method

2.1 Materials

2.1.1 Samples

Total three types of water samples from tea stall were collected and the total number was eighteen. Total six types of randomly selected water samples were collected.

Table 2.1: Area of sampling, number and types of samples

Area of sampling	**Sample type**	Sample number & name			Total
		Tan	ML	Dh	
Mohakhali	Non potable water	3	3	3	9
	Potable water	EF	DL	MM	9
		3	3	3	

Total= 18

2.1.2 Media

Different types of media were used for total viable count (TVC), enteric pathogen and coliform count (ECC), *Staphylococcus sp*. count. Media were the Nutrient agar, MacConkey agar, Thiosulfate-citrate-bile salts-sucrose agar, Xylose lysine deoxycholate agar, mFC agar and cetrimide agar respectively.

Media for bacterial isolation: the culture media used are following,

1) **Nutrient agar medium:**
   It is a common microbiological growth medium used for growth of non-fastidious bacteria. This media used for total microbial count.

2) **MacConkey agar medium**:
   It is a differential and low selectivity medium designed to grow Gram negative bacteria and also distinguish between lactose fermenting (e.g. *Klebsiella* and *Escherichia coli*) from non-fermenting bacteria (*Pseudomonas aeruginosa*, *Salmonella* species and *Proteus mirabilis*) (Oxoid 1998). It contains bile salts (inhibit the growth of most Gram positive bacteria, except *Enterococcus* and some species of *staphylococcus* i.e. *Staphylococcus aureus*), crystal violatedye (inhibits certain Gram-positive bacteria), neutral red dye (stains lactose fermenting microbes), lactose and peptone. Incubation time will be 24-48hrs at 37^{o}C. *E.coli* produces pink colonies on MacConkey agar.

3) **Thiosulfate-citrate-bile salts-sucrose agar (TCBS agar) :**

TCBS Agar is highly selective for the isolation of *V. cholerae* and *V. parahaemolyticus* as well as other *vibrios*. TCBS agar contains high concentrations of Sodium thiosulfate and Sodium citrate to inhibit the growth of *Enterobacteriaceae*. Inhibition of Gram-positive bacteria is achieved by the incorporation of ox gall, which is a naturally occurring substance containing a mixture of bile salts, and sodium chloride, a pure bile salt. Sodium thiosulphate also serves as a sulphur source and, in combination with ferric citrate, detects hydrogen sulphide production. Saccharose (sucrose) is included as a fermentable carbohydrate for the metabolism of *vibrio*. The alkaline pH of the medium enhances the recovery of *V. cholerae* and inhibits the growth of others. Thymol blue and bromothymol blue are included as indicators of pH changes.

4) **Cetrimide agar :**

Cetrimide agar is a type of agar used for the selective isolation of the gram-negative bacterium, *Pseudomonas aeruginosa*. As the name suggests, it contains cetrimide, which is the selective agent against alternate microbial flora.

5) **XLD agar medium:**

Xylose-Lysine Deoxycholate Agar (XLD Agar) is a selective medium recommended for the isolation and enumeration of *Salmonella Typhi* and other *Salmonella species.*

2.1.2.1 Enrichment broth:

a) **Alkaline Peptone Water**

It is an enrichment broth used for enrich the food sample to enrich the growth of *Vibrio spp*.if any *Vibrio* spp. present in food sample. The pH of the broth is higher than usual broth which is 8.6±0.2.

b) Selenite broth

This broth helps to enrich the growth of *Salmonella* spp. and *Shigella* spp. if any of these microorganisms present in the food sample. The broth is not autoclavable as selenite activity is destroyed by autoclave temperature.

2.1.2.2 Stock culture media:

Tryptone soya broth **G**lucose **G**lycerol (TGG)

This is a broth for stock culture of microorganisms. One litter broth requires 30gm tryptone soya broth, 5gm glucose and 100ml glycerol.

Skim milk–**T**ryptone soya broth–**G**lucose–**G**lycerol (STGG)

It is another broth for stock the isolates or microorganisms. The recipe is same as previous stock culture broth but this broth additionally requires 20gm skim milk in one litter.

2.1.2.3 Media for biochemical test:

- **MR-VP (Methyl Red- Voges Proskauer)broth**

  It is mixed acid fermentation test in which bacteria breakdown glucose and lowers the pH of the medium below 5.0. inoculation of isolates into MR-VP broth and after an incubation period methyl red indicator added to the culture broth and if pinkish circle is produced which indicate positive result.

- **VP broth**

  Some bacteria are capable of producing acetoin (acetyl methyl carbinol), a precursor in synthesis of 2, 3-butanediol. A pink colour will be developed after addition of 40% KOH and a 5% solution of alpha-naphthol into the media if acetoin is produced.

- **Simmons' Citrate Agar**

  Utilization of citrate by microorganisms is observed by changing in Simmons' citrate agar after inoculation and incubation of 24hrs.

- **Triple Sugar Iron Agar**

  TSI agar made with slant and butt. Utilization of three sugars such as sucrose, dextrose and lactose and hydrogen sulphide production and gas production is observed in this media. Hydrogen sulphide production is detected by blackening of media due to the presence of thiosulfate and ferrous sulphate in the media. Carbohydrate utilization pattern of microorganisms will determine the *enterobacteriaceae* and other Gram-negative intestinal-bacilli. Three types of results are observed in incubated inoculated TSI agar.

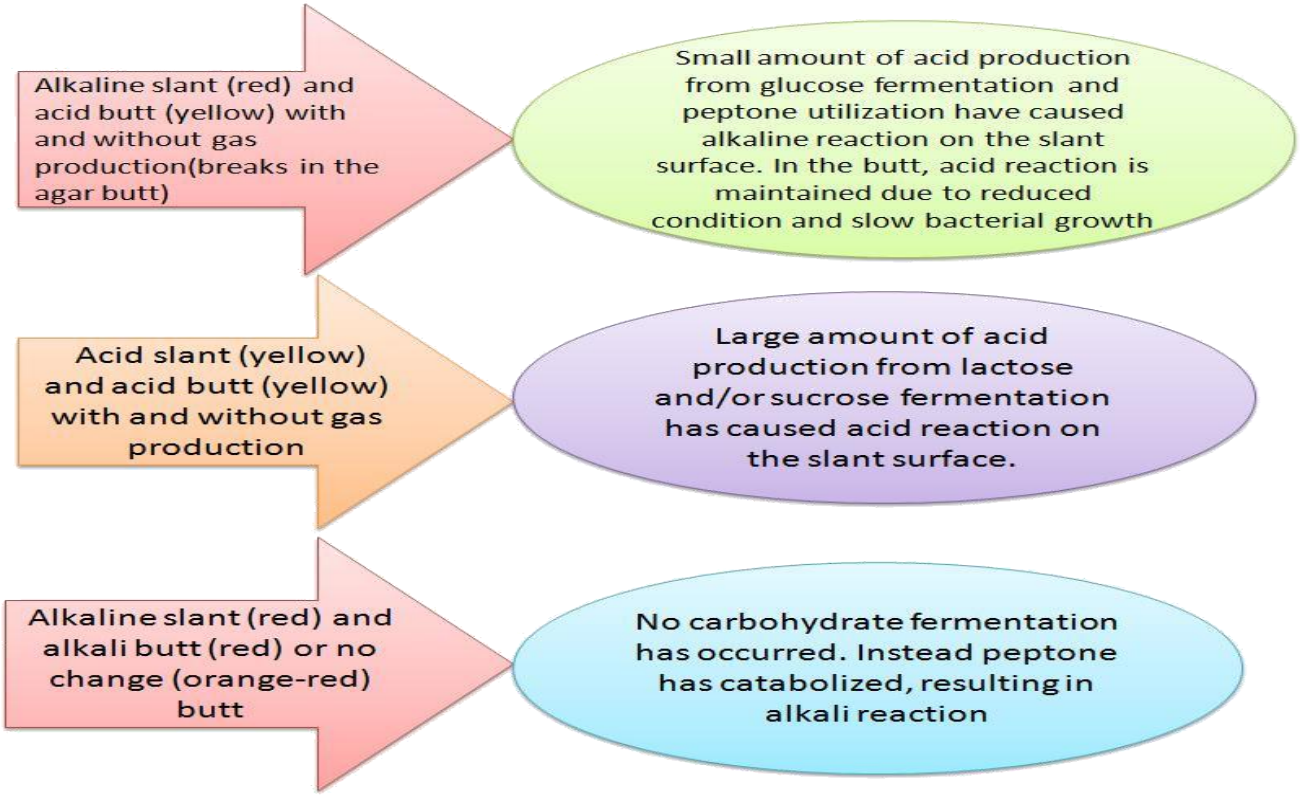

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Figure-2.1: TSI agar result interpretation diagram.

- **Motility Indole Urease Agar**

  Urease enzyme activity, indole production and motility of microorganisms are observed in this media.

- **Nitrate Broth**

  Nitrate reduction activity is observed in this broth; where change in broth colour after addition of nitrate test reagents detects the presence of nitrate utilization enzyme.

- **Phenol red dextrose broth**

  Utilization of dextrose is observed in this broth, where pH change in media cause colour change of the broth.

- **Phenol red lactose broth**

  Utilization of lactose is observed in this broth, where pH change in media cause colour change of the broth.

- **Phenol red sucrose broth**

  Utilization of sucrose is observed in this broth, where pH change in media cause colour change of the broth.

Some specific biochemical test media

- **Skim milk agar**

Caseins (proteinase) activity can be identified by this agar test.

- **Starch Agar**

  Alpha-amylase enzyme of bacteria breakdown starch into simple glucose molecules on starch agar and create a clear zone.

- **Blood Agar**

  Lysis of blood cells by microorganisms is observed in blood agar, alpha haemolysis, beta haemolysis and gamma haemolysis are type's blood cell lysis pattern by microorganisms.

- **Gelatine Agar**

  Gelatine is another protein some microorganisms are capable lyses gelatine by gelatinase enzyme, which create a clear zone or liquid gelatine form semisolid gelatine after incubation.

2.2 Methods

2.2.1. Sample collection

Sample collected in a sterile bottle which is sold in a non-potable condition. And potable water is collected in its remaining form and water was stored in refrigerator if it is not possible to spreading sample within 1-2 hour. Figure 2.2, 2.3 and 2.4 show the image of water sample from different places.

Fig 2.2: Non potable water

Fig 2.3: Potable water

Fig 2.4: Potable water

2.2.2 Sample preparation:

1ml of sample measured aseptically in three different sterilized test tubes and then 9ml saline (0.9%) solution in one test tube, alkaline peptone water 9 ml for *Vibrio* enrichment in second test tube and 9 ml selenite enrichment broth for *Salmonella* and *Shigella* culture were added. Alkaline peptone water and selenite broth enriched for 4-6 hours.

2.2.3 Plating of sample:

After collecting the sample, samples were diluted in saline water. The dilution factor was 01-02 times for drinking water. Saline solution with sample was serially diluted for two times. For each times dilution from 10^{-1} to 10^{-2} plated on duplicate plates of each agar media such as, in nutrient agar, macConkey agar, cetrimide agar, m-FC agar respectively. After 6 hours of incubation alkaline peptone water enriched 10^{-1} dilution streaked on TCBS agar media and selenite broth enriched sample streaked on XLD agar media.

2.2.4 Filtration method:

One hundred mililitres of each samples were poured through funnel & filter under laminar air flow cabinet and the filter was connected with vacuum pump. Then the membranes were placed on MacConkey agar and MFC agar respectively. The method for duplicate plate was repeated.

2.2.5 Observation of culture plate:

After 24 hours of incubation period growth of microbes were observed**.** Colony forming unit of every agar plate were counted for specific dilution. *Vibrio* species give large yellow colonies on TCBS, *Salmonella* species gives red colonies with black centre on XLD agar media, on the same media *Shigella* species gives red colonies and *E.coli* gives yellow colonies. *E.coli* species give yellow colour in MacConkey agar. Faecal *coliform* gives blue colour at MFC agar plate.Microbial growth also observedand noted after 48 hour incubation, and then different colonies from different selective media were sub cultured on specific media on the basis of colony morphology.But only on TCBS and XLD agar media were observed the presence of specific microorganisms according to microbial growth pattern.

2.2.6 Conservation:

After 24 hours of incubation of sub cultured colony morphology observed and again sub culture on nutrient agar for preservation in tryptone soya broth stock culture media for next biochemical test.

2.2.7 Microscopic observation of isolates

For evaluation of microscopic character, pure colony of each isolates was picked and Gram staining was performed according to Hacker's modified method (Detach, 1981). The size, shape, arrangement and Gram reaction properties of isolates were carefully observed.

2.2.8Analysing the Biochemical characteristics of isolates:

Gram staining, oxidase, catalase and other biochemical tests such as triple sugar iron test, motility indole urease test, Simmons' citrate test, nitrate test, methyl red and Voges-Proskauer test, carbohydrate (sucrose, dextrose, lactose) utilization tests were performed for each type of colony on nutrient agar.

2.2.9 Biochemical test:

- **Gram Staining:**

Gram staining is a differential staining method which was used to differentiate between Gram positive and Gram negative bacteria by their cell wall composition. Gram positive bacteria retain the violet colour upon staining with crystal violet dye and Gram negative bacteria are counter strained with safranin which appears pink under microscope.

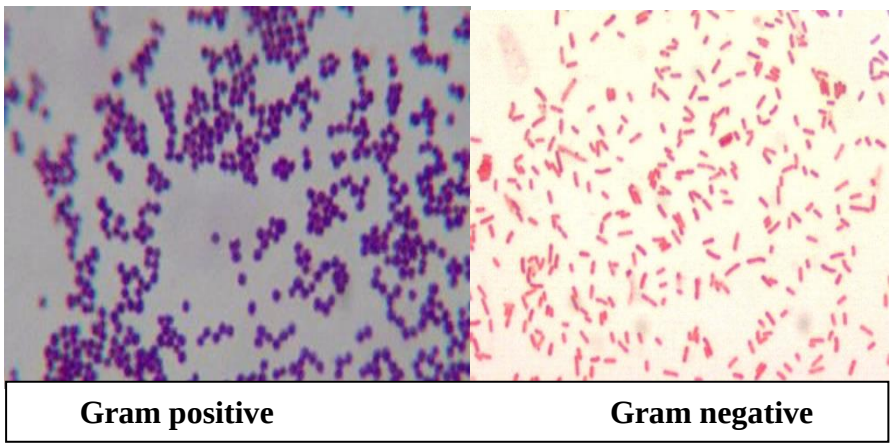


Fig 2.5: Gram Staining

❖ **Catalase test:**

Catalase is an enzyme that splits H_2O_2 into water and O_2. This test is performed to differentiate between groups of microorganism on the basis of catalase production. It is an easy biochemical test but it takes a very little time. Procedure of the test is given below:

1) One drop of hydrogen peroxide was placed on the glass slide.
2) A very small amount of bacterial colony was spread from nutrient agar plate to glass slide and mixed with hydrogen peroxide.
3) Bubble formation in took placed in 5-10 seconds that means result positive
4) If there was no bubbling that means a negative result.

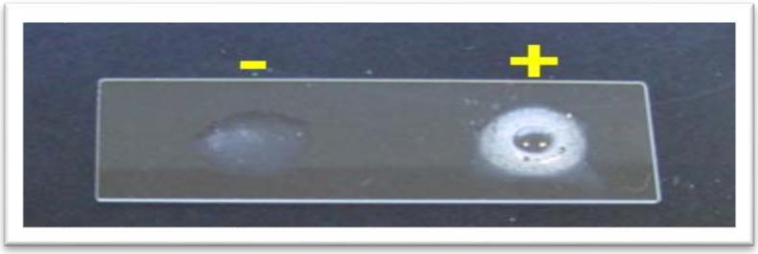


Fig 2.6: Catalase Test

❖ **Oxidase test :**

Oxidase test was performed to differentiate between enteric and non-enteric bacteria. A portion of the colony was picked up with a tooth pick and rubbed on a strip of a filter paper impregnated with oxidase reagent (1% aqueous solution of N'N'N'N'12 tetraniethyl-p-phenylenediaminedihyrochioride). Positive test is indicated by the presence of dark purple colour within 10 seconds**.**

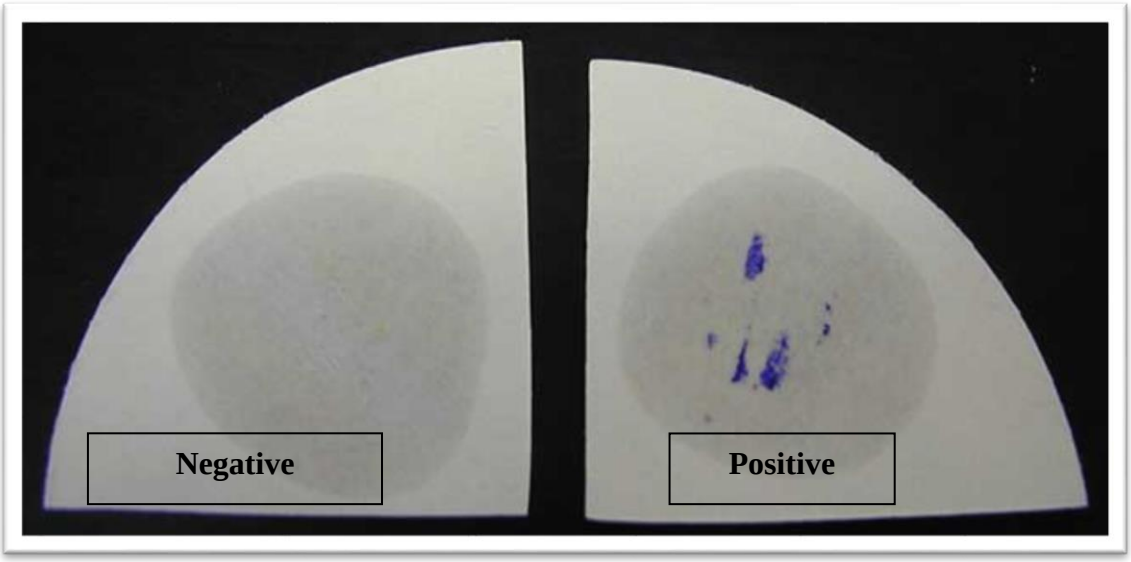
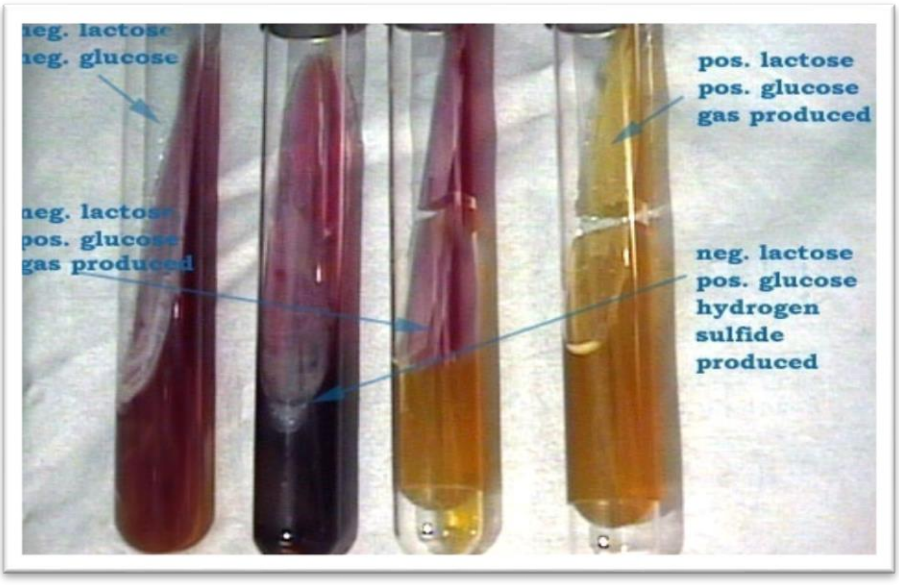


Fig 2.7: Oxidase test

❖ **Triple Sugar Iron (TSI) agar test**

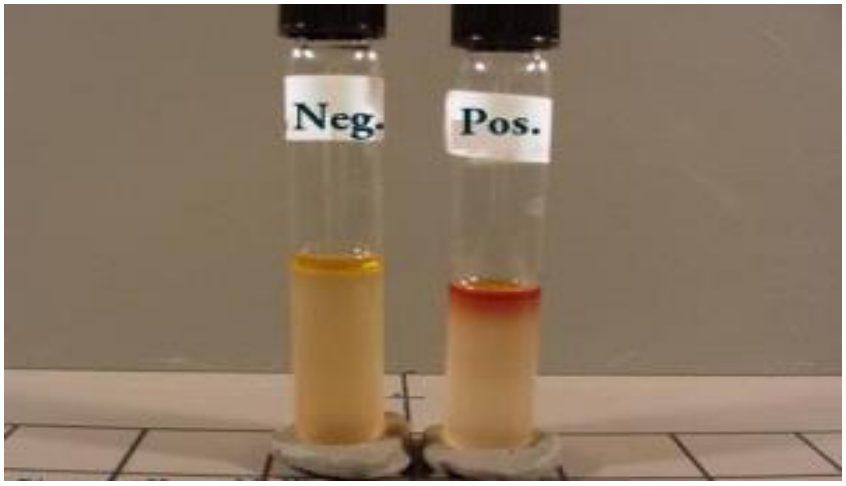
This test was performed to assess the mode of sugar utilization by stabbing the butt and streaking the bacteria over the slant of Triple Sugar Iron (TSI) agar media. Formation of acid from sugar in fermentative mode is indicated by yellowing of the butt and slant. If gas was formed during the fermentation, it was shown in the butt either by the formation of bubbles or cracking of the agar.



2.8: Triple sugar iron agar test

❖ **Motility Indole Urea (MIU) test**

The test was carried out in motility indole urea semisolid medium. One suspected isolated colony is touched with a straight wire and was stabbed into carefully down the tubes without touching the bottom. Following incubation, the tubes were observed for the presence of motile organisms which disperse through the medium leaving the stab line and made the tube turbid. Production of cherry red reagent layer after addition of Kovac's reagent in MIU medium demonstrates that the substrate tryptophan was hydrolysed which indicates indole positive reaction.



2.9: Motility Indole Urea (MIU) test

❖ **Citrate Utilization test**

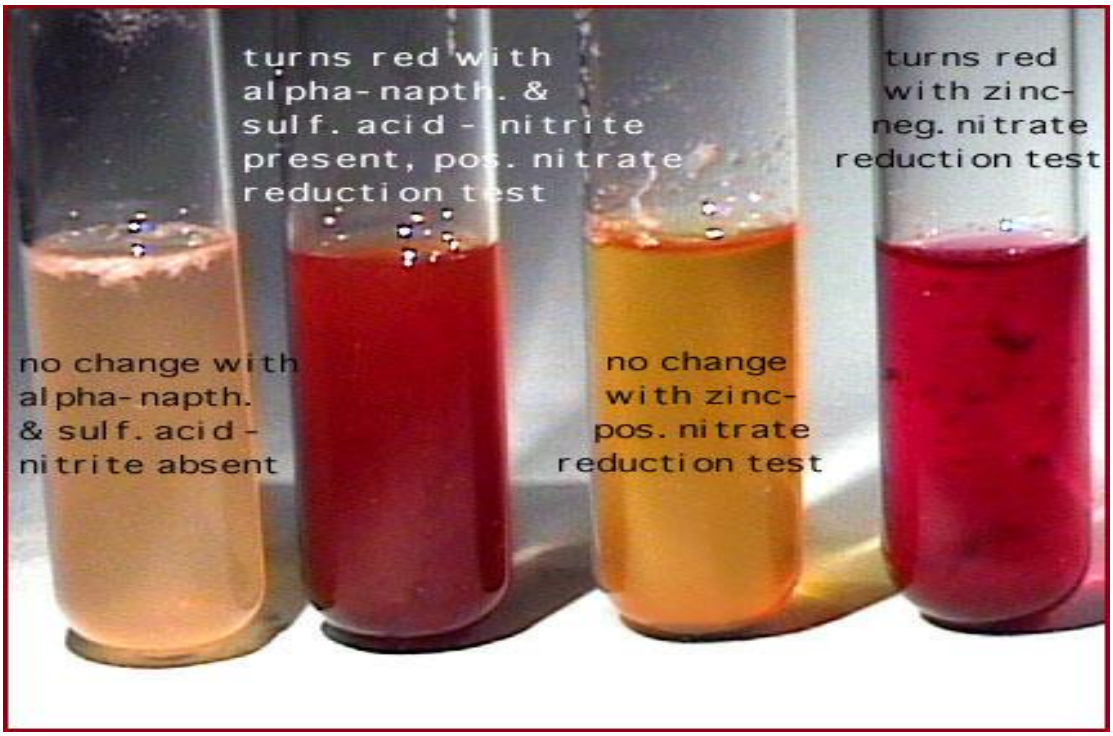
Citrate utilization by the isolates was observed by the growth of on slants of Simmons citrate agar. Following incubation, citrate positive culture was identified by the presence of growth on the surface of the slant and deep Prussian blue colouration of the medium. Citrate negative was identified by no growth and the green colouration of the medium.

❖ **Nitrate Reduction Test**

1) Nitrate broth was inoculated with an isolate from each sample plates and incubated for 48hours.

2) Then reagent A and reagent B were mixed carefully. If the bacterium produces nitrate reductase, the broth will turn a deep red within 5 minutes at this step (Fig-2.11).

3) If no colour change is observed, then the result is inconclusive. Add a small amount of zinc was added to the broth. If the solution remains colourless, then the both nitrate reductase and nitrite reductase are present. If the solution turns red, nitrate reductase is not present.



2.10: Nitrate reduction test

❖ **Carbohydrate utilization test**

1) The Durham tubes were inserted in an inverted position into all the tubes, fully filled with broth (lactose, dextrose and sucrose).

2) Each labelled carbohydrate broth (lactose, dextrose and sucrose) was inoculated aseptically with each of the seven bacterial isolates.

3) After inoculation into a particular sugar, the loop was sterilized in order to avoid cross contamination of the tube with other sugars.

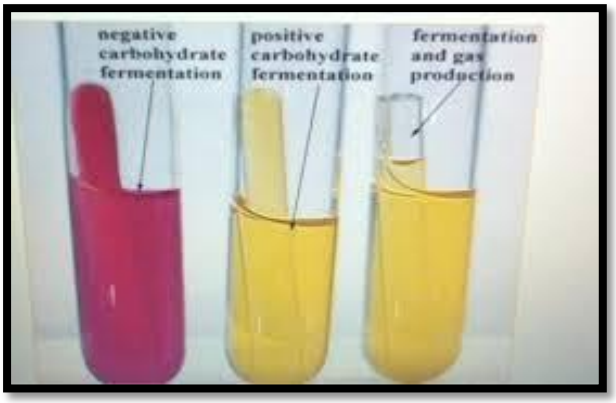
4) The tubes were incubated for 24 hours at 37^{O}C.

5) Following incubation, the tubes showed either of the results: acid production, acid And gas production or no fermentation at all.

6) The presence of acid and gas changes the medium into a yellow colour (Fig-2.12) indicating a positive result.

7) Gas production can be detected by the presence of small bubbles in the inverted Durham tubes.

8) The broth retaining the red colour is an indication of the absence of fermentation.



2.11: Carbohydrate utilization test

❖ **Methyl Red and Voges-Procure Test**

1) The bacterium to be tested was inoculated into potassium phosphate broth (MR-VP broth), which contained dextrose, peptone and potassium phosphate and incubated at 37^{O}C for 24 hours.

2) Over the 24 hours the mixed-acid producing organism might produce sufficient acid to overcome the phosphate buffer and remained acidic.

3) Half of this incubated broth transferred into another test tube for Voges-Procure Test

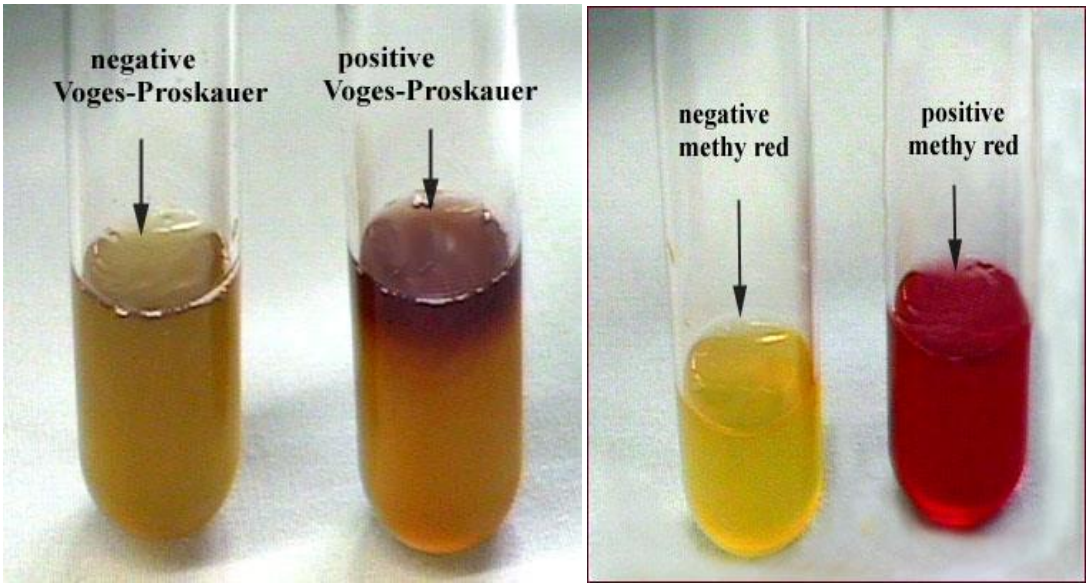
4) The pH of the medium was tested by the addition of five drops of MR reagent.

5) Development of red colour was taken as positive. MR negative organism produced orange colour.

6) Barritt's reagent A was added to the test broth and shaken.

7) Barrit's reagent B was added and the tube was allowed to stand for 15 min (Fig 2.13).

8) Appearance of red colour was taken as a positive test and no colour development taken as negative.



2.12: Methyl Red and Voges-Procure Test

❖ **Starch hydrolysis test**

1) A portion of the colony of a specific isolate streaked on starch agar.
2) Alpha amylase enzyme producing strain will produce a clear zone after overnight incubation.
3) The iodine solution was added to the culture medium a hallo zone around the colony indicate positive test. (Fig-2.13-14).

Fig 2.13: Positive reaction

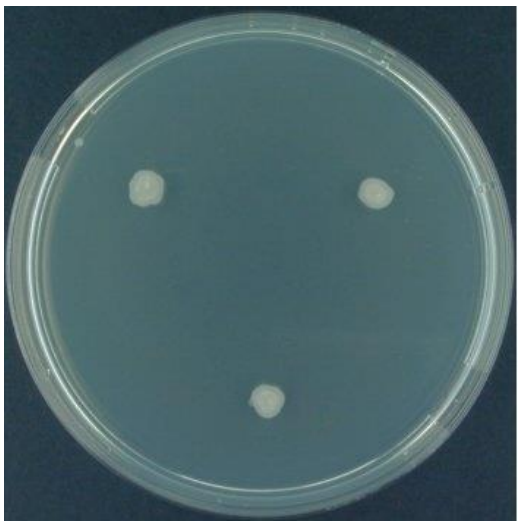


Fig 2.14: Negative reaction

❖ **Haemolysis test**

1) Specific bacterial colony was streaked on sheep blood agar.
2) After overnight incubation a clear zone appeared after beta haemolysis, greenish clear zone appeared if the bacteria broke blood cells partially which means alpha haemolysis and no clear zone will appeared (Fig-2.15) if the bacteria not able to break down blood cells which indicating gamma haemolysis.

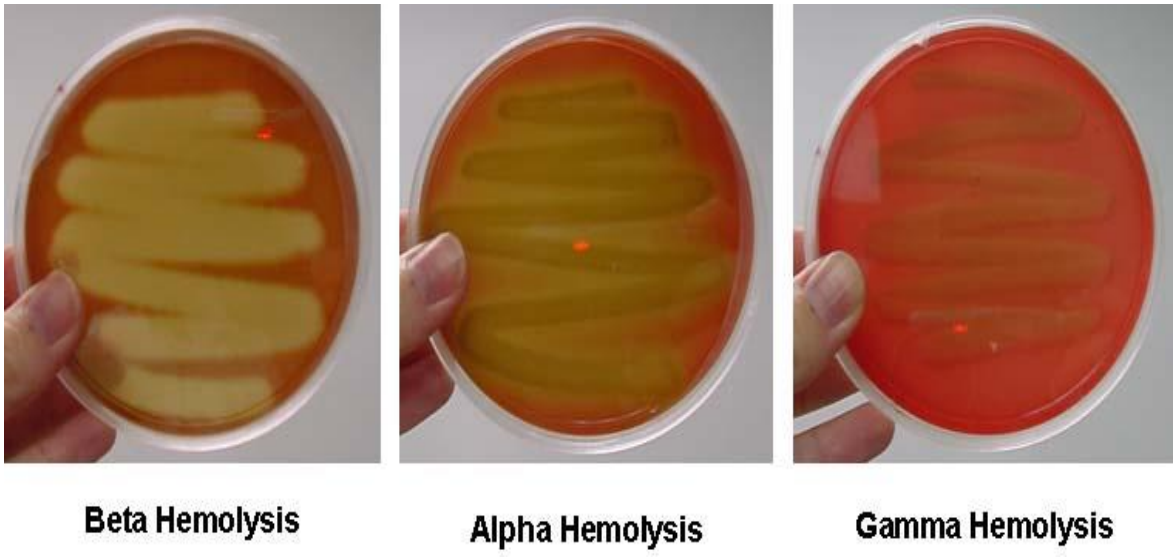


Figure 2.15: Haemolysis test

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2.2.10 Antibiotic Sensitivity Test

The antibiotic sensitivity test (AST) was used to find out the sensitivity of a bacterial strain against different types of antibiotic. For this purpose the bacterial isolate were enriched for 2 hours at 37°C in incubator followed by swabbing the bacterial strains on Nutrient Agar (NA) with a sterile swab stick so that a mat of bacteria was produced on the agar plate. Then with a sterile forceps an antibiotic disc was picked up from the cartridge and applied on the agar plate. The plate was again incubated overnight at 37°C in an incubator. Next day between 18-20 hours the zone of inhibition (clear zone) was measured with a ruler and compared with the Antibiotic disc zone diameter interpretation. Clear zone of specific size indicates susceptibility (Fig-2.16) of bacteria to the specific antibiotic and no clear zone indicates resistance to the antibiotic.

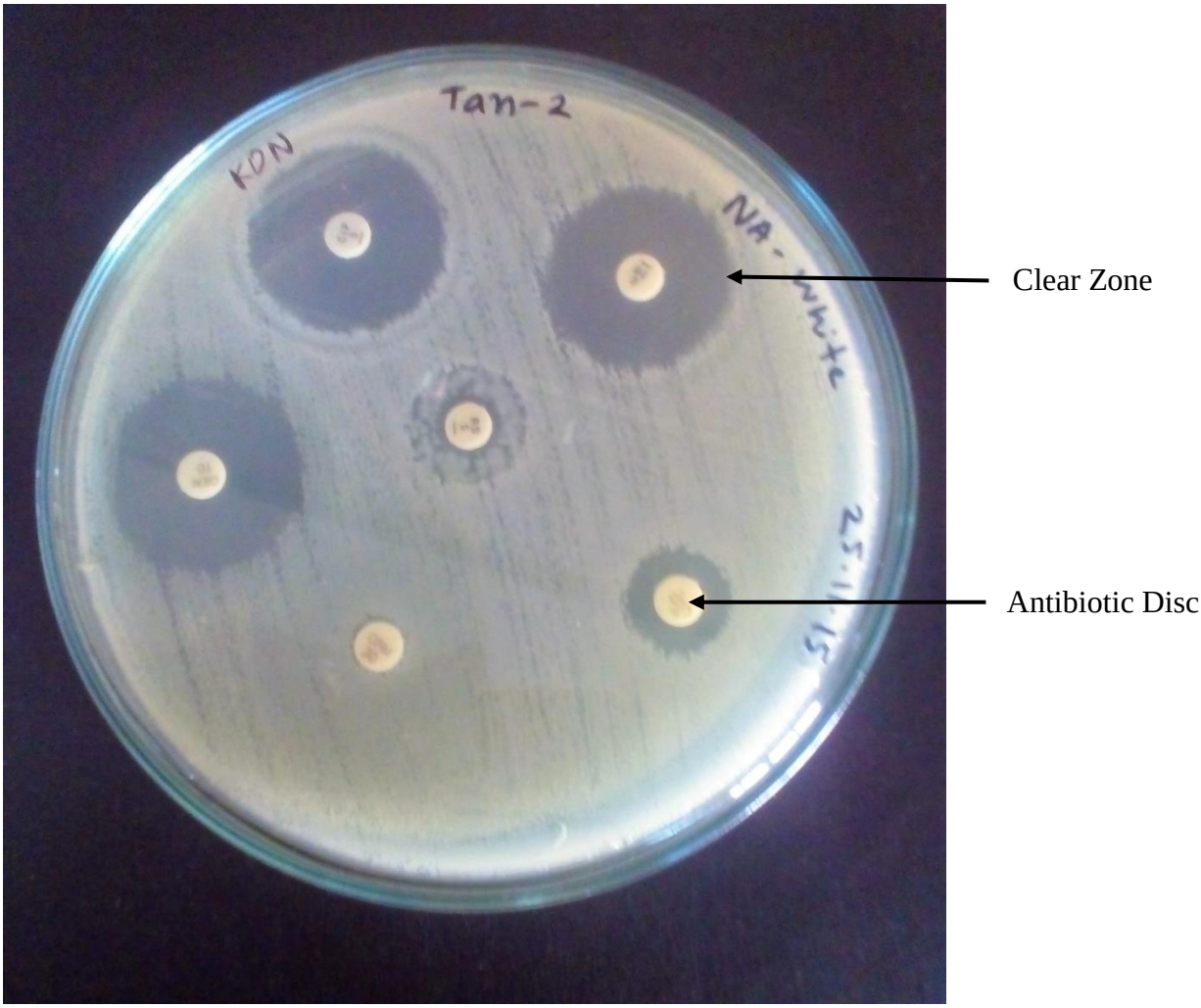

Figure 2.16: Antibiotic susceptibility test

2.2.11: Analysing the Biochemical test result for identification of bacteria:

Biochemical test results were noted on a chart and then all the results were put on online Advanced Bacterial Identification software (ABIS) on the basis of gram staining results on different category of bacteria such as *Enterobacteriaceae, Pseudomonas,Pasteurellaceae, Vibrio* and *Aeromonas, Compylobacter* and category of gram positive bacteria are *Streptococcus, Staphylococcus, Clostridium, Bacillus* and *Paenibacillus*. After putting the biochemical test result the software gives 4 most probable bacteria name which accurately match the test results. If detail option is applied after these probable results then it will suggest which test is positive/negative for these four bacteria among the biochemical tests which were performed and also suggest more biochemical tests for specification of bacteria species.

Chapter 3

Result

3.1.1. Qualitative Result

Selected water samples were placed on different selective and differential media. After 24 and 48hours, in some case 72 and 120hours of incubation microbial counts were recorded. Total count in nutrient agar (NA), coli-form count in MacConkey agar (Mac), *Pseudomonous* count in cetrimide and faecal coliform count in M-Fc agar were recorded. A table format of the results is presented next section.

3.1.2. Quantitative Result:

Some selective media were used to observe the presence of some specific microorganisms; TCBS agar for *Vibrio spp*. and XLD were used for *Slmonella spp.* and *Shigella spp*. The qualitative result is also presented in next sections.

In this case, 1 ml of drinking water was diluted 100 times. That's means 10 fold dilutions were done 2 times. After that 150 µl of water was spread in agar plate for colony count. After incubation period CFU were counted and recorded accurately. Here the results are presenting in table below:

Table 3.1- General calculation of Microbial load of potable and non-potable water sample from Mohakhali.

If the imaginary number of CFU is x in 150µl sample which was spreading in agar plate, when the dilution factor was 10^{-2}, then the count of CFU should be:

Amount of sample	**150µl sample**	**10^{-2} dilution**	**10^{-1} dilution**	**1ml (sample)**
CFU	x	x×10	$x\times10^2$	$x\times10^3$

Collection place: Mohakhali

Incubation time: 24

Table 3.2: Microbial load in different selective media for non-potable water sample

Samp. Name	Qualitative Result						Quantitative Result		pH of water
	Membrane filtration		NA (CFU/ml)	Mac (CFU/ml)	Cetrimide (CFU/ml)	m-FC (CFU/ml)	TCBS (CFU/ml)	XLD (CFU/ml)	
	m-FC (CFU/100 ml)	Mac (CFU/100 ml)							
Tan	2.1×10^2	3.1×10^2	9×10^3	1.33×10^3	NG	1.16×10^3	2.66×10	4.63×10^2	7.12
Dh	TNTC	4.7×10^2	3.71×10^4	1.06×10^4	NG	2.66×10^3	NG	NG	7.2
ML	NG	NG	4.95×10^4	3.33×10^3	NG	NG	NG	NG	7.06

****NG = negative growth;** Tan,Dh,ML were randomly selected sample from Mohakhali.

Collection place: Mohakhali

Incubation time: 24

Table 3.3: Microbial load in different selective media for potable water sample

Sample Num	Qualitative Result						Quantitative Result		pH of water
	Membrane filtration		NA (CFU/ml)	Mac (CFU/ml)	Cetrimide (CFU/ml)	m-FC (CFU/ml)	TCBS (CFU/ml)	XLD (CFU/ml)	
	m-FC (CFU/100 ml)	Mac (CFU/100 ml)							
EF	NG	1.6×10^2	1.20×10^4	NG	NG	NG	NG	NG	6.8
DL	NG	NG	1.01×10^3	NG	NG	NG	NG	NG	7.05
Mu	NG	NG	5×10^2	NG	NG	NG	NG	NG	7.0

****NG = negative growth; EF= Efad mineral drinking water; DL= Dalia mineral water;**

Mu = mum mineral drinking water

Table 3.4 (a): Colony characteristics of the isolates on MacConkey agar media

Collection Type	Sample Name	Colony characteristics				
		Size	**Form**	**Colour**	**Margin**	**Elevation**
Not Potable	**Tan**	Small	Circular	Colourless	Entire	Raised
	Dh	Large	Circular Dark pink		Entire	Raised
		Large	Circular Yellow		Entire	Raised
	ML	Small	Circular	Colourless	Entire	Raised
		Small	Circular	yellow	Entire	Raised
Potable	**EF**	Large	Circular	Yellow	Entire	Raised
	DL	Small	Circular	Yellow	Entire	Convex

Table 3.4 (b): Colony characteristics of the isolates on Nutrient agar media

Collection Type	Sample Name	Colony Characteristics				
		Size	Form	Colour	Margin	Elevation
Not Potable	Tan	Large	circular	Yellow	Entire	Raised
		Large	Circular	White	Entire	Raised
	Dh	Large	Circular	White	Entire	Raised
		Large	circular	Yellow	Entire	Raised
	ML	Large	circular	Brown	Entire	Raised
		Large	circular	Yellow	Entire	Raised
		Large	circular	White	Entire	Raised
Potable	EF	Large	circular	White	Entire	Raised
	DL	Large	circular	Yellow	Entire	Raised
	MU	Large	circular	Yellow	Entire	Raised

Growth of Bacterial colonies on different agar media

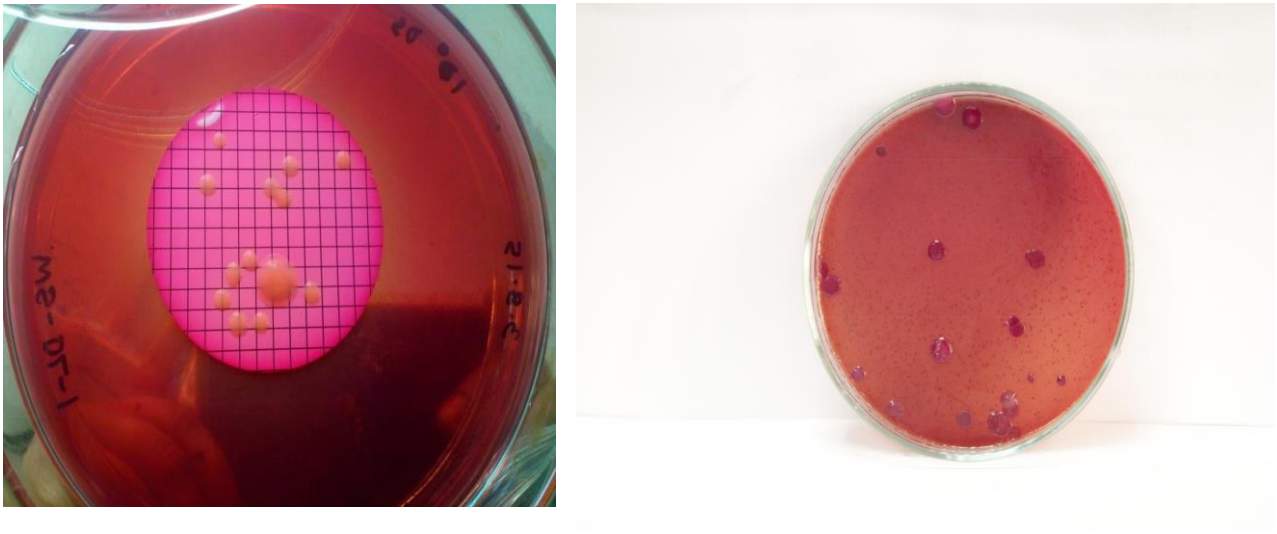

Figure 3.4.1: Bacterial colonies on MacConkey agar media. Pink colonies and colourless colonies indicate lactose fermenter and non-fermenter respectively.

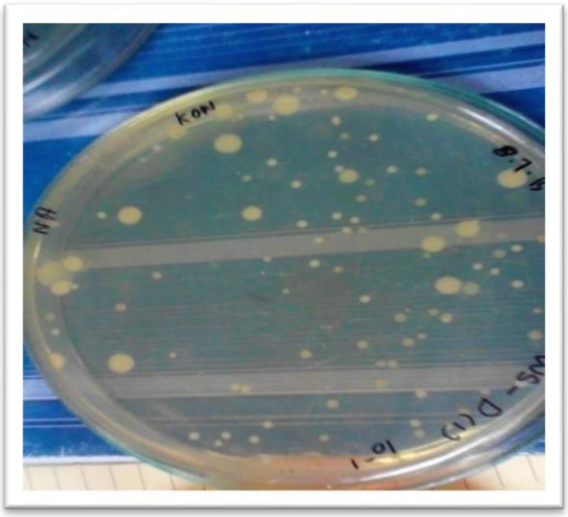

Figure 3.4.2: Bacterial colonies on nutrient agar

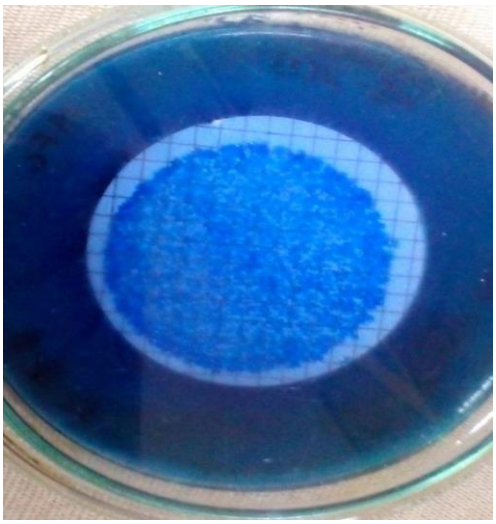

Figure 3.4.3: Bacterial colonies on m-FCagar media

Figure 3.4.4: Bacterial colonies on TCBS agar media.

Table 3.4 (C): Colony characteristics of the isolates on XLD agar media

Collection Type	Sample Name	Colony Characteristics				
		Size	**Form**	**Colour**	**Margin**	**Elevation**
Not Potable	**Tan**	Small	circular	Yellow	Entire	Raised
		Small	circular	Yellow with black centre	Entire	Raised
		Small	circular	Pink	Entire	Raised
		Small	circular	Pink with black centre	Entire	Raised

Table 3.4 (D): Colony characteristics of the isolates on m-FC agar media

Collection Type	Sample Name	Colony Characteristics				
		Size	**Form**	**Colour**	**Margin**	**Elevation**
Not Potable	**Tan**	Small	circular	Dark blue	Entire	Raised
		Small	Circular	White	Entire	Raised
	Dh	Large	Circular	White	Entire	Raised
		Large	circular	Yellow	Entire	Raised
		Large	circular	Brown	Entire	Raised

Table 3.4 (E): Colony characteristics of the isolates on TCBS agar media

Collection Type	Sample Name	Colony Characteristics				
		Size	**Form**	**Colour**	**Margin**	**Elevation**
Not Potable	**Tan**	Small	circular	Yellow	Entire	Convex
		Small	circular	White	Entire	Raised

3.5 Antibiotic susceptibility test to identify the MAR (multiple antibiotic resistant) bacteria

Total thirty-one isolates of bacteria were selected for antibiotic susceptibility test. Eleven antibiotics were analysed for each type of bacteria to see the sensitivity and resistance toward antibiotics. In this study thirty-one isolates individually were tested one time for seven types of antibiotics. Two tables are prepared according to Gram negative and Gram positive bacteria and showed sensitivity, resistance and intermediate according to the clear zone diameter.

Some bacteria have shown no clear zone which means they were resistant to the specific antibiotic and some shown very small diameter of clear zone this also an indicator of antibiotic resistance. If clear zone diameter is larger than resistant diameter scale and less than susceptible diameter than this result called intermediate, which means the specific bacteria is neither resistant nor susceptible to the specific antibiotic. The clear zone diameter scale when matches the susceptibility diameter scale then the bacteria is sensitive to that specific antibiotic. The standard diameter scale is organized as a chart by antibiotic manufacturing companies and the test outcome is then matched with this chart to interpret the result.

3.5.1 Antibiotic susceptibility of gram negative bacteria

Total seven Gram negative microorganisms were analysedfor antibiogram test (Table-3.6). Seven types of antibiotic disk were used against each bacterium to check the resistance or sensitivity.Ampicillin (Amp), Amikacin (AK), cefoxitin (CX), chloramphenicol(C), gentamicin (CN), kanamycin (K), nofloxacin (NOR) were used to test. Which isolates showed resistance to three or more antibiotics those isolates were chosen for biochemicaltest.

3.5.2 Antibiotic susceptibility of gram positive bacteria

In total twenty four isolates were chosen for Ab test (Table -3.7; 3.8). Seven types of antibiotic disks were used to test. Ampicillin (Amp), cefepime (CPM), cefoxitin (CX), chloramphenicol(C), gentamicin (CN), ciprofloxacin (CIP), Rifampicin (RD) were used to isolates showed resistance to three or more antibiotics were chosen for further biochemical test.

Table 3.6: Antibiotic susceptibility of gram negative bacteria

Sample name	Antibiotic name						
	Amp	AK	CX	C	CN	K	NOR
mFc-blu-Tan-2	**R**	**S**	**S**	**S**	**S**	**S**	**R**
mFc-wblu-Tan-3	**R**	**S**	**R**	**S**	**S**	**S**	**S**
Mac-pink-Tan-3	**R**	**S**	**R**	**S**	**S**	**S**	**S**
mFC-blu-ML-1	**S**	**S**	**R**	**R**	**S**	**S**	**S**
Mac-pink-ML-1	**R**	**S**	**R**	**S**	**S**	**S**	**S**
mFC-blu-ML-2	**S**	**R**	**S**	**R**	**S**	**S**	**S**
Mac-pink-ML-3	**R**	**S**	**R**	**S**	**S**	**S**	**S**

****S= sensitive R= resistant**

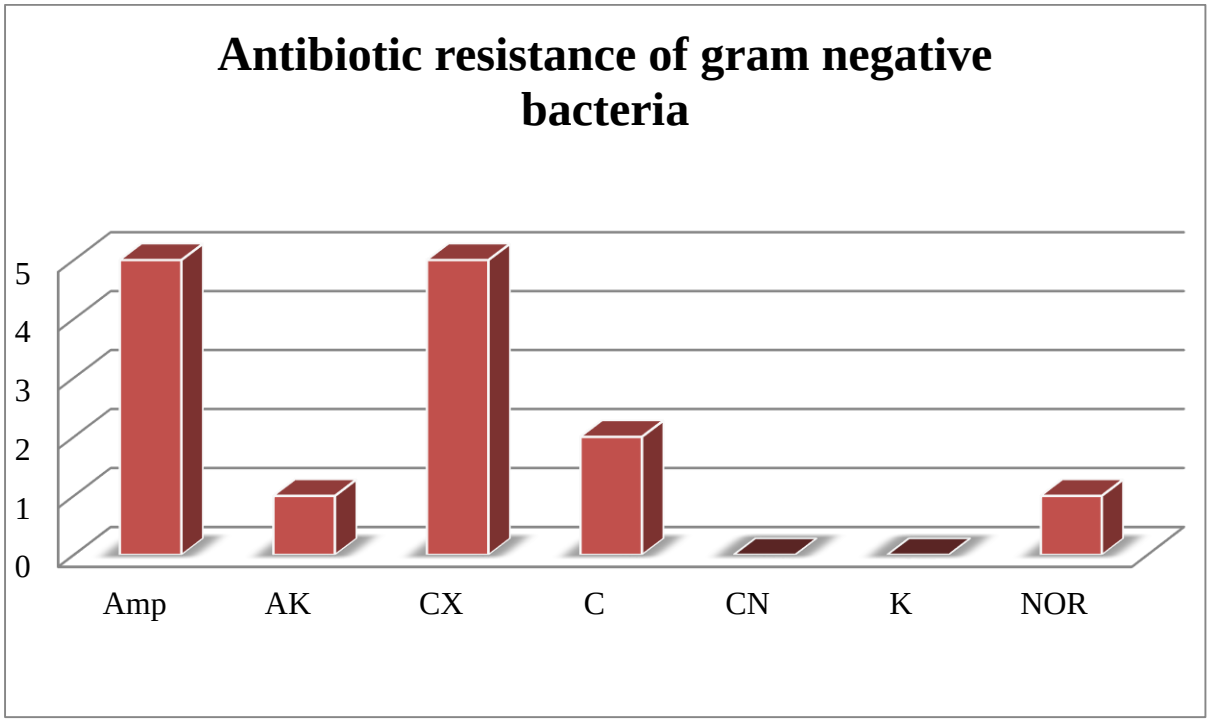

Figure 3.5.1(a): Antibiotic resistancy of gram negative bacteria

Ampicillin (Amp), Amikacin (AK), cefoxitin (CX), chloramphenicol(C), gentamicin (CN), kanamycin (K), nofloxacin (NOR)

Table 3.7: Antibiotic susceptibility of gram positive bacteria

Sample Name	Antibiotic name						
	Amp	CPM	CX	C	CN	CIP	RD
NA-W-DL-1	R	S	S	S	S	S	R
Mac-Green-EF2	R	R	S	S	S	S	R
NA-Yel-Tan-1	S	S	S	S	R	S	R
NA-W-Tan-1	S	S	S	S	S	S	R
Mac-Y-EF-2	R	S	R	R	S	S	R
NA-Y-EF-1	R	S	R	S	S	S	R
Mac-pin-Dh-1	R	R	R	S	S	S	R
NA-W-EF-1	S	R	R	S	R	S	S
NA-Y-Dh-1	S	S	S	S	S	S	R
Mac-Y-Dh-1	R	S	R	R	S	S	S
TCBS-W-Tan-3	R	R	S	R	S	S	R
NA-Y-Tan-2	R	R	R	R	S	R	S
NA-W-Tan-2	S	R	S	S	S	S	S
XLD-Y-Tan-2	R	S	R	R	R	S	S
XLD-Black-Tan-2	R	R	S	S	S	S	R
NA-W-Tan-3	S	S	S	S	S	S	R
mFC-Blu-tan-3	S	S	R	S	S	S	R
NA-W-ML-2	R	R	R	S	S	S	R
NA-Y-ML-2	S	S	S	S	S	S	S
NA-OFW-ML-2	S	S	S	S	S	S	S
NA-Colorless-ML-1	R	S	S	S	S	S	R
NA-W-ML-1	S	S	S	S	S	S	R
NA-W-ML-3	R	R	S	S	S	S	R
NA-W-DL-3	S	R	S	S	R	S	R

S= sensitive; R=resistant

Here, seven isolate are selected for biochemical test which have shown the highest resistance against antibiotic. The results of biochemical test are shown below by table.

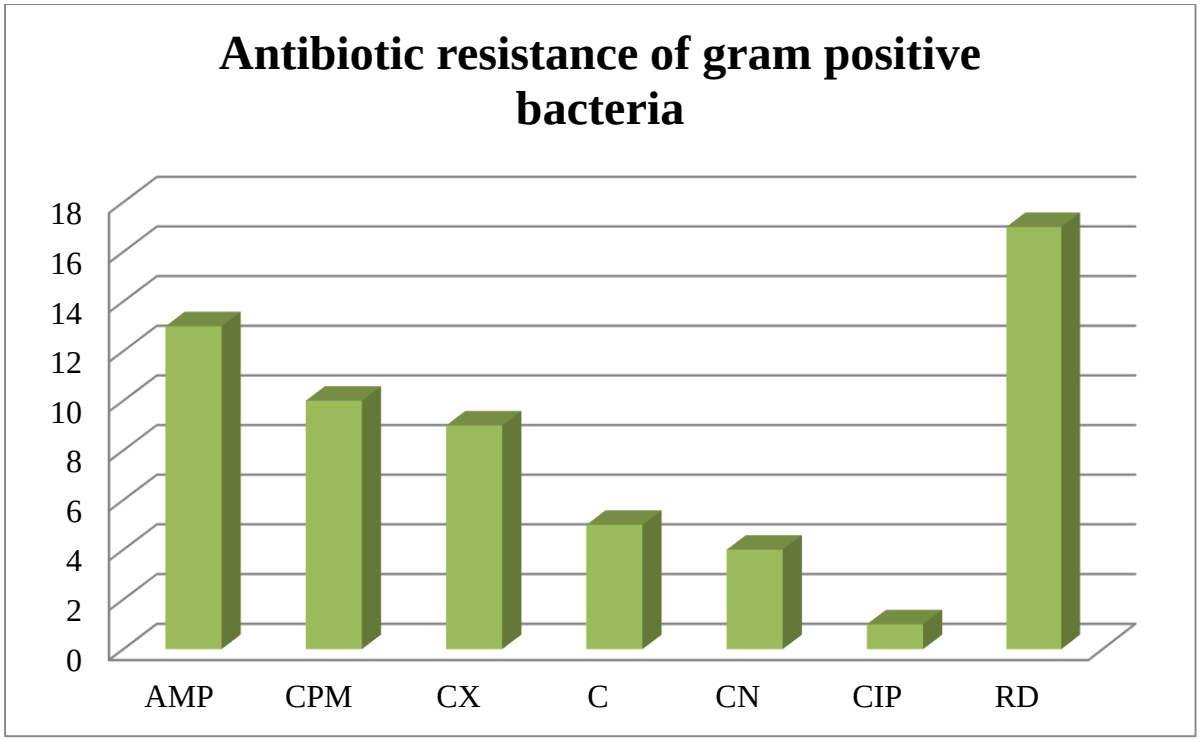

Figure 3.5.2(a): Antibiotic resistancy of gram positive bacteria

Ampicillin (Amp), cefepime (CPM), cefoxitin (CX), chloramphenicol(C), gentamicin (CN), ciprofloxacin (CIP), Rifampicin (RD)

Table 3.8: Results of biochemical tests of the isolates collected from different agar

Sample name	TSI (Slant/butt)	Nitrate reduction	Citrate utilization	Indole activity	MR	VP	Haemolysis (Blood agar)	Sucrose	Lactose	Dextrose	Urease production	Motility	Casein hydrolysis (Milk agar)	Starch hydrolysis (Starch agar)	Probable Species
Mac-pink- D1	R/Y; G+	_	+	_	+	_	α	+ ; G-	+ ; G+	+ ; G-	+ve	-ve	-ve	-ve	1.*Pragia fontium*~ 82% 2.*Erwinia tracheiphila*~88%
XLD-yellow-Tan2	Y/Y; G+	_	_	_	+	+	α	+ ; G+	+ ; G+	+ ; G+	+ve	-ve	-ve	-ve	1.*Citrobacter rodentium*~83% 2.*Yersinia aleksiciae* ~ 75%
NA-yellow-tan2	R/Y; G-	+	_	_	+	+	α	+ ; G+	--; G+	+ ; G+	-ve	-ve	-ve	-ve	1.*Tatumella punctata* ~ 89% 2.*Enteric Group 68* ~ 87%
TCBS-white-Tan3	R/R; G-	+	+	_	+	+	γ	+ ; G-	+ ; G+	+ ; G-	-ve	-ve	-ve	-ve	1.*Vibrio sinaloensis* ~87% 2.*Vibrio fluvialis* ~ 81%
Mac-yellow-D1	Y/Y; G-	+	+	_	+	+	α	+ ; G-	+ ; G-	+ ; G-	-ve	-ve	-ve	-ve	1.*Tatumella citrea* ~ 87% 2.*Tatumella punctata* ~ 87%
NA-white-EF1	Y/Y; G-	+	_	_	+	_	α	+ ; G+	--; G+	+ ; G+	-ve	-ve	-ve	-ve	1.*Yersinia bercovieri* ~ 95% 2.*Yersinia mollaretii* ~ 95%
Mac-yellow-EF2	Y/Y; G-	+	+	_	+	+	α	+ ; G-	--; G-	+ ; G-	-ve	-ve	-ve	-ve	1.*Tatumella punctata* ~ 89% 2.*Tatumella citrea* ~ 84%

R=red; Y= yellow; G+ = gas production positive; G- = No gas production; (+ve) / (+) = positive growth; (-ve) / (-) =negative growth

Chapter 4

Discussion

Discussion:

4.1. Quantitative result

Based on this research study, it has been observed that, total viable count of bacteria and coliform count of water were higher in non-potable drinking water sold at tea stall. This is due to street side dust, unhygienic handling and processing, vehicle exhaust. However in potable mineral water growths of microbes were unacceptable. Because those bottled water are commercialized by well-known reputed companies. According to those companies the water is highly purified but in this study we found different result. Quality controls of those products were not appropriate. All research works were done under laminar hood against air flow so the chances of contamination were negative.

4.2. Qualitative result

Two agar media TCBS agar and XLD agar were used to observe the presence of certain microorganisms in water sample. Thiosulfate Citrate Bile Salts Sucrose Agar is highly selective for *Vibrio* species isolation and the formula developed by Kobayashi and his colleagues, (Kobayashi *et al.*, 1963). The growth of intestinal flora suppressed at high pH of 8.5-9.5 of TCBS agar(Applied Microbiology, 1970). Different components allow the optimum growth and metabolic activities of *Vibrio* spp. such as, one percent sodium chloride and sodium thiosulphate provides a source of sulphur and in combination with ferric citrate to detect the production of hydrogen sulphide. The source of carbohydrate is sucrose also in combination with bromothymol blue and thymol blue indicators the *Vibrio* spp. give different colour. Yellow-brown colonies were produced by *V. cholerae* biotype Eltor by fermenting sucrose and pH shifting. Light bluish colonies were produced by *V.parahaemolyticus*. Small yellow colonies are produced by certain species of *Proteus* and enterococci which are easily distinguishable. In this study most of the colonies on TCBS were yellow, brownish-yellow also green colonies were also observed. After biochemical tests it was observed that almost all were non-*Vibrio spp.* but not *Vibrio cholera* varieties.

Xylose-Lysine-Desoxycholate agar (XLD) is selective medium for the isolation of *Salmonellae* and *shigellae* from clinical specimens and foods.

For isolation and primary differentiation of *Salmonella* and *Shigella* from non-pathogenic bacteria the media relies on xylose fermentation, lysine decarboxylation and production of hydrogen sulphide. Almost all enteric bacteria are capable of fermenting xylose except *Shigella,Providencia* and *Edwardsiella. Shigella*can be identified due to the xylose negative reaction.

Salmonella spp. is differentiated from non-pathogenic xylose fermenter by decarboxylation oflysine, which by altering the pH to alkaline and mimicking the *Shigella* reaction but producing hydrogen sulphide differentiates it. Lysine-positive coliforms cannot reverse the pH to alkaline condition due to high level acid produced by fermentation of lactose and sucrose and also non-pathogenic hydrogen sulphide producers do not decarboxylate lysine. To inhibit the growth of coliforms without decreasing the growth of *shigellae* and *Salmonellae* sodium desoxycholate is incorporated into the medium. Sodium desoxycholate is incorporated as an inhibitor in the medium. The concentration used allows for the inhibition of coliforms without decreasing the ability to support *shigellae* and *Salmonellae (*Taylor and Harris, 1967*).*

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4.3. Biochemical test results of isolates according to ABIS software

ABIS has shown most probable four bacteria and among those two highly probable bacteria were put into biochemical test chart in the result chapter of this thesis. The genera were approximately same but the species were not, more specific biochemical tests are required to confirm the species.

4.4. Microbial load of drinking water

In this study three types of microbial loads were observed such as, total viable count (TVC), coliform and enteric pathogen count (CEC).Two types of water were examined for microbial load from six street side tea stall of Mohakhali.

In case of non-potable drinking water in almost every sample *coliform* were found. Growth of blue colonies in m-Fc agar indicates the presence of faecal *coliform*. It was found in every sample of non-potable water.

But in case of potable mineral water *faecal coliform* count was zero. There were no growths in m-Fc agar. As well as no growth at cetrimide agar also confirm the absence of *Pseudomonous spp.*

Biological treatment processes in the waste water treatment plants may result in a selective increase of antibiotic-resistant bacteria and therefore increase the occurrence of multidrug-resistant organisms (Zhang, Marrs, Simon, and Xi, 2009) Although microorganisms in drinking water are reduced by chlorination, they may survive the treatment process and enter the distribution system (Faria, Vaz-Moreira, Serapicos, Nunes, and Manaia, 2009). Moreover, the presence of antibiotic resistance in microorganisms has been previously reported (Mulamattathil, Esterhuysen, and Pretorius, 2000; Lin and Biyela, 2005; Kinge and Mbewe, 2010). Six selected sample's microbial quality was analysed from tea stalls. After observing microbial growth and biochemical tests, the test results were decided by using ABIS. According to this result further analysis were done.

Conclusion

Conclusion:

The World Health Organization has estimated that up to 80% of all sickness and disease in the world is caused by inadequate sanitation, polluted water or unavailability of water and at least 5 million deaths per year can be attributed to water borne diseases (Karn et al., 2001).

The most common forms of freshwater habitats of Bangladesh are hoar, baod, beel, lake, pond, rivers, and floodplains. The sediment/water phases are not permanently separated. Mineral and organic particles of the lake water, and enteric and other bacteria adsorbed by these particles, can pass to sediments column as a result of diving, rowing, walking in water, use of motor boats, navigation, sand extraction etc. Passage of bacteria from bottom sediment to water may also take place as a result of changes in salinity or concentration of organic matter. It is believed that enteric bacteria are able to metabolize elutes of bottom sediments, and develop and multiply in such aquatic ecosystems free of predators. In addition, survival of these microorganisms in surface is higher than in bottom sediments, and water may be a reservoir of these bacteria.

Due to urbanization people are inclined towards the outside life. They have to stay in their workplace for several times, in term of Bangladesh job seeker include employee, students of different institution also patient of various hospital buy their water from different tea stall or glossary shop. Rickshaw puller, students, many people are drink water from tea stall. So the quality of drinking water of tea stall plays a major role in our life. If the water is contaminated with pathogenic microbes it'll cause serious water born disease. The goal of this study was to check the contamination of drinking water which was sold at tea stall. From this study it was found that there were several types of organism found in water those decrease the water quality. MAR bacterial organism also found in drinking water what means a threat to us. In this study it is also found that in case of experimental area potable mineral water is more safe then non potable drinking water which was sold at tea stall.

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APPENDIX

APPENDIX-I

Media composition

The composition of the media used in the present study has been given below. Unless otherwise mentioned, all the media were autoclaved at 121^{o}C for 15 min.

1. Nutrient Agar (Himedia, India)

Ingredients	Amounts (g/L)
Peptic digest of animal tissue	5.0
Beef extract	1.50
Sodium chloride	5.0
Yeast extract	1.50
Agar	15.0

2.M-FC Agar Base (Himedia, India)

Ingredients	Amounts (g/L)
Tryptose	10.0
Proteose peptone	5.0
Yeast extract	3.0
Lactose	12.50
Bile salts mixture	1.50
Sodium chloride	5.0
Aniline blue	0.10
Agar	15.0

3. Nutrient Broth (Oxoid, England)

Ingredients	Amount (g/L)
Lab-lemco powder	**1.0**
Yeast extract	**2.0**
Peptone	**5.0**
Sodium chloride	**5.0**

4. Cetrimide agar (Merck, India)

Ingredients	Amount (g/L)
Pancreatic digest of gelatine	**20.0**
Magnesium chloride hexahydrate	**1.4**
Potassium sulphate anhydrous	**10.0**
Cetrimide	**0.3**
Agar-Agar	**13.0**

5. Tryptone soy broth, (Oxoid, England)

Ingredients	Amount (g/L)
Pancreatic digest of Casein	**17.0**
Papaic digest of soybean meal	**3.0**
Sodium chloride	**5.0**
Di-basic potassium phosphate	**2.5**
Glucose	**2.5**

6. **MacConkey agar (Oxoid, England)**

Ingredients	Amount (g/L)
Peptone	**20.0**
Lactose	**10.0**
Bile salts	**5.0**
Sodium chloride	**5.0**
Neutral red	**0.075**
Agar	**12.0**

7. **Simmon's citrate agar (Oxoid, England)**

Ingredients	Amount (g/L)
Magnesium sulphate	**0.2**
Ammonium dihydrogen phosphate	**0.2**
Ammonium phosphate	**0.8**
Sodium citrate	**2.0**
Sodium chloride	**5.0**
Agar	**15.0**
Bactobrom thymol blue	**0.08**

8. **Peptone Water**

Ingredients	Amount (g/L)
Peptone	**10.0**
Sodium chloride	**5.0**

9. **MR-VP broth**

Ingredients	Amount (g/L)
Peptone	**7 g**
Dextrose	**5 g**
Potassium phosphate	**5 g**

10. Triple sugar iron agar (Himedia, India)

Ingredients	Amount (g/L)
Peptic digest of animal tissue	**10.0**
Sodium chloride	**5.0**
Lactose	**10.0**
Sucrose	**10.0**
Dextrose	**1.0**
Ferrous sulphate	**0.20**
Sodium thiosulfate	**0.30**
Casein enzymatic hydrolysate	**10.0**
Yeast extract	**3.0**
Beef extract	**3.0**

11. Thiosulphate Citrate Bile Salts Sucrose agar (Difco, USA)

Ingredients	Amount (g/L)
Proteose peptone	**10.0**
Sodium thiosulfate	**10.0**
Sodium citrate	**10.0**
Yeast extract	**5.0**
Oxgall	**8.0**
Sucrose	**20.0**
Sodium chloride	**10.0**
Ferric citrate	**1.0**
Thymol blue	**0.04**
Agar	**15.0**

12. Phenol red (Lactose, Dextrose, Sucrose) Broth

Ingredients	Amount (g/L)
Trypticase	**0.4**
Lactose	**0.2**
Sucrose	**0.2**
Dextrose	**0.2**
Sodium chloride	**0.2**
Phenol red	**0.00072**
Final pH	**7.3**

APPENDIX-II

Buffers and reagents

1. **Phosphate buffered saline (PBS)**

PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 2.0 g of KH_2PO_4 in 800 ml of distilled water. The pH was adjusted to 7.4 with HCl. The final volume was adjusted to 1 litre by distilled water. The solution was sterilized by autoclaving and was stored at room temperature.

2. **Kovac's reagent**

5 g of para-dimethylaminobenzaldehyde was dissolved in 75 ml of amyl alcohol. Then concentrated HCl was added to make the final volume 25 ml. This reagent was covered with aluminium foil and stored at 4^oC.

3. **Methyl red reagent**

0.1 g of methyl red was dissolved in 300 ml of 95% ethyl alcohol. Then distilled water was added to make the final volume 500 ml. This reagent was covered with aluminum foil and stored at 4^oC.

4. **Barritt's reagent**

Solution A

5 g of alpha-naphthol was dissolved in 95% ethanol. This solution was covered with aluminium foil and stored at 4^oC.

Solution B

40 g of KOH was dissolved in distilled water. The solution became warm. After cooling to room temperature, creatine was dissolved by stirring. Distilled water was added. This solution was covered with aluminium foil and stored at

5. Oxidase reagent

100 mg of N,N,N^1,N^1-tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in 10 ml of distilled water and covered with aluminium foil. Then the solution was stored at 4^{o}C.

APPENDIX-III

Instruments

The important equipments used through the study are listed below:

Autoclave	SAARC
Freeze (-20°C)	Siemens
Incubator	SAARC
Micropipette (10-100µl)	Eppendorf, Germany
Micropipette (20-200µl)	Eppendorf, Germany
Oven, Model:MH6548SR	LG, China
pH meter, Model: E-201-C	Shanghai Ruosuaa Technology company, China
Refrigerator (4^{o}C), Model: 0636	Samsung
Safety cabinet Class II Microbiological	SAARC
Shaking Incubator, Model: WIS-20R	Daihan Scientific, Korea
Vortex Mixture	VWR International
Water bath	Korea
Weighing balance	ADAM EQUIPMENT™, United Kingdom