Study of the microbiological status of mineral drinking water of Dhaka city

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

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Declaration

I, hereby declare that the thesis work entitled "**Study of Microbiological Status of Mineral Drinking water of Dhaka City"** submitted to the Department of Mathematics and Natural Science, BRAC University in partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology is a record of work carried out by me under supervision and able guidance of my supervisors Dr. Mahboob Hossain associate professor and Co-ordinator of Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University .It is further declared that the research work presented here is original and the contents of this report in full or parts have not been submitted to any other university and institution for any degree or diploma.

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Dedicated to

My parents, All My Loved Ones

And

My supervisor

Dr. Mahboob Hossain

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ABBREVIATIONS

mg =Milligram

gm =Gram

L =Liter

p1 = Microliter

pH = Negative logarithm of hydrogen ion concentration

% =Percentage

spp.=Species

Conc. = Concentration

Cont. = Control

^{0}C = Degree centigrade

et al. = Associates

E .coli= Escherichia coli

Etc = Etcetera

Fig = Figure

HPC = Heterotrophic Plate Count

I = Intermediate

MCA = Mac Conkey Agar

ML = Microbiology Laboratory

Ml = Milliliter

MR = Methyl Red

NA = Nutrient Agar

No. = Number

R = Resistant

S = Sucrose

S = Susceptible

TCC = Total Coliform Count

TVC = Total Viable Count

VP = Voges-Proskauer

Vol. = Volume

- = Negative

+ = Positive

% = Percentage

WHO = World Health Organization

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Abstract

The objectives of this study were to assess the overall quality of mineral water quality samples that obtained from different sources in and around Dhaka city. For achieving the above mentioned objectives, standard questionnaire, methods of heterotrophic plate count (HPC) and total coliform count (TCC) were applied. Moreover, isolated colony from mineral water samples were characterized by using biochemical and antimicrobial susceptibility tests for *Staphylococcus aureus*, *S. intermedius, S. felis* and *S. saccharolyticus* were taken for antibiotic susceptibility test. Qualitative assessment of mineral water indicated that a good number of people preferred bottled water to tap water. HPC was found the lowest in some mineral water samples such as 1.0×10, 1.05×10^2, 2.0×10, 1.5×10, 1.05×10^2, 3.0×10 but also found the highest count in some mineral water samples. They were: 6.00×10^2, 6.40×10^2, 8.00×10^2, 1.50×10^2 and 5.50×10^2. Among all organism as per interested, *S. intermedius, S. aureus, S. felis* and *S. Saccharolyticus* were taken for antibiotic susceptibility test. In respect to antimicrobial susceptibility testing most of the *Staphylococcus spp.* isolates were susceptible to erythromycin, tetracycline, norfloxacin and ciprofloxacin. Furthermore, a few *Staphylococcus spp.* isolates were intermediate resistant to pencillin and oxacillin. However, most of the *Staphylococcus spp.* isolates were resistant to cefixime. The results indicate that mineral water serves as a reservoir of various bacteria and that people drinking the water might get diseases. This study emphasizes the need for elaborated microbiological examinations of mineral drinking water commonly used in Dhaka city.

CHAPTER 1

Introduction

1. INTRODUCTION

1.1Background

Water is essential to life, but many people do not have access to clean and safe drinking water and many die of waterborne bacterial infections. The consumption of mineral water has been substantially increasing all over the world in since decade (Doria, 2006). The increase also has happened in the countries where tap water is used as drinking water. The reasons could be attributed by the number of factors. The factors are primarily concerned with safety and health benefits. An adequate, safe and accessible supply must be available to all. Improving access to safe drinking-water can result in significant benefits to health. Every effort should be made to achieve a drinking water quality as safe as possible. Public and environmental health protection requires safe drinking water, which means that it must be free of pathogenic bacteria. According to the World Health Report (2002), every year more than 3.4 million people die as a result of water related diseases indicating these as the leading cause of disease and death around the world. In the disease-prone, humid, tropical region of Bangladesh, outbreaks of diarrheal diseases, often on an epidemic scale, are not unusual and the possible role of water-borne pathogens in these outbreaks has been emphasized. Among waterborne diseases of bacterial origin typhoid fever, bacillary dysentery and diarrhea are common in Bangladesh.

Despite the availability and promotion of the use of safe water sources, water-related diseases remain an important cause of mortality and morbidity in Bangladesh and it is suggested that intake of contaminated water acts an important mode of pathogen transmission. Even if disinfection is practiced in water supply systems, but failure of the disinfection system due to poor management could result in serious health hazards and post contamination. It is estimated that the 1% of drinking water is getting polluted with various organic and inorganic matters. The organic matters which are responsible for the contamination of water are fecal wastes of poultry and livestock farms, pesticides, herbicides, and many industrial wastes, minerals (including toxic metals such as lead, copper etc) and biological agents such as bacteria, virus, fungus, algae etc. Enterobacteriaceae in the water of river Manzanares at Cumana (Venezuela) and a high degree of enteric species of organisms was present in water (Mieres and Bastardo, 1975). Chemical contaminants of the most of the water sources are man-made. In urban area the major contaminants that contribute to chemical contamination of the water sources include industrial

effluents, sludge from sewage treatment plants, raw untreated sewage from urban populations and industry, suspended solids, biodegradable organics (proteins, carbohydrates and fats), pathogens (bacteria, virus, protozoa, helminthes), priority pollutants (highly toxic chemicals), refractory organics (pesticides, phenols, surfactants), heavy metals and dissolved in organics (nuisance chemicals). In rural area water pollution is also caused by silts and other suspended solids such as soil, wash off plowed fields, agriculture run-offs, and eroded river banks when rains, sewage and wastes of houses. In China, about 90% of surface waters and over 60% of drinking water sources in urban areas have been polluted by different extents of organic substances, ammonia nitrogen, phenols, pesticides and pathogenic microorganisms (Wang *et al.,* 2000). In South Asian countries, rivers such as in the Kathmandu valley, the Yamuna River at Delhi, and peripheral rivers (mainly Buriganga River) of Dhaka was more severely polluted by urban activities destined to unplanned urbanization and industrialization, inadequate sewerage, and lack of effective pollution control measures (Karn *et al.,*2001).

With over 70 percent of the planet are covered by ocean but these bodies of water are now polluted in many ways. This contaminated water is used for human being, poultry, and livestock consumption without treatment and causes various problems and diseases. The role of water as a carrier of disease producing agents was not recognized before the middle of nineteenth century and prior to the establishment of bacteriology as a science. Drinking of this contaminated water causes various enteric diseases of human, poultry and livestock. It is therefore essential to ensure the supply of safe drinking water for poultry and livestock where one fifth of populations of this country are lacking in safe drinking water.

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 diarrhea, dysentery and cholera (Pelezar and Reid, 1978). Water borne diseases constitute a major health burden in Bangladesh. According to Bangladesh health and injury report on children under 5 in 2005, children die every year from diarrhea (Bangladesh Health and Injury Survey Report, 2005). In the humid, tropical region of Bangladesh, outbreaks of diarrheal diseases, often on an epidemic scale, are not unusual and the possible role of waterborne pathogens in these outbreaks has been emphasized (Khan et al., 1992). Water can contain and support very large and genetically diverse bacterial populations that may include pathogenic

strains. The release of bacteria with resuspended sediments can create elevated concentrations of bacteria in water far above regulatory thresholds. Current microbial water quality models that are used to support management decisions ignore this bacterial input by sediment. It has been known for some time that substantial populations of fecal-coliforms and *E coil* are harbored in fresh water both in surface and ground water. However, the relative importance of sediments as bacterial habitats and as a source of water-borne fecal-coliforrns and *E. coil* has not been recognized until recently when a large number of publications have shown that in many cases the resuspension of sediment, rather than runoff from surrounding lands, can create elevated *E. coli* concentrations in water. The survival of enteric bacteria in aquatic ecosystems has received considerable attention because of the dangers that pathogenic members of the group pose to humans.

The present study was conducted to identify fecal coliforms and pathogenic bacteria e.g. *Escherichia coli*, *Enterobacter*, *Staphylococcus aureus*, *Pseudomonas and Vibrio species (V. cholerae*, *V. parahaemolyticus*, *V. mimicus* and *V.alginolyticus*) from mineral water in Dhaka city. The detection of pathogenic bacteria in water will help in controlling water borne infection in this region. 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. It was estimated that nearly 1.5 billion people lack safe drinking water and that at least 5 million deaths per year can be attributed to waterborne disease.

In Bangladesh, people are mostly illiterate and unconscious about their health and hazards. This is because of the poor level of literacy among the people. Most of the people live in village where there is not much arrangement to make them aware about their health hazards. Therefore, religious and cultural beliefs make the health of these people more hazardous. On the other hand, people living in the city are quite conscious about their health's and hazards. They know the benefits of precautions taken for maintaining good health. These precautions are primarily concerned with food and drinking water. In Bangladesh, drinking water is available in two forms. One form is bottled water and other form is non-bottled water.

Bottled water entered into the market of Bangladesh after 1988 flood when hepatitis broke out widely and people began to regard tap water as unsafe. Research shows that the people living in the city have some general and specific beliefs on health benefits related to bottled drinking water. The beliefs are bottled water is purer than tap water, health benefits of bottled water are

substantial, bottled water is properly processed etc. Therefore, people living in the city are much serious about the use of bottled drinking water in Bangladesh.

Health effects associated with water supplies in developing countries are evaluated to be based on four bacterial indicators of tropical drinking-water quality (fecal coliforms, *Escherichia coli*, *Enterococci* and fecal *Streptococci*) and their relationship to the prevalence of diarrheal disease in Cebu, Philippines (Moe *et al.*, 1991).The contaminated water or inadequate supply of safe drinking water causes various gastrointestinal diseases like diarrhea, dysentery and water-borne diseases like cholera, typhoid. It is now evident that most of the enteric diseases of human and animals are transmitted through contaminated food and water (Johnson *et al.,* 2003). So to get rid from suspended biological agents and to ensure the supply of pure drinking water, water must need prior treatment or purified before consumption. From this view point of public health, it is highly imperative that potable water supply system should be safe.

Water may be polluted at its sources by excreta or sewage, which is almost certain to have pathogenic microorganisms. Potable water system can become polluted with coliform and pathogenic bacteria due to lack of hygiene and sanitation. As a result, microbiological examination of water should routinely be carried out to monitor and control the quality and safety of drinking water. Although the concept of safe water is under consideration in Bangladesh, unfortunately science based little information is available. Therefore, the present study was conducted to determine the quality (both qualitative and bacteriological assessments) of bottled water available in Dhaka city.

1.2 Literature review

1.2.1 Bottled Water Industry in Bangladesh:

Mordor Intelligence (August, 2015) has the world's most inclusive research on the Bottled Water market industry in Bangladesh. It is a fast growing sector and an important source of earnings for the nation. Domestic Bottled Water Market focuses on the development and enhancement of plants, factories and market (storage and distribution) in the country. There have been remarkable progresses in the field of Bottled Water in the past 5 years. Bangladesh's focus on

improving water facilities have resulted in growth of Bottled Water in the country. Changing inhabitant's perception, travelling and need of treated water in the country have impacted the Bottled Water industry in positive ways.

1.2.2 Sources of pollution and its effects on mineral water:

Bottled water used by the people of developing countries like Bangladesh is also not free from germs and contamination. A study was conducted in Bangladesh on four brands of bottled water regarding the quality of the water and it found that all four brands were judged to be unsatisfactory by accepted health standards (Khan et al., 1992). About 80% of the total tube-wells pump underground drinking water that is contaminated with arsenic. Surface water is also not free from arsenic. Specific beliefs of the people are concerned with plastic bottles (Ward et al., 2009). The bad side of the bottled water is that in the developing countries bottled water is contaminated by the bacteria quite frequently even there is assurance on the label and thus it is to be boiled before drinking (Masaak and Hiroaki, 1998). Study also showed that it is safer to consume filtered drinking water after filtration system and boiled than tap source water (Chan et al., 2007). But all those plastic bottles use a lot of fossil fuels and pollute the environment.

1.2.3 Microbial population on bottled water:

Over the past two decades the consumption of bottled water, commonly known as "mineral water" has increased substantially in Bangladesh. The most significant impetus for this phenomenon can be attributed to the frequent outbreaks of diarrheal and other diseases resulting from microbial and chemical contamination of drinking water sources (Chakraborti *et al.*, 2010; Islam *et al.*, 2006). A common perception is that bottled water is safe for consumption. This is largely because bottled water is marketed as "pure and clean" by manufacturers, and therefore, consumers prefer bottled water over other drinking water sources. Bottled water has also been marketed as ideal for infants and immune compromised individuals in order to avoid to exposure to potential pathogens which are detrimental to human health (Warburton *et al.*, 1992).

Despite the perceived purity, the microbiological quality of bottled water has been questioned over the years (Kassenga 2007, Rosenberg 2003). Several research studies have reported the

presence of fecal indicator and heterotrophic bacteria with levels exceeding drinking water guidelines (Bartram *et al.*, 2004; Kassenga 2007;Svagzdiene *et al.*, 2010). Potential pathogens such as *Aeromonas* spp. (Venieri *et al.*, 2010), *Staphylococcus aureus* (Leclerc *et al.*, 1995), *Pseudomonas* spp. (Svagzdiene , 2010), *Shigella* spp. (Khan *et al.*, 1992), *Salmonella* spp. (Warburton *et al.*, 1994), *Vibrio cholerae* (Blake *et al.*, 1997) have been detected in bottled water. Local newspapers in Bangladesh too, have expressed their concerns that some brands of bottled water may not be safe for consumption (Jamir 2009). There are suspicions that, with some exception, the bottles are filled with water of unsatisfactory quality. Unfortunately, no comprehensive study has been conducted to date to determine the quality of the bottled water being marketed in Bangladesh.

Pathogenic microorganisms in bottled water can multiply during storage and can reach a level which can be detrimental to consumers (Korzeniewska *et al.*, 2005; Messi *et al.*, 2002). For example, *Escherichia coli*, *Pseudomonas* spp., and *Salmonella* spp. have been demonstrated to survive and multiply in bottled water (Warburton *et al.*, 1994). To ensure that bottled water is safe for drinking, quality standards have to be strictly enforced. According to the European Community Directive (European Community 1980), total coliforms *E. coli*, *Enterococcus* spp., *Pseudomonas aeruginosa* and parasites should not be detected in 250 mL of bottled water, whereas, World Health Organization (WHO 2004) recommends the number of fecal coliforms should be zero in water used for drinking (WHO 2004).

1.2.4 Pathogens of mineral water

Some of the major bacterial pathogens spread via contact with contaminated water. The sources of contamination are most often animal or human focal wastes or infected humans and other animals. Fresh water can serve both as reservoir for the pathogenic bacteria and as a vehicle of transmission of disease (Benenson, 1995). Water is also a natural reservoir of common bacteria, water-borne human pathogens such as *Shigella* species. *Salmonella* species, *E. coli* 0157:H7, enterotoxigenic *E. coli*, *Vibriocholerae*01, and *Vibrio parahaemolyticus* (APHA, 1992) and opportunistic pathogens such as *Aeromonas hydrophila* and *Pseudomonas aeruginosa* (Auerbach et al., 1987; Graevenitz, 1985).

Escherichia coli

Escherichia coli is present in large numbers in the normal intestinal flora of humans and animals, where it generally causes no harm. However, in other parts of the body, *E. coli* can cause serious disease, such as urinary tract infections, bacteraemia and meningitis. A limited number of entero pathogenic strains can cause acute diarrhea. Several classes of enteropathogenic *E.coli* have been identified on the basis of different virulence factors, including enterohaemorrhagic *E.coli* (EHEC), enterotoxigenic *E. Coli* (ETEC), enteropathogenic *E. Coli* (EPEC), enteroinvasive *E. Coli* **(**EIEC). EHEC serotypes, such as *E. coli* 0157:H7 and *E. coli* 0111, cause diarrhoea that ranges from mild and non-bloody to highly bloody, which is indistinguishable from haemorrhagic colitis. ETEC produces heat-labile or heat-stable enterotoxin, or both toxins simultaneously, and is an important cause of diarrhea in developing countries, especially in young children. Symptoms of ETEC infection include mild watery diarrhea, abdominal cramps, nausea and headache. Infection with EPEC has been associated with severe, chronic, non-bloody diarrhea, vomiting and fever in infants. EIEC causes watery and occasionally bloody diarrhea where strains invade colon cells by a pathogenic mechanism similar to that of *Shigella* (Nataro and Kaper, 1998). Humans are the major reservoir of EPEC, ETEC and EIEC strains. On the other hand, livestock, such as cattle, sheep, goats, pigs and chicken are a major source of EHEC strains. Infection is associated with person-to-person transmission, contact with animals, food and consumption of contaminated water. Person-to-person transmissions are particularly prevalent in communities where there is close contact between individuals, such as nursing homes and day care centers (O'Connor et al., 2002). Recently, a fifth category of *E. coli*, recognizable by its aggregative or "stackedbrick" type of adherence to cultured mammalian cells, has been recognized as yet another category of diarrhea genic *E. coli* in children in different parts of the world. Because of its characteristic aggregative type of adherence, this *E. coli* has been referred to as entero aggregative *E. coli* (EAggEC). The diagnosis of EAggEC is dependent upon cell culture adherence assays, both of which are expensive and cumbersome and are beyond the reach of the majority of clinical laboratories. Recently a research in Thailand showed that EAggEC forms bacterial clump which is visible as a thick scum in a liquid culture e.g. Mueller-Hinton broth, nutrient broth or Luria broth (Nataro and Kaper, 1998).

Vibrio spp.

The genus Vibrio is in the family *Vibrio naceae*. Members of the *Vibrio* genus are straight or curved Gram-negative, non spore-forming rods. *V.vulnificus*is similar phenotypically to *V. parahaemolyticus.* All *vibrios* are ubiquitous in the marine environment. There are 30 species in the genus *Vibrio*; thirteen of these are pathogenic to humans, including *V. cholerae*, *V.mimicus* , *V. fluvialis*, *V.parahaemolyticus*, *V. alginolyticus*, and *V. vulnificus*, all of the pathogenic. *Vibrios* have been reported to cause foodborne and waterborne diseases, although *V. cholera* 01, *Vibrio parahaemolyticus*, and V. vulnificus are considered the most significant agents (Atlas, 1997).

Staphylococcus spp.

The *Staphylococcus* genus includes at least 40 species. Of these, nine have two subspecies and one has three subspecies. Most are harmless and reside normally on the skin and mucous membranes of humans and other organisms. Members of the *Staphylococcus* frequently colonize the skin and upper respiratory tracts of mammal sand birds. Some species specificity has been observed in host range, such that the *Staphylococcus* species observed on some animals appear more rarely on more distantly related host species. *Staphylococcus* can cause a wide variety of diseases in humans and animals through either toxin production or penetration. *Staphylococcal* toxins are a common cause of food poisoning, as they can be produced by bacteria growing in improperly stored food items. The most common sialadenitis is caused by *staphylococci*, as bacterial infections.

Pseudomonas spp.

Pseudomonas is a genus of Gram-negative, aerobic gammaproteobacteria, belonging to the family Pseudomonadaceae containing 191 validly described species. The members of the genus demonstrate a great deal of metabolic diversity, and consequently are able to colonise a wide range of niches. Their ease of culture *in vitro* and availability of an increasing number of *Pseudomonas* strain genome sequences has made the genus an excellent focus for scientific research; the best studied species include *P. aeruginosa*in its role as an opportunistic human pathogen, the plant pathogen *P. syringae*, the soil bacterium *P. putida*, and the plant growth-promoting *P.Fluorescens*. Infectious species include *P. aeruginosa*, *P. oryzihabitans*, and *P.plecoglossicida*. *P. aeruginosa* flourishes in hospital environments, and is a particular problem

in this environment, since it is the second-most common infection in hospitalized patients (nosocomial infections) (Zam *et al., 2010*). This pathogenesis main part be due to the proteins secreted by *P. aeruginosa*. The bacterium possesses a wide range of secretion systems, which export numerous proteins relevant to the pathogenesis of clinical strains.

The review of literatures related to the present study is briefly presented under the following headings:

1.2.5. Qualitative assessment of bottled water samples

Majumder *et al*., (2011) stated that bottled water has become one of the most popular drinks in Bangladesh. Nowadays it is easily available in the market. Due to lack of confidence on municipal supply water, people now prefer to drink bottled water instead of tap water, mostly when they are outside the home, and in many occasions. The results also revealed the ill performance and poor drinking water quality of the purification systems of the investigated water samples. 28% of the respondents considered that the quality of bottled water was satisfactory, in contrast to the 26% of the respondent, who judged that the quality of bottled water was not that satisfactory. However, the highest (34%) number of the respondents did not know whether the quality of the bottled water is good or not satisfactory. Recommendation was suggested for new treatment systems of the investigated suspicious water to prevent human illness.

1.2.6 Microbiological assessment of water samples from different sources

Zam *et al., (*2010) stated that, microorganisms are ubiquitous in the environment Drinking water along with food, air and soil is one of the numerous possible sources of microbes. This project focused on the level of heterotrophic microorganisms in bottled drinking water. Regulatory bodies such as Food and Drug Administration (FDA), Environmental Protection Agency (EPA), World Health Organization (WHO) and Health Canada do not specify a maximum allowed limit for the heterotrophic bacteria counts in bottled drinking water available in the market. However, according to the United States Pharmacopeia (USP) not more than 500 CFU/mL of microbial contaminants should be present in the water used for drinking. In this study, different brands of

packaged water (from 0.5L plastic bottles to 20L carboys) were analyzed for their microbiological quality, using different culture media. Heterotrophic microbiological count varies between less than 10 and 72,000 CFU/mL for ten different brands of bottled water. Whereas, the average heterotrophic microbial count for the tap water and USP water samples was 170 CFU/mL and less than 10 CFU/mL respectively. Morphological studies indicated the presence of five different kinds of colonies in the bottled water samples. There were no cases of fecal contamination or the presence of *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella*. Bottled water is not expected to be free from microorganisms but the CFU observed in the samples was surprisingly high which indirectly reflects the poor sanitary practices during the packaging of the product. Since the significance of non-pathogenic heterotrophic microorganisms in relation to health and diseases is not entirely understood, there is an urgent need to establish a maximum limit for the heterotrophic count in the bottled water that should be tested and regulated periodically.

Okagbue *et al.,* (2002) analyzed sixty processed bottled water samples supplied by three companies in Zimbabwe, for microbial safety. The authors observed that four (6.7%) and seven (11.7%) samples to exceeded the recommended maximum level of total viable and coliform counts, respectively.

Malaney and Weiser (1962) isolated coliform bacteria from pond water of Central North, Central Southeastern and Southwestern Ohio.

Kroger and Noll (1969) cultured water samples of 180 wells and isolated high proportion of *E. coli* with other members of family Enterobacteriaceae.

Lin *et al.,* (1974) conducted a bacteriological study of spoon river water in order to determine its hygienic quality. They found 200 coliforms / 100 ml water.

Cooper (1975) isolated different species of *Salmonella* organisms from wastewater of domestic origin. In evaluating the public health impact of wastewater reuse it was imperative that the sanitarians should have knowledge about the occurrence of these agents.

Dragas and Tratnik (1975) examined 1950 samples of drinking water and swimming pool for the presence of enteropathogenic *E. coli*. They observed that 21.5% of water samples were microbiologically unsafe and contained a high MPN of *E. coli*.

Mieres and Bastardo (1975) studied the presence of enterobacteria in the water of the river Manzanares in Venezuela. They found a higher percentage of enteropathogenic organisms in water.

Badge *et al.*, (1982) stated that the growth of aquatic microorganism was affected by a great variety of physical and chemical factors. The authors observed that the rate of change in bacterial density in water was related to a variety of factors such as (a) assailable organic nutrient in water (b) organisms competing for dominance in the microbial flora, (c) water pH and (d) storage temperature.

Ho *et al.*, (1988) reported that, in countries with well developed infrastructures, infectious diarrhea from water borne sources still remains a problem. It has been estimated that 200,000 children are hospitalized annually in the US alone at a cost of $1 bn.

Bern *et al.*, (1992) concluded that contaminated drinking water causes a large percentage of diarrheal disease globally. They observed that children under five experienced about one billion episodes of diarrheal disease each year, with over 3.3 million deaths.

Edberg *et al*., (1996) conducted to determine the numbers and types of bacteria found in three water sources-bottled water, water cooler water, and tap water-and to determine their virulence characteristics. A wide variety of water types were collected and each was analyzed for Heterotrophic Plate Count (HPC) bacteria, *Pseudomonas aeruginosa,* and total coliforms. For each isolate, virulence characteristics were determined by enzyme analysis (10 associated with virulence), antibiotic susceptibility testing (natural and semi-synthetic antibiotics), and acid liability (survival at pH 3.5) .and cytotoxicity testing (HEp-2 cells). Results showed that all water sources had a normal bacterial content. Only 2 per cent of bottled water sources had *P. aeruginosa.* Total coliforms were isolated only from bottled water that used mixed (water alternating with milk) filling lines. Environmental bacteria did not produce significant enzymes associated with virulence, were not acid resistant, were susceptible to semi-synthetic antibiotics, and did not produce appreciable cytotoxicity. These natural aqueous bacteria were adapted to a water environment, did not grow well at conditions analogous to the human host, and did not have the characteristics associated with virulence. Future drinking water revisions and changes to the treatment processes should be directed towards the elimination of specific pathogens and to the prevention of exogenous sources of contamination rather than the elimination of natural water microbial populations.

Sundstrom and Herberk (1997) stated that from time immemorial, water has been both man's benefactor and his curse. The authors considered water as a renewable resource delineating its supply, consumption, quality and analysis.

Karn *et al.,* (2001) stated that in South Asian countries such as Nepal, India, and Bangladesh, pollution of river water was more severe and critical near urban stretches due to huge amounts of pollution load discharged by urban activities. The Bagmati River in the Kathmandu valley, the Yamuna River at Delhi, and peripheral rivers (mainly Buriganga River) of Dhaka suffer from severe pollution these days. They observed that during dry season average of biochemical oxygen demand (BOD) in all these rivers is in the range of 20-30 mg/liter and total coliform are as high as 104-105 MPN/100 ml.

Kassenga (2007) reported that the consumption of bottled and plastic-bagged drinking water in Tanzania had increased largely because of the deteriorating quality of tap water. It is uncertain whether these water products are safe for drinking. In this study, the microbiological quality of bottled and plastic-bagged drinking water sold in Dar es Salaam, Tanzania, was investigated. One hundred and thirty samples representing 13 brands of bottled water collected from shops, supermarkets and street vendors were analyzed for total coliform and fecal coliform organisms as well as heterotrophic bacteria. These were compared with 61 samples of tap water. Heterotrophic bacteria were detected in 92% of the bottled water samples analyzed. Total and fecal coliform bacteria were present in 4.6% and 3.6%, respectively, of samples analyzed with a tendency for higher contamination rates in plastic-bagged drinking water. Microbiological quality of tap water was found to be worse compared with bottled water, with 49.2% and 26.2% of sampling points showing the presence of total coliform and fecal coliform organisms, respectively. The results suggest caution and vigilance to avert outbreaks of waterborne diseases from these types of drinking water.

Sultana *et al., (*2009) studied the whole concepts adopted for microbiological quality is that no water intended for human consumption shall contain *E. coli* in 100 ml sample. Our study was aimed to investigate the extent of bacterial contamination among protected and unprotected water sources and potable quality of drinking water was assessed from seven (07) selected areas in Khulna city, Bangladesh. The experiment was conducted during May-June 2007. Water from the mains (pump water) and corresponding residences (households) were tested parallel for the

presence of coli form organism. Analysis of bacteriological quality of pump water and household's water showed that 36.36% pump water and 42.86% of household water were contaminated with fecal-coliform and coliform of non-fecal origin. Our study concludes that 71.43% drinking water sources of Khulna city are unsafe & not potable. We suggest that regular quality control measures to be adopted to ensure safe drinking water.

Cabral (2010) stated that water is essential to life, but many people do not have access to clean and safe drinking water and many die of waterborne bacterial infections. In this review a general characterization of the most important bacterial diseases transmitted through water—cholera, typhoid fever and bacillary dysentery— is presented, focusing on the biology and ecology of the causal agents and on the diseases' characteristics and their life cycles in the environment. The importance of pathogenic *Escherichia coli* strains and emerging pathogens in drinking water transmitted diseases is also briefly discussed. Microbiological water analysis is mainly based on the concept of fecal indicator bacteria. The main bacteria present in human and animal feces (focusing on their behavior in their hosts and in the environment) and the most important fecal indicator bacteria are presented and discussed (focusing on the advantages and limitations of their use as markers). Important sources of bacterial fecal pollution of environmental waters are also briefly indicated. In the last topic it is discussed which indicators of fecal pollution should be used in current drinking water microbiological analysis. It was concluded that safe drinking water for all is one of the major challenges of the 21st century and that microbiological control of drinking water should be the norm everywhere. Routine basic microbiological analysis of drinking water should be carried out by assaying the presence of *Escherichia coli* by culture methods. Whenever financial resources are available, fecal coliform determinations should be complemented with the quantification of *enterococci.* More studies are needed in order to check if ammonia is reliable for a preliminary screening for emergency fecal pollution outbreaks. Financial resources should be devoted to a better understanding of the ecology and behavior of human and animal fecal bacteria in environmental waters.

Islam *et al*., (2010) studied the bacteriological quality of treated water of different sources .It was determined by presumptive coliform count. In source-wise distribution of samples, 50% of mineral water, 87.5% of filtered water and 100% of tap water samples were exceeded the drinking water guideline value of WHO. Microorganisms in tap water comprised *Escherichia coli* spp. (60%), *Klebsiella*spp. (40%), *Enterobacter*spp. (20%), *Pseudomonas* spp. *(70%),*

Proteus spp. *(10%), Staphylococcus* spp. *(40%)* and *Salmonella* spp. (0%). Furthermore, there was no correlation between faecal coliform and the presence of Salmonella species. Results obtained from this investigation revealed that municipal tap water of Dhaka city was contaminated with a number of enteric bacteria such as *E. coli.* This organism was considered as a good bio indicator model for surveillance studies of antimicrobial resistance. So, only antibiotic resistance pattern of *E. coli* was determined .

Moniruzzaman*et al.*, (2011) analyzed the microbiological status of water from dispensers in different roadside and restaurants of Dhaka city and Savar area. Seven samples from Dhaka and 8 samples of Savar were checked .The heterotrophic plate count was in a range of 1.0x103CFU mL-1 to 2.0x104 CFU mL-1 (from new bottles), 1.0x103 to 1.5x104 CFU mL-1 (after dispensation) and 1.5x103 CFU mL-1 to 1.0x105 CFU mL-1 (from serving glass). In several of the samples, the HPC was higher than the count in water from new bottle or after dispensation, suggesting added contamination from the serving glass. 80% of the samples were contaminated with total and fecal coliform bacteria, which render these waters unacceptable for human consumption. The sample were found to contain gram negative bacteria like *E. coli ,Shigella* spp*, Klebsiella* spp*, Enterobacter* spp, *Pseudomonas* spp and *Salmonella* spp , which are potential pathogens and thus pose a serious threat to public health. This study elucidates the importance of monitoring the bottling companies and the restaurants and put them under strict regulations to prevent future outbreak of any water borne diseases caused by consumption of dispensed water.

Nazir*et al.*, (2005) found out the effective antibiotic(s) against *Escherichia coli* and to observe the relationship between the plasmids to the antibiotic resistant pattern found by antibiotic sensitivity tests. For these forty water samples were collected from different sources for isolation and identification of pure *E. coli*. The overall recovery rate of *E. coli* from water samples was 45%. The pure cultures were subjected to observe the antibiotic resistant pattern by commonly used ten antibiotic disks. All the isolates were found resistant to Penicillin G (94.45%) but 50% isolates were resistant to Amoxicillin. The isolates were highly sensitive to other antibiotics as Ciprofloxacin (88.89%), Chloramphenicol (72.22%), Norfloxacillin (88.33%) and Tetracycline (61.11%). The isolates exhibited moderate sensitivity to Ampicillin (44.44%), Gentamicin (77.78%) and Streptomycin (3.33%).

1.3 Water Quality Parameters

Contamination of water-bodies is a major concern in today's era. The biological wealth of a water body is mainly dependent on its water quality and it is of major issue of concern to mankind toady. Decrease in water quality (unfit for human consumption) is also attributed to the fact that today most water bodies are been loaded with toxic material and chemicals, human and industrial waste, organic matter and religious rituals of Idol immersions. Twenty Water samples were collected from different area of Dhaka city and were analyzed for various water quality parameters such as pH, conductivity and following standard methods.

1.4 Aims and objectives of the present study

Due to increased demand and consumption of bottled water in Dhaka, there has been a growing concern about the microbiological quality of this product. The present study has been undertaken with the following objectives:

1. To find out the microbial growth estimation of the mineral drinking water samples of different brands available in Dhaka city.

2. To assess the bacteriological quality of bottled water samples to know about the safety

CHAPTER 2

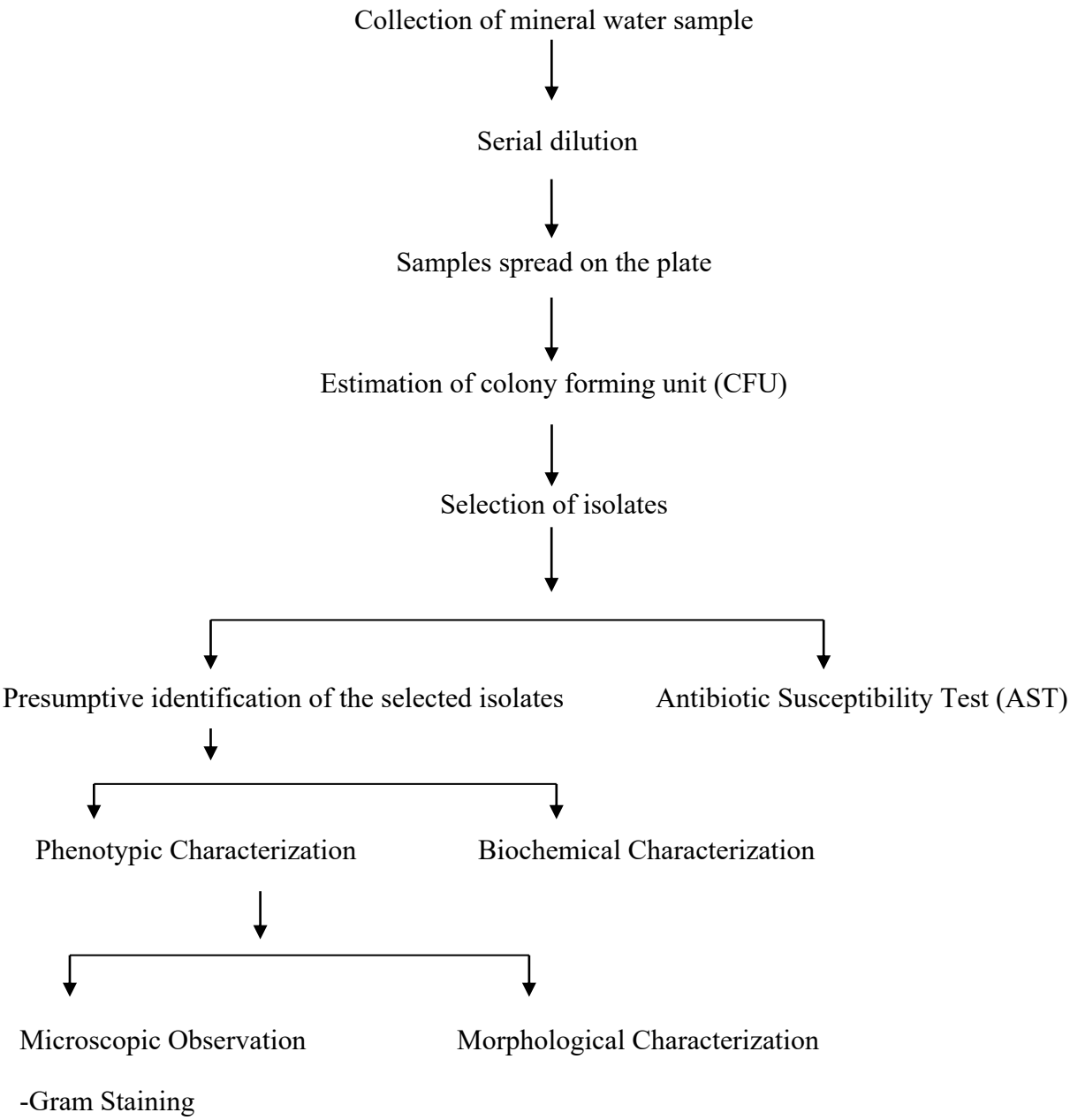
Materials and Methods

2. Materials and Methods

2.1 Study place

This research work was carried out at the Microbiology, Biotechnology and Molecular Biology Laboratory of the Department of Mathematics and Natural Sciences of BRAC University.

2.2 Flow Diagram of the Study Design



2.3 Collection of Samples

Ten Mineral water samples were collected from different places of Dhaka city from October 2015 to January 2016. The samples were aseptically collected from many shops. Samples were labeled in the field and transported to the laboratory and were processed in the Laboratory of Microbiology, BRAC University within 3 hours of collection. Then different physicochemical parameters (PH and conductivity) were measured by using PH meter and conductivity meter.

2.4 Isolation of bacteria water sample

Three different selective agar media MacConkey, XLD and M-FC agar media were used for isolation of *Escherichia coli*, *Enterobacter*, *Staphylococcus aureus*, *Pseudomonas* and Vibrio species respectively. Nutrient agar media are used for total heterotrophic count of organisms.0.2microlitre and 10^{-1}dilutions of the samples were spread on Nutrient agar plate and different selective media. All the plates were then incubated at 37 °C for 20 to 36 hours. M-Fc plate was incubated in 45°C. After incubation, every plate was observed carefully. According to 'Microbiological Laboratory Manual' by Cappuccinos and Sherman(1999), colony morphology of various isolates were examined and recorded on the basis of size, form, pigmentation, margin ,elevation and opacity.

2.5 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 (Doetsch, 1981). The size, shape, arrangement and Gram reaction properties of isolates were carefully observed.

2.6 Biochemical characteristics of the isolates

According to 'Microbiological Laboratory Manual' by Cappuccion and Sherman (1999), following biochemical tests were performed for the identification of bacteria.

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. 3% H_2O solution was added to each of the slides and a portion of the bacterial colony was mixed with it. Production of bubble indicates presence of catalase enzyme in the bacteria.

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 tetramiethyl-p-phenylene diaminedi hyrochioride). Positive test is indicated by the presence of dark purple color within 10 seconds.

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 showm in the butt either by the formation of bubbles or cracking of the agar.

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 for 18-24 hrs at 37°C, 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 has been was hydrolyzed which indicates indole positive reaction.

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 coloration of the medium. Citrate negative was identified by no growth and the green coloration of the medium.

Methyl Red (MR) and Voges-Proskaur (VP) tests

MR-VP broth was inoculated and incubated at 37 °C for 48 hours. After incubation, the broth culture was divided into two portions. In one portion, methyl red reagent was added. A distinct red colour and yellow colour indicate positive and negative reaction respectively. In the other portion of the broth, Barritt's reagent A (6% a-naphthol) and B (40% KOH) were added and formation of pink color indicates positive reaction.

Nitrate Reduction Test:

The nitrate reeducates test is a test to determine the ability or inability of some microorganisms to reduce nitrate (NO_3^-) to nitrite (NO_2^-) or beyond the nitrite stage using anaerobic respiration. Inoculate nitrate broth with a heavy growth of test organism using aseptic technique. After that, incubated at an appropriate temperature for 24 to 48 hours. Then Added 5 drops of reagent A and 5 drops of reagent B to each broth. If Nitrate Reduction Positive: (Red after sulfanilic acid + alpha-naphthylamine; no color after zinc)or Nitrate Reduction Negative: (No color after sulfanilic acid + alpha-naphthylamine followed by Red after zinc).

Gelatin hydrolysis test

The gelatin hydrolysis test detects the ability of bacteria to produce gelatinases. This test aids in the identification of *Serratia*, *Pseudomonas*, *Flavobacterium*, and *Clostridium*. It distinguishes the gelatinase positive, pathogenic*Staphylococcus aureus* from the gelatinase negative, nonpathogenic *Staphylococcus epidermidis* . Gram-positive, spore-forming, rod-shaped, aerobic or anaerobic bacteria such as Bacillus *anthracis*, *B.cereus*, *B.subtilis*, *Clostridium perfringens* and *C. tetani* are also positive for gelatin hydrolysis. The test can be used to differentiate genera

of gelatinase-producing bacteria such as *Serratia* and *Proteus* from other members of the family *Enterobacteriaceae*.

Blood agar test:

This test provides information on what hemolytic enzymes a bacterium possesses. By providing a culture medium enriched with red blood cells, it is possible to determine whether a bacterium can destroy the cells and whether it can digest the hemoglobin inside. A nutrient medium augmented with the addition of 5% sheep blood routinely is used. Incubate for the appropriate length of time. For this test 24 hours is sufficient. Observe the medium surrounding colonies in the plate. If the culture showing a darkening or discoloration of the medium in the vicinity of growth demonstrates then it is alpha-hemolysis. If the cultures showing clear halos around colonies and under growth then it is exhibiting beta-hemolysis.the result can be gamma hemolysis.

Casein hydrolysis test (plate):

Casein hydrolysis test was done to determine whether the bacteria produce the exoenzyme casesase and hydrolyse enzyme. The whole procedure and the used equipments should be sterilized. After inoculated the organism on the plate either a straight line or a zig-zag it was incubated at 25° or 37° C. For result hold the plate up to the light to see the zones. If the result is Positive reactions may be recorded as strong +ve or weak +ve reactions. There is no reagent or indicator in the agar. A zone of clearing around the growth area identifies the presence of the enzyme caseinase.

Starch hydrolysis:

The purpose was to see if the microbe can use *starch*, a complex carbohydrate made from glucose, as a source of carbon and energy for growth. Use of starch is accomplished by an enzyme called alpha-amylase. Inoculum from a pure culture is streaked on a sterile plate of starch agar. The inoculated plate is incubated at 35-37 °C for 24 hours. Iodine reagent is then added to flood the growth. Presence of clear halos surrounding colonies is positive for their ability to digest the starch and thus indicates presence of alpha-amylase. Iodine reagent is added after incubation to flood the surface of the plate. The dropper was placed above the plate and adds the reagent to the culture. The starch in the plate is changed to blue-brown by the iodine reagent. Areas where starch has been digested by bacterial growth exhibit clear halos in the midst of the dark plate, indicating a positive alpha-amylase, or starch hydrolysis test. Plates were containing bacteria without alpha-amylase was uniformly dark, a negative result.

Mannitol salt agar:

Mannitol salt agar contains the sugar mannitol. As with the other sugar fermentation tests, we are able to determine the ability of a microbe to metabolize mannitol by observing color changes of the pH indicator phenol red in the medium. If the microbe metabolizes mannitol, phenol red is changed to yellow by the acidic products. Mannitol salt agar is thus selective in prohibiting the growth of bacteria unable to tolerate 7.5% NaCl and differential in the use of mannitol by those that can grow. Most frequently, it is used to select for Gram positive cocci, particularly Staphylococci.

2.7 Antimicrobial Susceptibility

Susceptibility and resistance of different antibiotics was measured in vitro by employing the Kirby-Bauer method (Bauer *et al.,* 1996). This method allowed for the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that resulted from diffusion of the agent into the medium surrounding the disc. A suspension of test organism was prepared in nutrient broth by overnight culture for 24 hours at 37 ºC. The broth were streaked using by sterile glass spreader homogenously on the medium. Antibiotic disc were applied aseptically to the surface of the inoculated plates at an appropriate special arrangement with the help of a sterile forceps on Mueller-Hinton agar plates. The plates were then inverted and incubated at 37º C for 24 hours. The diffusion discs with antimicrobial drugs were placed on the plates and incubated for 24 hours at 37 ºC. The antibiotics discs used were: erythromycin (15 µg), pencillin(10 µg),doxycycline(30 µg),vancomycin(30 µg), oxacillin(1 µg),trimethoprim-sulfametnoxazole(25 µg),gentamicin(10 µg), tetracyclin(30 µg), chloramphenicol(30 µg),moxifloxacin(5 µg), norfloxacin (10 µg),nitrofurantion(300 µg), ,ciprofloxacin (5 µg) ,rifampin(5 µg),cefixime(5 µg),minocycline(30 µg),levofloxacin(5 µg),clindaycin(2 µg). Sterile glass spreader was used to spread the culture homogenously on the medium. Antibiotic disc were applied aseptically to the surface of the inoculated plates at an appropriate special arrangement with the help of a sterile forceps. The plates were then inverted and incubated at 37°C for 24 hours. After incubation, the plates were examined and the diameters of the zone of complete inhibition were observed. Here is the table of antibiotics and diameter of zone inhibition:

Table2.1: list of antibiotics and diameter of zone inhibition.

Antibiotics name	Disc concentration	Diameter of zone of Inhibition		
		Resistant <or = nm	Intermediate nm	Susceptible = or>nm
1.Erythromycin	15 µg	≤13	14-22	≥23
2.Pencillin	10 µg	≤19	20-27	≥28
3.Vancomycin	30 µg			≥15
4.Trimethoprim-sulfamethnoxazole	25 µg	≤10	11-15	≥16
5.Gentamicin	10 µg	≤12	13-14	≥15
6.Oxacillin	1 µg	≤10	11-12	≥13
7.Tetracycline	30 µg	≤14	15-18	≥19
8.Chloramphenicol	30 µg	≤12	13-17	≥18
9.Moxifloxacin	5 µg	≤20	21-23	≥24
10.Norfloxacin	10 µg	≤12	13-16	≥17
11.Nitrofurantion	30µg	≤14	15-16	≥17
12.Ciprofloxacin	5 µg	≤15	16-20	≥21
13.Rifampin	5 µg	≤16	17-19	≥20
14.Doxycycline	30 µg	≤12	13-15	≥16
15.Cefixime	5 µg	≤15	16-18	≥19
16.Minocycline	30 µg	≤14	15-18	≥19
17.Levofloxacin	5 µg	≤15	16-18	≥19
18.Clindamycin	2 µg	≤14	15-20	≥21

2.8 Maintenance and preservation of isolates

The isolation and purification of bacterial isolates from different water samples were investigated. Typical and atypical colonies were picked up and streaked on nutrient agar plate. After 24 hours of incubation at 35°C, all the isolates were inoculated individuals containing nutrient agar slant with sterile paraffin and preserved at 4°C.

CHAPTER 3

Results

3. Results

In the present study, mineral water samples were collected from different places in two different season of Dhaka city based on human activities from August 2015 to January 2016 (rainy to winter season). Different physicochemical parameters (PH and conductivity) were measured and were inoculated into three different and selective agar media, MacConkey, XLD, M-FC Agar and also Nutrient agar to detect the presence of pathogenic bacteria and total count respectively. The result section can be divided into four parts:

- ✓ Bacterial load analysis of water samples collected from different areas in Dhaka city.
- ✓ Physiochemical quality analysis of collected samples.
- ✓ Cultural and biochemical examinations of isolates.
- ✓ Antibiotic test result of isolated organisms.

Table3.1: Microbial load in different samples in the different media in January 2016

Sample no.	Sample name	Growth on Nutrient agar (CFU/ml)	Growth on Mac-Conkey agar (CFU/ml)	Growth on Membrane Faecal Coliform agar (CFU/ml)	Growth on Xylose Lysine Deoxycholate agar (CFU/ml)
1	MWa1	6.00×10^2	NIL	NIL	NIL
2	MWf2	6.40×10^2	NIL	NIL	NIL
3	MWm3	1.0×10	NIL	NIL	NIL
4	MWp4	8.00×10^2	NIL	NIL	NIL
5	MWb5	1.50×10^2	NIL	NIL	NIL
6	MWs6	2.0×10	NIL	NIL	NIL
7	MWv7	1.5×10	NIL	NIL	NIL
8	MWac8	1.05×10^2	NIL	NIL	NIL
9	MWe9	3.0×10	NIL	NIL	NIL
10	MWj10	5.50×10^2	NIL	NIL	NIL

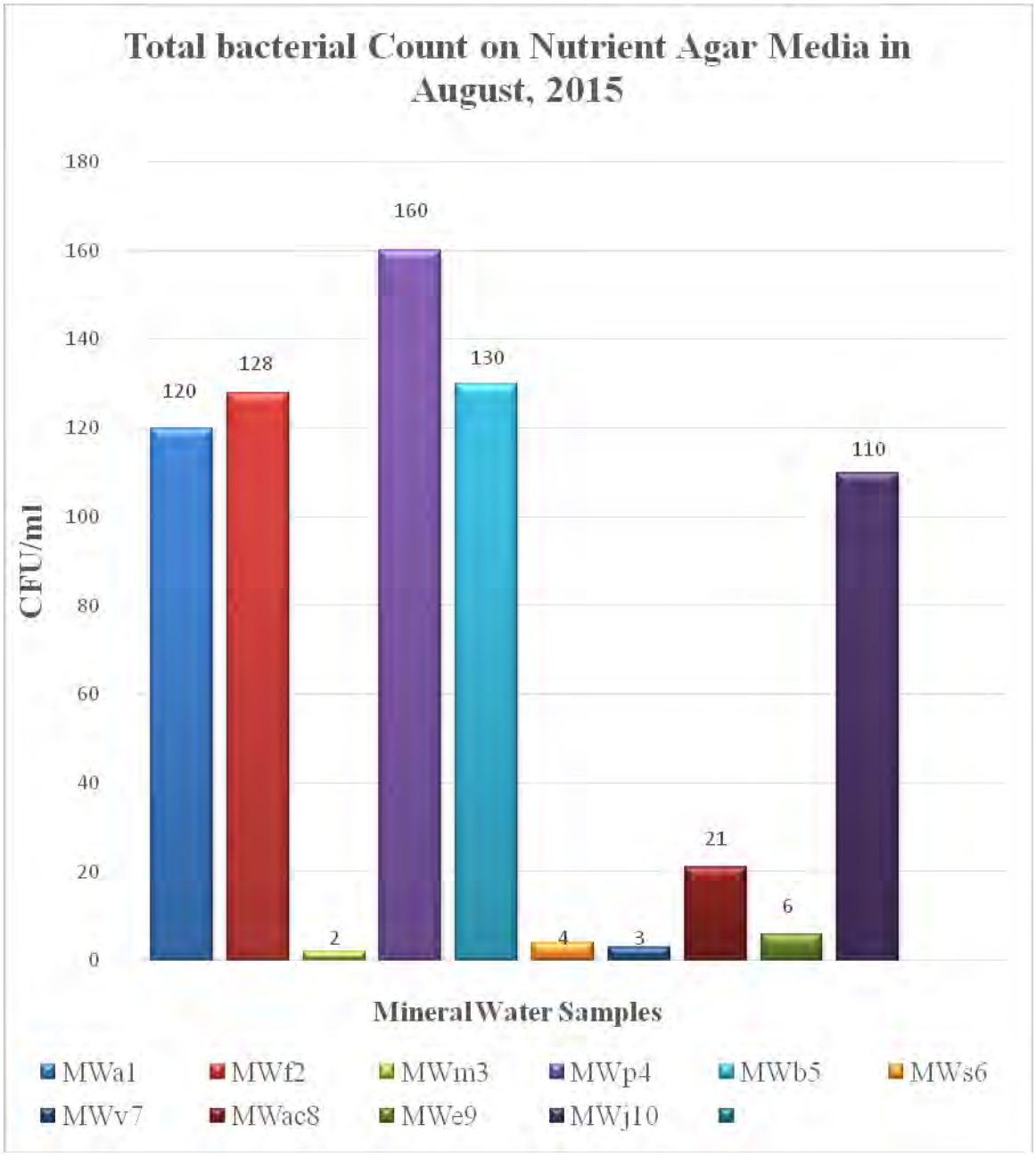


Fig 3.1: Total bacterial count on Nutrient agar media in August 2015

Table 3.2: Microbial load in different samples in the different media in January 2016

Sample no.	Sample name	Growth on Nutrient agar (CFU/ml)	Growth on Mac-Conkey agar (CFU/ml)	Growth on Membrane Faecal Coliform agar (CFU/ml)	Growth on Xylose Lysine Deoxycholate agar (CFU/ml)
1	MWa1	5.00×10^2	NIL	NIL	NIL
2	MWf2	6.00×10^2	NIL	NIL	NIL
3	MWm3	1.5×10	NIL	NIL	NIL
4	MWp4	7.50×10^2	NIL	NIL	NIL
5	MWb5	2.00×10^2	NIL	NIL	NIL
6	MWs6	3.0×10	NIL	NIL	NIL
7	MWv7	2.5×10	NIL	NIL	NIL
8	MWac8	$1.5\ 0 \times 10^2$	NIL	NIL	NIL
9	MWe9	4.0×10	NIL	NIL	NIL
10	MWj10	4.50×10^2	NIL	NIL	NIL

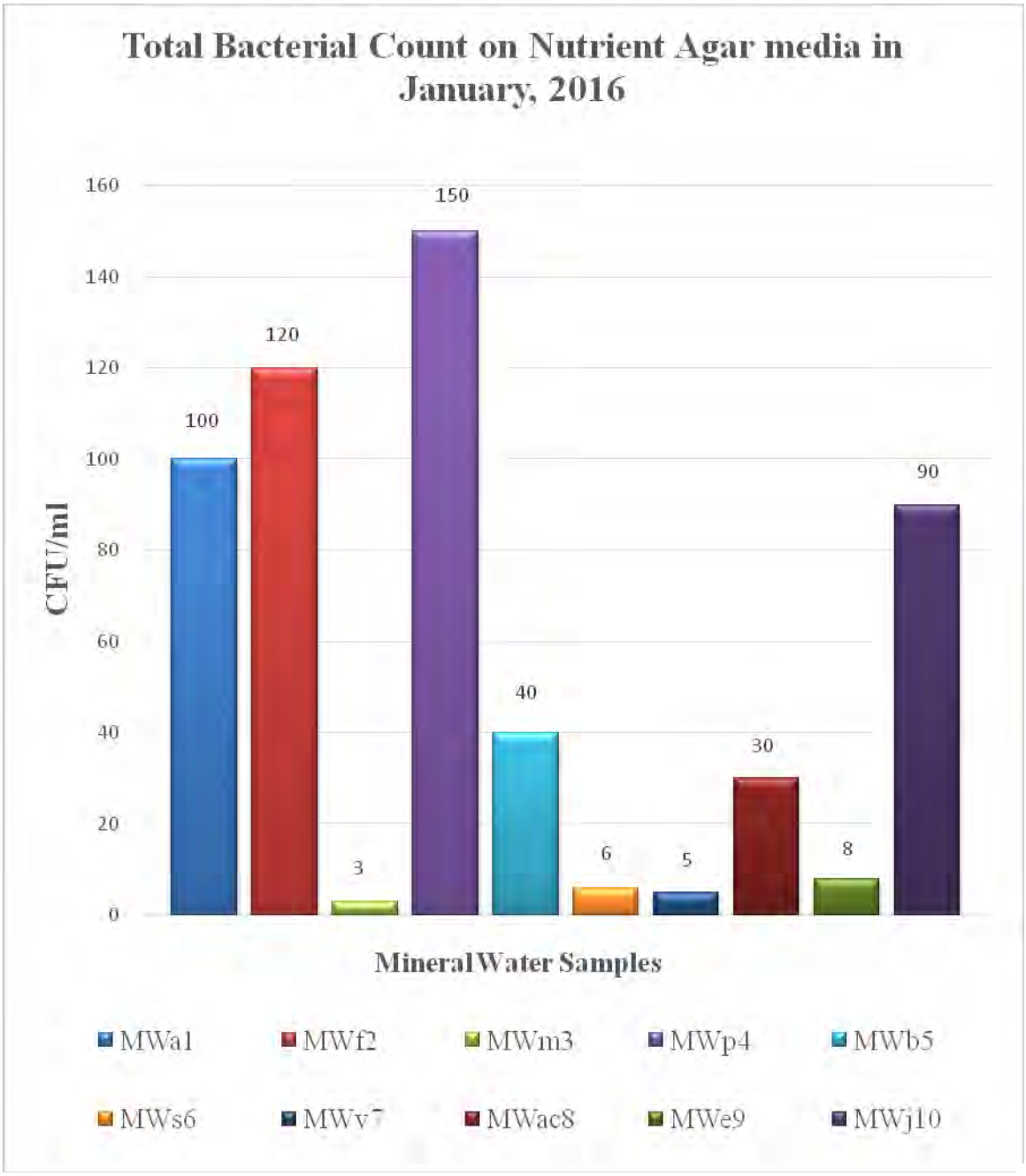


Fig 3.2: Total bacterial count on Nutrient agar media in January 2016

Table3.3: Water quality parameter

Sample No	Sample name	CONDUCTIVITY		PH
		20MS	2MS	
1	MWa1	0.09	0.018	6.9
2	MWf2	0.26	0.196	6.7
3	MWm3	0.25	0.193	7.4
4	MWp4	0.22	0.164	7.4
5	MWb5	0.24	0.182	7.1
6	MWs6	0.62	0.548	6.8
7	MWv7	0.19	0.127	6.8
8	MWac8	0.20	0.129	7.0
9	MWe9	0.26	0.192	6.9
10	MWj10	0.21	0.143	6.9

While comparing the overall result it was found that the bacterial count only found on nutrient agar media. There was no growth on MacConkey agar,M-FC agar and XLD agar media. Microbial count on nutrient agar ranged between 1.0×10 cfu/ ml and 8.00×10^2 cfu/ml. Maximum count was observed in the sample of mineral water during rainy season (table 3.1). Microbial count on nutrient agar ranged between 1.5×10cfu/ ml and 7.50×10^2 cfu/ml. Maximum count was observed in the sample of mineral water during winter season (table 3.2).The physical properties of the water samples are given in table 3.3.In winter, the water temperature ranged between 25 and 27°C, while in rainy season that was around 29°C. The results indicate a favorable temperature for bacterial growth throughout the time period. The maximum pH (7.4) was detected in the samples, while the minimum pH (6.7) was seen in the samples (table3.3). According to the United State Public Health (USPH), drinking water standards are pH 7.0.

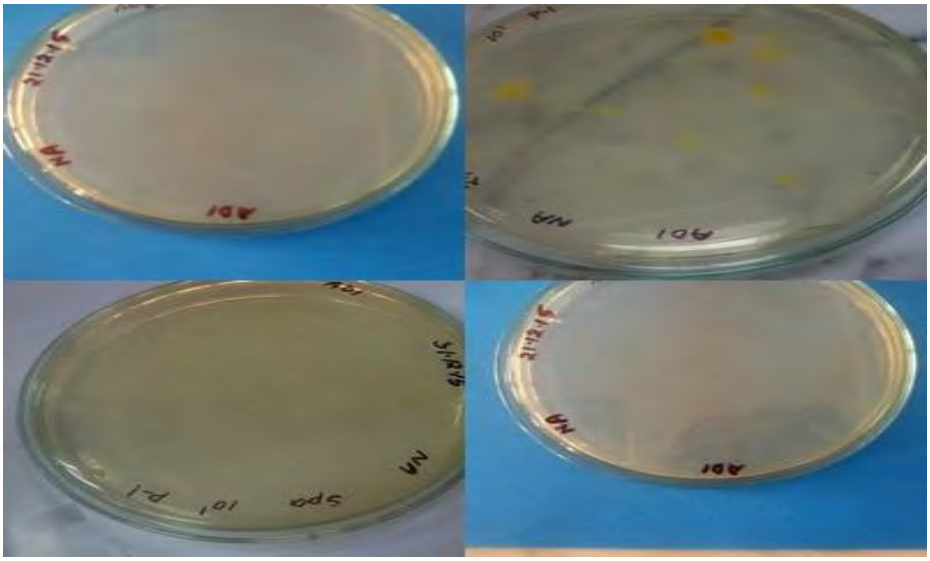


Fig3.3: Bacterial colonies on in Nutrient agar (NA) media.

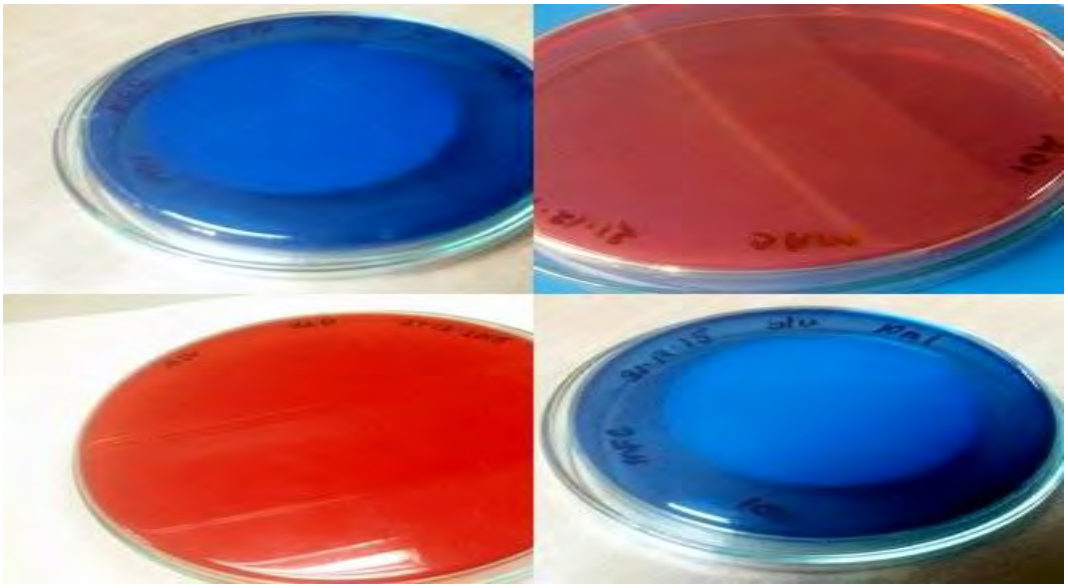
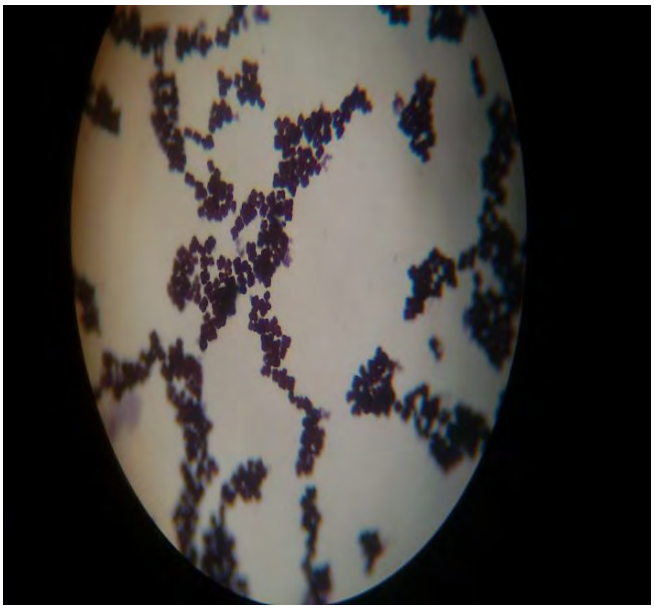


Fig 3.4: No bacterial colonies were grown on the selective media, Membrane Faecal-coliform, Xylose Lysine Deoxycholate agar and MacConkey agar media

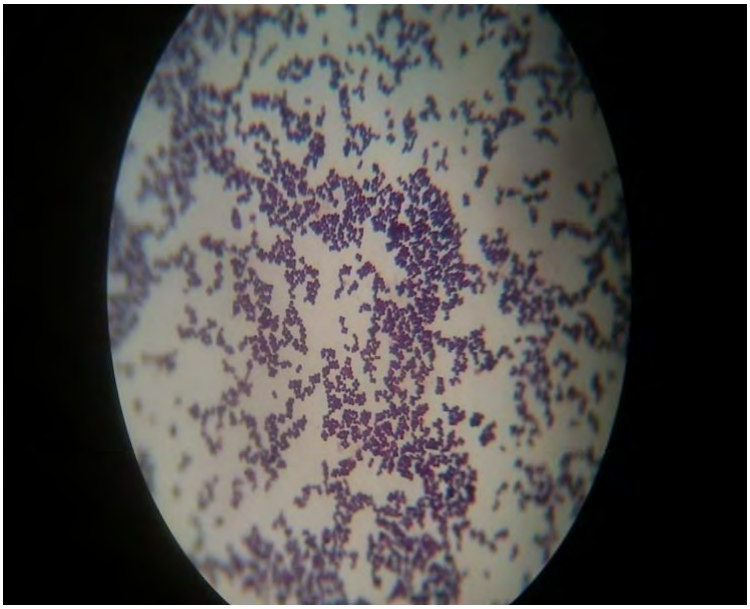
Table3.4: Colony characteristics of the isolates on Nutrient agar media

Sample ID	Isolate no	Isolate sample name	Colony characteristics				
			Size	**Form**	**Colour**	**Margin**	**Elevation**
MWa1	1	Mwa1	Medium	Circular	Yellow	Entire	Convex
MWf2	2	MWf2	Large	Circular	White	Entire	Convex
MWp4	3	$MWp^{r}4$	Large	Irregular	yellow	Entire	Convex
MWp4	4	$MWp^{l}4$	Large	Circular	0range	Entire	Raised
MWb5	5	MWb5	Large	Circular	yellow	Entire	Convex
MWs6	6	MWs6	Medium	Circular	yellow	Undulate	Convex
MWv7	7	MWv7	Large	Irregular	yellow	Entire	Convex
Mwac8	8	$Mwac^{r}8$	Large	Circular	yellow	Entire	Convex
Mwac8	9	$Mwac^{l}8$	Small	Circular	white	Entire	Raised
MWj10	10	MWj10	Medium	Circular	yellow	Entire	Convex
Mwe9	11	$Mwe^{r}9$	Medium	Circular	yellow	Entire	Convex
Mwe9	12	$Mwe^{l}9$	Small	Circular	white	Entire	Convex

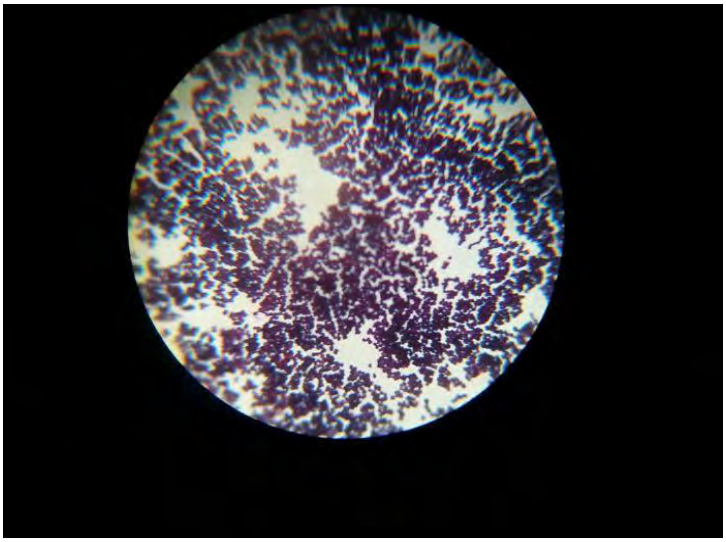
Colony characteristics of the isolates on nutrient agar medium are given in a table 3.4. From that table, the samples of colony were named with new isolates sample number and name. After that these isolates colony were used for biochemical tests to find organism and also antibiotic test for sensitivity.



(a)



(b)



(c)

Fig 3.5: (a), (b) and (c) -Unknown organisms were showing gram positive and purple colour by Gram staining method (100X).

Gram staining revealed that all the isolates organisms were *Staphylococcus spp.* For this biochemical test was observed to find out organism's name.

Table 3.5: Results of biochemical tests of the isolates collected from Nutrient agar

Isolate no	Isolate Sample name	Gram stain		TSI				MIU			Oxidase	Catalase	Citrate	lactose	MR	VP	Nitrate reduction	Gelatin hydrolysis	Blood agar	Caseinhydro lysis	Starch hydrolysis	Mannitol	Presumptive organism
		+/-	shape	BUTT	SLANT	H_2s	Gas	Motilit	Indole	Urease													
1.	**Mwa1**	-	cocci	K	K	-	-	+	+	+	-	+	-	-	+	-	+	-	-	+	-	-	***S. intermedius***
2.	**MWf2**	+	cocci	A	A	-	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	***S. auricularis***
3.	**MWp^{r}4**	+	cocci	A	A	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	***S. aureus***
4.	**MWp^{1}4**	+	cocci	K	K	-	-	-	+	+	-	+	-	-	+	-	+	-	-	-	-	-	***S. auricularis***
5.	**MWb5**	+	cocci	A	A	-	-	-	+	+	-	+	-	+	+	-	+	-	-	-	-	-	***S. hominis***
6.	**MWs6**	+	cocci	A	A	-	-	-	-	-	-	+	-	+	+	-	+	-	-	-	-	-	***S. felis***
7.	**MWv7**	+	cocci	A	A	-	-	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	***S. saccharolyticus***
8.	**MWac^{r}8**	+	cocci	A	A	-	-	-	+	+	-	+	-	+	+	-	+	-	-	+	-	-	***S. saccharolyticus***
9.	**MWac^{1}8**	+	cocci	K	K	-	-	+	+	-	-	+	-	-	+	-	+	-	-	-	-	-	***S. intermedius***
10.	**MWj10**	+	cocci	K	K	-	-	+	-	-	-	+	-	-	+	-	+	-	-	-	-	-	***S. intermedius***
11.	**MWe^{r}9**	+	cocci	K	K	-	-	+	-	-	-	+	-	-	-	-	+	-	-	+	-	-	***S. saccharolyticus***
12	**MWe^{1}9**	+	cocci	A	A	-	-	-	+	+	-	+	-	+	+	-	+	-	-	-	-	-	***S. felis***

K=Alkaline reaction, A=Acidic reaction, + =Positive reaction; - = Negative reaction

All the biochemical tests results of twelve isolated organisms were observed and the entire organisms were identified as different genus of *Staphylococcus spp.* (abis online software). Out of twenty samples of different areas mineral water were found to contain *S. intermedius, S. aureus, S. felis, S. auricularis, S. hominis* and *S. saccharolyticus.* Out of 20 samples in two different seasons three different samples showed the growth of *S. intermedius* and *S. saccharolyticus,* two different samples showed the growth of *S.auricularis* and *S. felis.* Another two different samples showed the growth of *S.aureus and S.hominis.* Of the 12 isolates antibiotic resistance pattern of 7 isolates could be performed (shown in table no 3.6). Some of the biochemical test results are given in the figure:

Fig 3.6: Triple iron salt agar test

In the figure 3.6, TSI result was observed left one was control, middle one was acid butt, acid slant (yellow butt, yellow slant): lactose and/or sucrose has been fermented and right one was alkaline butt, alkaline slant (red butt, red slant): neither glucose, lactose, nor sucrose has been fermented.

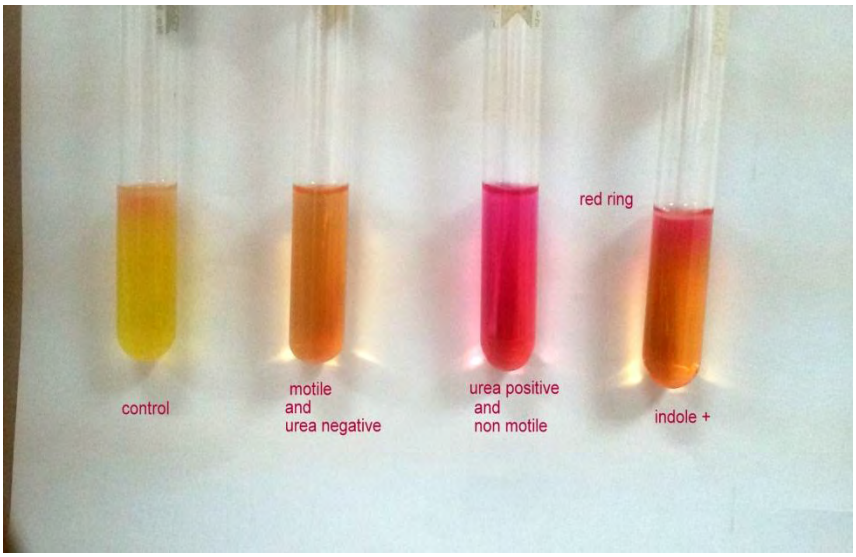


Fig 3.7: Motility, Indole and Urea test.

In the figure 3.7, MIU result was observed. Motility positive: entire medium becomes opaque (semisolid medium allows bacterial movement) and Motility negative: Culture grows on the stabbing line only. Indole positive: red ring on addition of Kovac's reagent and Indole negative: No ring. Urea hydrolysis is observed if the entire medium turns red.

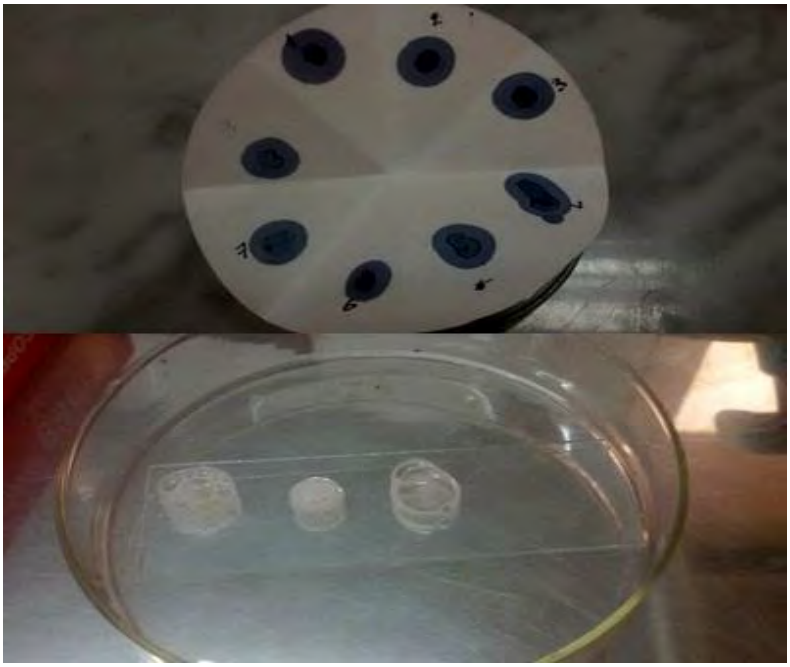


Fig 3.8: Oxidase and Catalase test. Upper =oxidase negative and downwards= catalase positive.

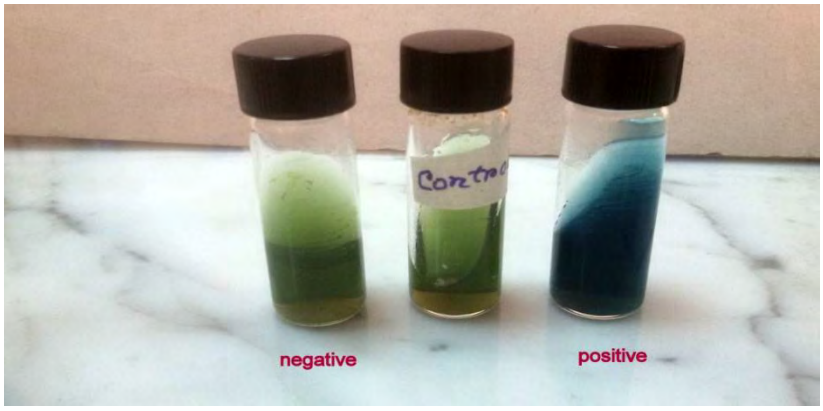


Fig 3.9: Simmon's citrate test. Left =negative and right = positive

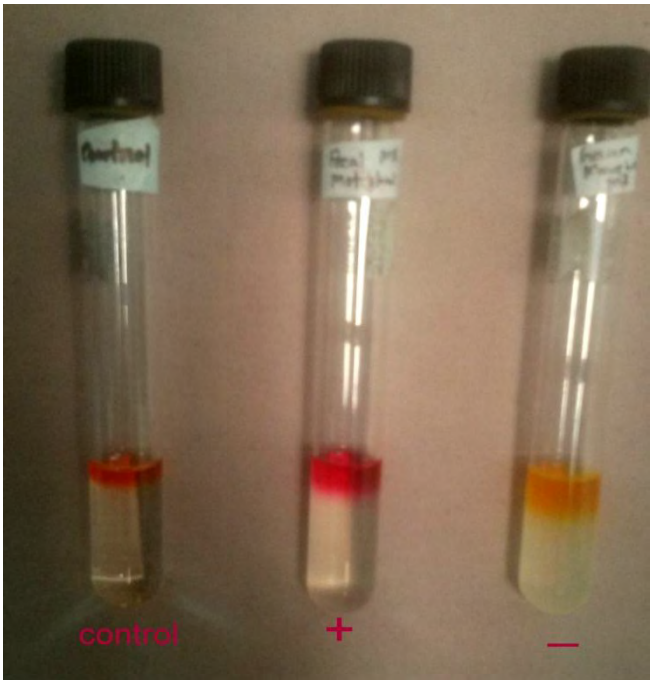


Fig 3.10: Methylred (MR) test on MR-VP test.

In the figure 3.10, A positive (middle one) MR test was demonstrated by the development of a stable red colour on the surface of the medium after the addition of methyl red indicator. A negative MR test (right) was demonstrated by the development of a yellow colour on the surface of the medium.

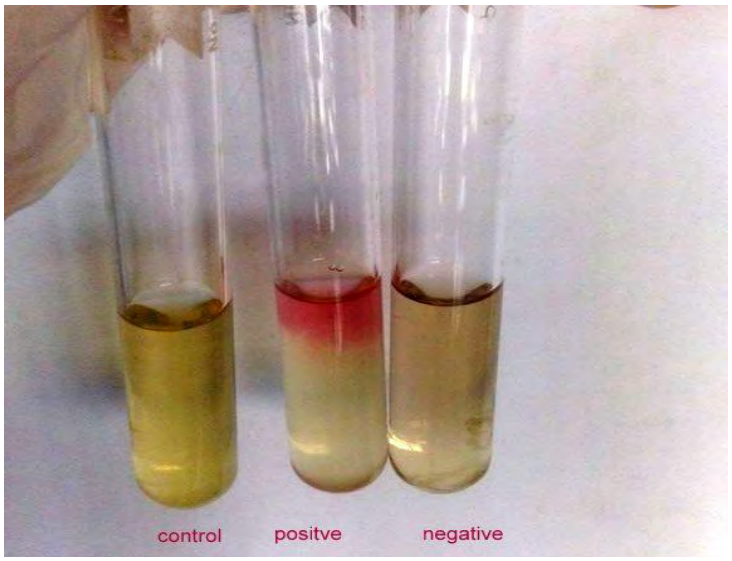


Fig 3.11: Voges-Proskauer test in MR-VP test

In the figure 3.11, a positive VP test was demonstrated by the development of a pink-red colour on the surface of the medium 15 minutes to one hour after the addition of the reagents (middle one). A negative VP test was demonstrated by the appearance of a yellow colour on the surface of the medium. Development of a copper-like colour is also interpreted as negative (right side).

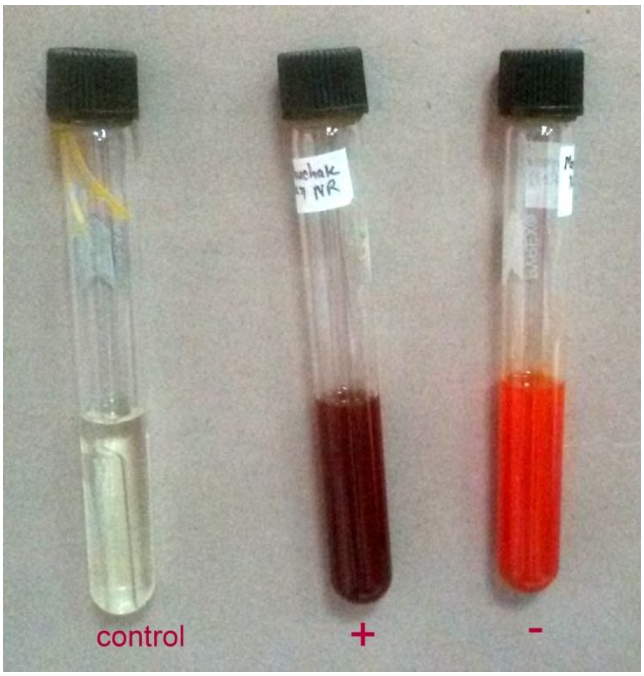


Fig3.12: Nitrate reduction test. Middle =Positive (Red after sulfanilic acid + alpha-naphthylamine; no colour after zinc). Right= Negative (No colour after sulfanilic acid + alpha-naphthylamine followed by Red after zinc)

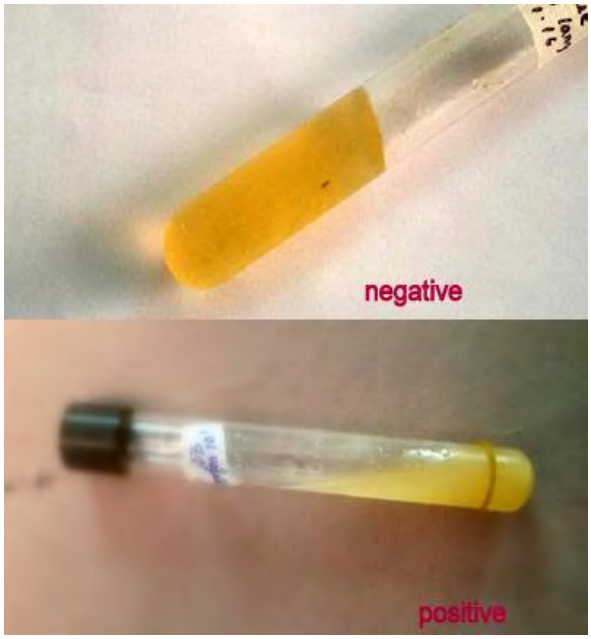


Fig 3.13: Gelatin hydrolysis test.Upper= negative (solid) and downwords= positive (liquids)

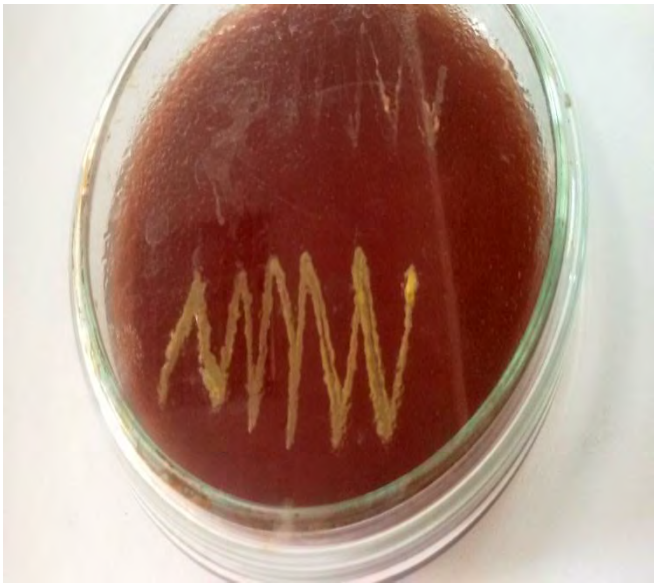


Fig 3.14: Blood agar test (no hemolysis found)

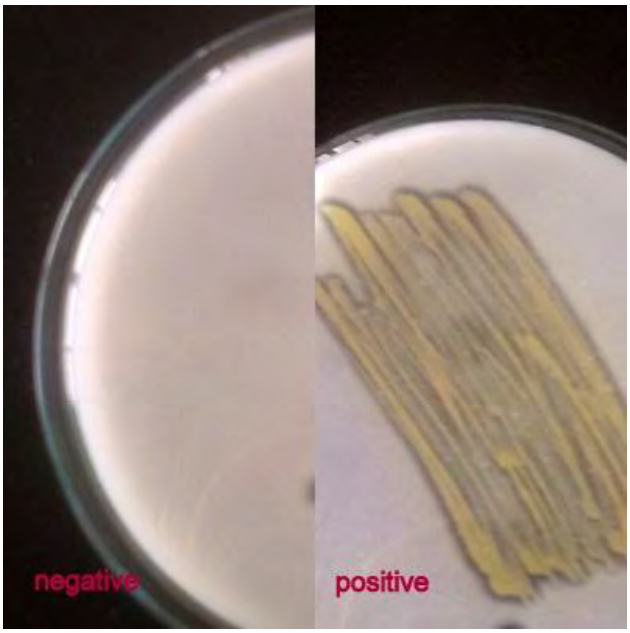


Fig 3.15: Casein hydrolysis test.

In the figure 3.15, a zone of clearing around the growth area identifies the presence of the enzyme caseinase (right side). Left = negative.

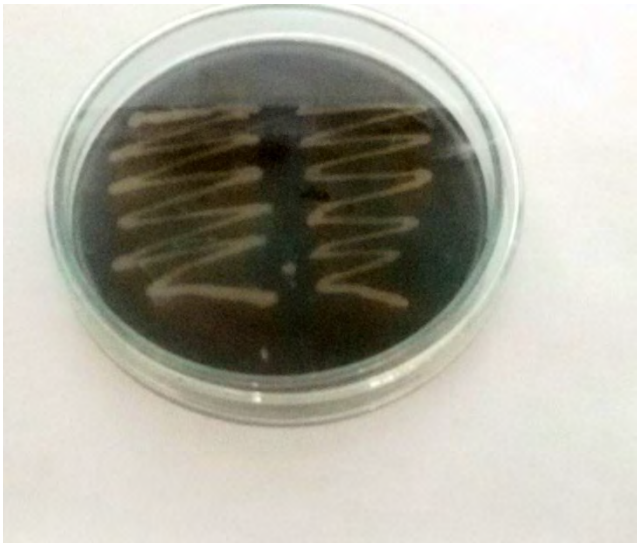


Fig 3.16: Starch hydrolysis test. (Negative result = no hydrolysis)

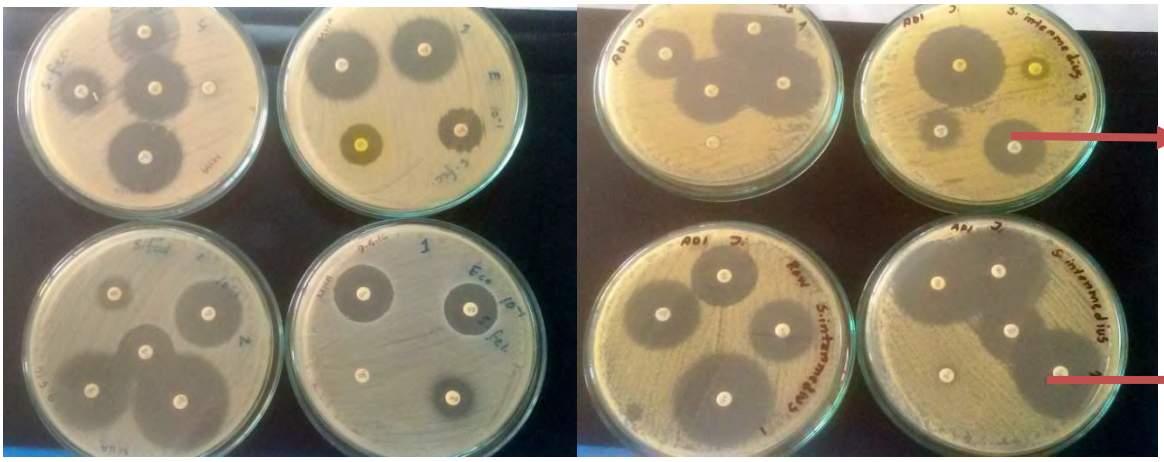
Fig 3.17: Mannitol salt agar test

In the figure 3.17, mannitol was fermented by a bacterium, acid is produced, which lowers the pH and results in the formation of a yellow area surrounding an isolated colony on MSA (left on). Left on was showed a non fermenting bacterium that withstands the high salt concentration would display a red to pink area due to peptone breakdown.

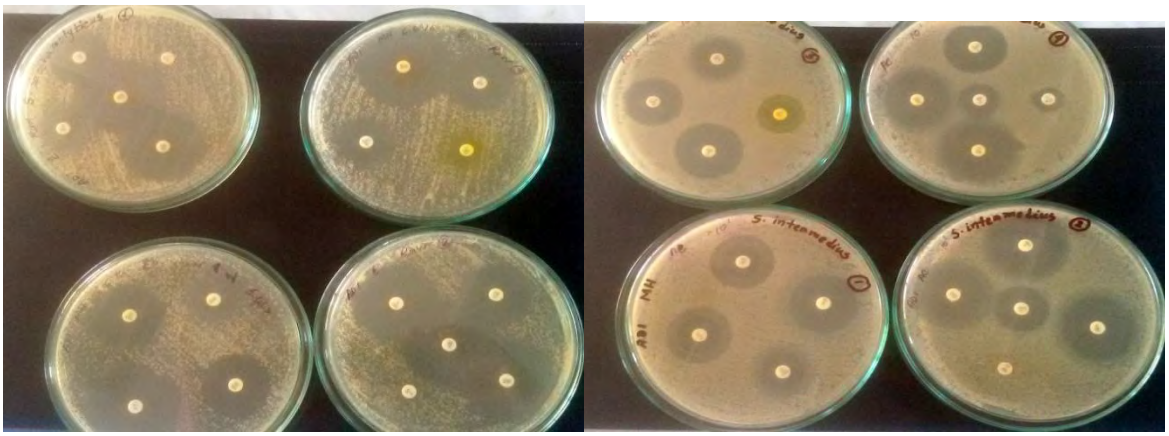
Table 3.6: Antimicrobial sensitivity pattern of *different organism* isolated from mineral water

Antibiotics name	Isolated samle name													
	MWa1		**MWp^{r}4**		**MWs6**		**MWac^{l}8**		**MWj10**		**MWe^{r}9**		**MWe^{l}9**	
	S. intermedius		*S. aureus*		*S. felis*		*S. intermedius*		*S. intermedius*		*S. saccharolyticus*		*S. felis*	
	Zone of diameter (mm)	Interpretation	Zone of diameter(mm)	interpretation	Zone of diameter(mm)	interpretation	Zone of diameter(mm)	interpretation	Zone of diameter(mm)	interpretation	Zone of diameter(mm)	interpretation	Zone of diameter(mm)	interpretation
1.Erythromycin	42	S	32	S	38	S	28	S	31	S	15	I	18	I
2.Pencillin	38	S	25	I	40	S	29	S	41	S	45	S	26	I
3.Vancomycin	31	S	21	S	31	S	30	S	30	S	32	S	24	S
4.Trimethoprim-sulfamethnoxazole	26	S	25	S	25	S	20	S	35	S	30	S	0	R
5.Gentamicin	38	S	26	S	33	S	22	S	31	S	30	S	31	S
6.Oxacillin	18	S	11	I	0	R	0	R	0	R	0	R	26	S
7.Tetracycline	40	S	29	S	40	S	30	S	41	S	40	S	14	I
8.Chloramphenicol	43	S	29	S	44	S	32	S	42	S	42	S	35	S
9.Moxifloxacin	40	S	35	S	40	S	35	S	40	S	38	S	42	S
10.Norfloxacin	26	S	25	S	23	S	27	S	20	S	25	S	30	S
11.Nitrofurantion	14	R	20	S	14	R	20	S	10	R	10	R	20	S
12.Ciprofloxacin	35	S	29	S	31	S	32	S	29	S	30	S	34	S
13.Rifampin	45	S	22	S	20	S	24	S	43	S	45	S	20	S
14.Doxycycline	40	S	29	S	38	S	30	S	40	S	40	S	20	S
15.Cefixime	20	S	0	R	0	R	15	R	0	R	0	R	0	R
16.Minocycline	40	S	30	S	41	S	35	S	42	S	43	S	24	S
17.Levofloxacin	32	S	28	S	30	S	30	S	28	S	30	S	35	S
18.Clindamycin	40	S	20	I	30	S	18	I	38	S	25	S	19	I
*S= Susceptible *R= Resistant *I= Intermediate														

Of the 12 isolates antibiotic resistance pattern of 7 isolates could be performed (shown in table no 3.6) among all organism as per interested, *S. intermedius, S. aureus, S. felis*and*S. Saccharolyticus* were taken for antibiotic test. In respect to antimicrobial susceptibility testing most of the *Staphylococcus spp.* isolates were susceptible to erythromycin, tetracycline, norfloxacin and ciprofloxacin. Furthermore, a few *Staphylococcus spp.* isolates were intermediate resistant to pencillin and oxacillin. However, most of the *Staphylococcus spp.* isolates were resistant to cefixime.

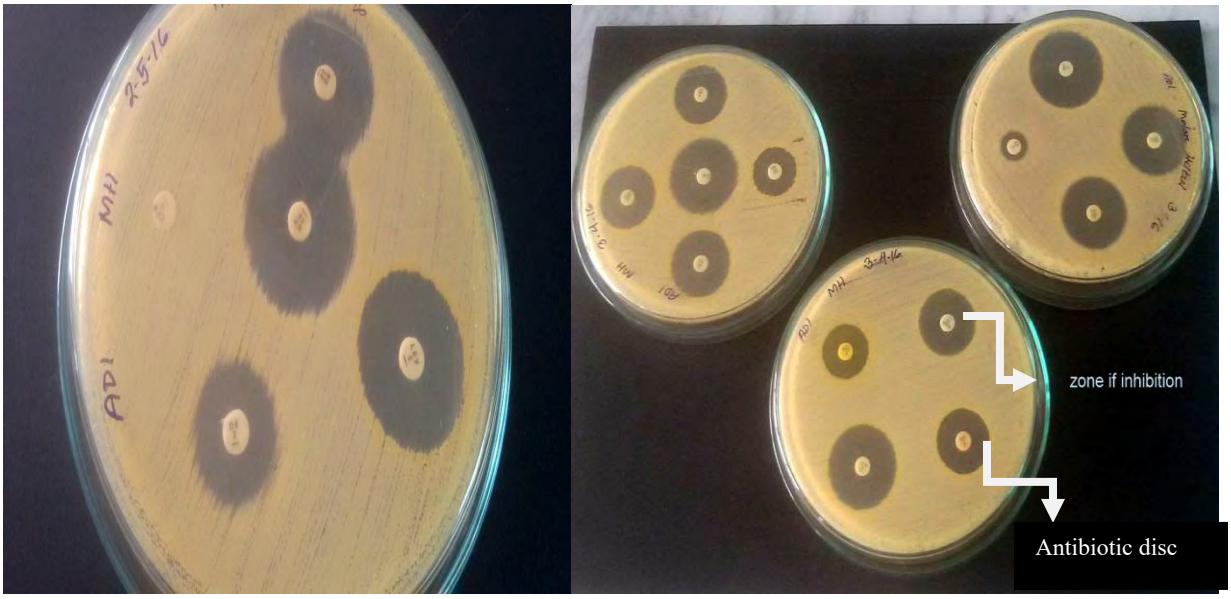


(a) Sample of MWe^{r} 9 **(b) Sample of MWj10**

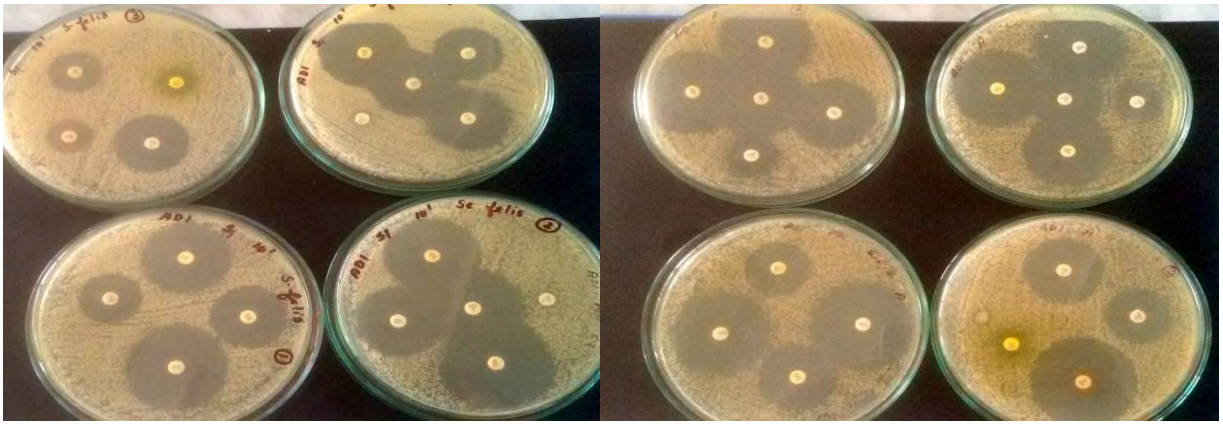


(c) Sample of MWe^{1}9 **(d) Sample of MWac^{1}8**

Fig 3.18: Antimicrobial susceptibility testing of *S. felis*(a), *S. intermedius* (b), *and S. saccharolyticus* (c,d) isolated by disc diffusion method.



(f) Sample of **MWp^{r}4**



(g)Sample of MWs6 **(h) Sample of MWa1**

Fig 3.19: Antimicrobial susceptibility testing of *Staphylococcus aureus (f),S. intermedius* (g)and *S. felis (*h) isolated by disc diffusion method.

CHAPTER 4

Discussion & Conclusion

4. DISCUSSION:

Water is considered as an inevitable element of life. Around 75% of the earth is surrounded by water, but only 1% of water can be used as a source of drinking water for animal and human being. Consumption of contaminated water may cause various gastrointestinal diseases like diarrhea, dysentery and other water borne diseases like cholera, typhoid of human, poultry and livestock. 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 born diseases (Karn et al., 2001). Bottled water generally receives no further treatment by the consumer before consumption, so its microbiological safety and quality are of paramount importance. The microbiological quality and safety of bottled water is influenced by the microbiological status of the source water and the level of hygiene in the extraction and bottling process.

The primary objective of this research work was to assess the overall quality of bottled water (both qualitative and bacteriological) available in Dhaka city of Bangladesh. Qualitative assessment of bottled water indicated that a good number of people preferred bottled water rather than tap water in this study. The age group belonging to 12-24 years preferred bottled water mostly (60%). However, 66.7% undergraduate student preferred bottled water for their daily consumption. In this study, the cause of preference of bottled water was health consciousness, which is 70% of the total peoples. The criteria to be good bottled water were taste (43.3%). The bottled water quality was satisfactory to 40% of the peoples in this study on the basis of people's satisfaction, perception and expenditure on bottled water quality. On the other hand, the percentage of dumping of bottled water after consumption refuse was 33.3% (Sharmin, 2012). Moreover, monthly expenditure on bottled water was less than taka 300 in 80% of the total people. The findings of the study are more or less similar to the findings of Majumder *et al*., (2011).

After performing all required test (microbial culture and biochemical), the results of the study was revealed. Nutrient agar plates were used for the calculations of total viable count (TVC). Bacterial colonies of different morphology and colour were observed. Heterotrophic plate count (HPC) and total coliform count (TCC) are commonly used to assess the general microbiological

quality of mineral water. But no TCC was observed in the present study. The HPC of bottled water of different brands such as MWa1, MWf2,MWm3, MWp4, MWb5, MWs6, MWv7, MWac8, MWe9,MWj10 were 6.00 $\times 10^2$, 6.40 $\times 10^2$, 1.0 $\times 10$, 8.00 $\times 10^2$,1.50 $\times 10$,2.0 $\times 10$,1.5 $\times 10$,1.05 $\times 10^2$,3.0 $\times 10$,5.50 $\times 10^2$ cfu/100 ml respectively in this study. HPC was found the lowest (1.0×10) in MWm3 mineral water but the highest (8.0×10^2) in MW4 bottled water in this study(table 3.1). The HPC of mineral water of different brands such as MWa1, MWf2,MWm3, MWp4, MWb5, MWs6, MWv7, MWac8, MWe9,MWj10 were 6.00 $\times 10^2$,1.5 $\times 10$,7.50 $\times 10^2$, 2.00 $\times 10^2$,3.0 $\times 10$,2.5 $\times 10$,1.5 0×10^2 ,4.0 $\times 10$,4.50 $\times 10^2$ cfu/100 ml respectively in this study. HPC was found the lowest (1.5×10) in MWm3 bottled water but the highest (7.50 $\times 10^2$) in MW4 bottled water in this study(table 3.2).In this study, MWm3 bottled water was found to be best in terms of microbiological quality when compared with other brands of mineral water available in Dhaka city of Bangladesh. According to the world health report (2002), drinking water quality specifications world-wide recommend HPC limits 50 cfu/ml in mineral water.

A total of 12 isolates were identified as different species of *Staphyloccous spp.* on the basis of cultural and biochemical characteristics from mineral water samples used in this study. The colony characteristics of the isolated colony were differing from each other. All the biochemical tests of twelve isolated organisms were observed and all the organism were identified as different genus of *Staphylococcus spp.* (abis online software). Out of twenty samples of different areas mineral water were found to contain *S. intermedius, S. aureus, S. felis, S. auricularis, S. hominis* and *S. saccharolyticus.* Out of twenty samples in two different seasons three different samples showed the growth of *S. intermedius* and *S. saccharolyticus,* two isolated samples showed the growth of *S.auricularis* and *S. felis.* Another two different samples showed the growth of *S.aureus and S.hominis.* Among them seven organisms were taken from different isolated samples like *S. intermediu , S. aureus, S. felis and S. saccharolyticus* for antibiotic test.

The fermentation reaction by the isolates Of *Staphylococcus aureus* in basic sugars (lactose and mannitol) was positive. Moreover, MR reaction and catalase tests were also positive for *Staphylococcus aureus.* The result of sugar fermentation tests corresponds to the findings of Beutin *et al.,* (1997) and Sandhu *et al*., (1996). These respective authors reported that although *Staphylococcus aureus* ferments sugars. Variation of the results might be due to genetic factors and nature of inhabitant of the organisms. Malaney and Weiser (1962) isolated *Staphylococcus*

aureus from water. Dragas and Tratnik (1975) stated that 21.5% of mineral were contained *Staphylococcus aureus* Lin *et al.* (1974) and Mieres and Bastardo (1975) isolated *Staphylococcus aureus* from mineral water. They however found that *Staphylococcus aureus* was present in majority of the improved water sources. Likewise in the present study *Staphylococcus aureus* was detected and found absent in bottled water. The findings of the present study obviously demonstrated that protection of mineral water sources is very important and the avoidance of contamination can promote hygienic quality of mineral water supplies,

Staphylococcus aureus was able to acquire resistance easily; therefore it is a good bio indicator model for surveillance studies of antimicrobial resistance. Antimicrobial resistance testing was performed by disc diffusion method using 18 different antibiotics. In antimicrobial susceptibility test, mos of the organisms were intermediate resistant to pencillin, oxacillin, clindamycin and susceptible resistant to erythromycin, vancomycin, trimethoprim-sulfametnoxazole, gentamicin, tetracycline, norfloxacin chloramphenicol, moxifloxacin, nitrofurantion, ciprofloxacin, rifampin, minocycline, levofloxacin and resistance to cefixime, nitrofurantion . These findings are in partial agreement with Islam *et* al., (2010). These findings are in partial agreement with Nazir *et al*., (2005). Such high incidence of multidrug resistant might be due to indiscriminate use of antibiotics, which may eventually superseded the drug resistant microorganisms from antibiotic saturated environment. In Bangladesh, for many years antibiotic is randomly used for treatment purposes. People are not aware about the schedule use of antibiotics. Thus, resistant strains might be emerged by genetic recombination against one or more antimicrobial agent(s).

The major limitation of this study was conducted with only limited number of samples. This study has several imperfections. There was an unavailability of many materials that was needed while working with samples. So results which were obtained may have dissimilarity with the results of other studies. Further research is needed to be carried out for better results. So, the study should also be performed at different location of Dhaka city along with the consideration of total suspended solid (TSS), turbidity, and temperature variation.

Conclusion

Therefore, from the findings of the present study, it may be concluded that a number of people preferred bottled water rather than tap water for their daily consumption. MWm3 bottled water was found to be superior in terms of microbiological quality to other brands of bottled water available in Dhaka city of Bangladesh. However, it does not confirm that the microbiological quality of this sample is good because it may contain toxic preservative. Only HPC were found in municipal mineral water. The isolates of *Staphylococcus sp.* were isolated and characterized from samples using different cultural , morphological examination, biochemical tests .However, the following tasks may be scheduled for further study-Molecular characterization of *Staphylocoocus spp.* isolated from bottled mineral water by using PCR, PCE-RFLP, sequencing and so on Genome analysis to have an idea about the genes responsible for pathogenicity and multidrug resistant of *Staphylocoocus spp. isolated* from mineral water.

Chapter5

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Appendix

Appendix- I

Media compositions

The composition of all media used in the study is given below.

Nutrient Agar

Component	**Amount (g/L)**
Peptone	5.0
Sodium chloride	5.0
Beef extract	3.0
Agar	15.0
Final pH	7.0

Mannitol Salt Agar

Component	**Amount (g/L)**
Proteose peptone	10.0
Beef extract	1.0
Sodium chloride	75.0
D-mannitol	10.0
Phenol red	0.025
Agar	15.0
Final pH	7.4 ± 0.2 at 25°C

Xylose-Lysine-Deoxycholate Agar

Component	Amount (g/L)
Yeast extract	3.00
L-lysine	5.00
Lactose	7.50
Sucrose	7.50
Xylose	3.50
Sodium chloride	5.00
Sodium deoxycholate	2.50
Sodium thiosulfate	6.80
Ferric ammonium	0.80
Phenol red	0.08
Agar	15.00

MacConkey Agar

Component	Amount (g/L)
Peptic digest of animal tissue	1.5
Casein enzymic hydrolysate	1.5
Pancreatic digest of gelatin	17.00
Lactose	10.00
Bile salts	1.50
Crystal violate	0.001
Neutral red	0.03
Agar	15.00

M-FC Agar

Component	Amount (g/L)
Tryptose	10.00
Proteose peptone	5.00
Yeast extract	3.00
Lactose	12.50
Bile salts mixture	1.50
Sodium chloride	5.00
Aniline blue	0.10
Agar	15.00

Starch Agar

Component	Amount (g/ L)
Beef extract	3.0
Soluble starch	10.0
Agar	12.0

Simmon's Citrate Agar

Component	Amount (g/L)
Magnesium sulphate	0.2
Ammoniun dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.0
Bactobromothymol blue	0.08

Methyl red VogusPrekaure (MRVP) Media

Component	Amount (g/L)
Peptone	7.0
Dextrose	5.0
Dipotassium hydrogen phosphate	5.0
Final pH	7.0

Triple Sugar Iron Agar

Component	Amount (g/L)
Bio-polytone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Phenol red	0.0125
Agar	13.0
Final pH	7.3

Physiological Saline

Component	Amount (g/L)
Sodium Chloride	9.0

Motility Indole Urease (MIU) Agar

Component	**Amount (g/L)**
Tryptone	10
Phenol red	0.1
Agar	2.0
Sodium chloride	5.0
pH (at 25°C)	6.8 ± at 25°C

Gelatin Broth

Component	**Amount (g/L)**
Peptone	5.0
Beef extract	3.0
Gelatin	120.0
Final pH	6.8 ± 0.2 at 25°C

Nitrate Reduction Broth

Component	**Amount (g/L)**
Beef extract	3.0
Gelatin peptone	5.0
Potassium nitrate	1.0

Blood Agar Base

Component	**Amount (g/L)**
Beef heart infusion from (beef extract)	500.0
Tryptose	10.0
Sodium chloride	5.0
Agar	15.0
Final pH	6.8 ± 0.2 at 25°C

Mueller Hinton Agar

Component	**Amount (g/L)**
Beef, infusion from	300.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000
Final pH	(at 25°C) 7.3±0.1

MacFarlane turbidity standard no. 5

Sulfuric acid	0.18 M
Barium chloride	0.048 M
Distilled water	1000 ml

Appendix – II

Reagents

Crystal Violet (100 ml)

To 29 ml 95% ethyl alcohol, 2 g crystal violet was dissolved. To 80 ml distilled water, 0.8 g ammonium oxalate was dissolved. The two solutions were mixed to make the stain and stored in a reagent bottle at room temperature.

Safranin (100ml)

To 10 ml 95% ethanol, 2.5 g safranin was dissolved. Distilled water was added to the solution to make a final volume of 100 ml. The final solution was stored in a reagent bottle at room temeperature.

Gram's iodine (300 ml)

To 300 ml distilled water, 1 g iodine and 2 g potassium iodide was added. The solution was mixed on a magnetic stirrer overnight and transferred to a reagent bottle and stored at room temperature.

Kovac's Reagent (150 ml)

To a reagent bottle, 150 ml of reagent grade isoamyl alcohol, 10 g of p-dimethylaminobenzaldehyde (DMAB) and 50 ml of HCl (concentrated) were added and mixed. The reagent bottle was then covered with an aluminum foil to prevent exposure of reagent to light and stored at 4°C.

Methyl Red (200 ml)

In a reagent bottle, 1 g of methyl red powder was completely dissolved in 300 ml of ethanol (95%). 200 ml of destilled water was added to make 500 ml of a 0.05% (wt/vol) solution in 60% (vol/vol) ethanol and stored at 4°C.

Barrit's Reagent A (100 ml)

5% (wt/vol) a-naphthol was added to 100 ml absolute ethanol and stored in a reagent bottle at 4°C.

Barrit's Reagent B (100 ml)

40% (wt/vol) KOH was added to 100 ml distilled water and stored in a reagent bottle at 4°C.

Oxidase Reagent (100 ml)

To 100 ml distilled water, 1% tetra-methyl-*p*-phenylenediaminedihydrochloride was added and stored in a reagent bottle covered with aluminum foil at 4°C to prevent exposure to light.
Catalase Reagent (20 ml 3% hydrogen peroxide)

From a stock solution of 35 % hydrogen peroxide, 583 µl solution was added to 19.417 ml distilled water and stored at 4°C in a reagent bottle.

Urease Reagent (50 ml 40% urea solution)

To 50 ml distilled water, 20 g pure urea powder was added. The solution was filtered through a HEPA filter and collected into a reagent bottle. The solution was stored at room temperature.

Nitrate Reagent A (100 ml)

5Nacetic acid was prepared by adding 287 ml of glacial acetic acid (17.4N) to 713 ml of deionized water.In a reagent bottle, 0.6 g of N, N-Dimethyl-α-naphthylamine was added along with 100 ml of acetic acid (5N) and mixed until the colour of the solution turned light yellow. The reagent was stored at 4°C.

Nitrate Reagent B (100 ml)

In a reagent bottle, 0.8 g of sulfalinic acid was added along with 100 ml acetic acid (5N)^{a}to form a colourless solution and stored at 4°C.

Appendix – III

Gadgets

List of gadgets that were used during the study

Instrument	Manufacturer
Weighing Machine	Adam equipment, UK
Incubator	SAARC
Laminar Flow Hood	SAARC
Autoclave Machine	SAARC
Sterilizer	Labtech, Singapore
Shaking Incubator, Model: WIS-20R	Daihan Scientific Companies, Korea
Spectrophotometer, UV mini – 1240	Shimadzu Corporation, Australia
NanoDrop 2000 Spectrophotometer	Thermo Scientific, USA
Microscope	A. Krüssoptronic, Germany
UV Transilluminator, Model: MD-20	Wealtec Corp, USA
-20°C Freezer	Siemens, Germany
Magnetic Stirrer, Model: JSHS-180	JSR, Korea
Vortex Machine	VWR International
Microwave Oven, Model:MH6548SR	LG, China
pH Meter: pHep Tester	Hanna Instruments, Romania
Micropipette	Eppendorf, Germany
Disposable Micropipette tips	Eppendorf, Ireland
Refrigerator (4^{O}C) Model: 0636	Samsung
Conductivity meter (digital)	CD-4302
Membrane filter unit	Mo-a.s-2.0 Dynair