Bethlehem University

Biotechnology Master Program

Identification and Molecular Characterization of Thermo Tolerant Fecal Coliforms and Bacterial Pathogens "*E.coli*, *Salmonella* spp and *Shigella* spp" in Ein-Elbalad Spring in Battir

By

Joseph Ibrahim Danho

In partial fulfillment of the requirements for the degree of

Master of Science in biotechnology

July, 2016

The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

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Graduate Advisory Committee:

Committee Chair Name, (student supervisor)	Date
Dr. Ghassan Handal, Bethlehem University	
Internal Committee Member Name.	Date
Dr. Omar Dar-Issa, Bethlehem University	
External Committee Member Name.	Date
Dr. Motaz Kutob, Al-Quds University	

Approved for the Faculties

Dean of Faculty of Scientific Research and Higher studies	Dean of Faculty of Science
Palestine Polytechnic University	Bethlehem University
Date:	Date:

"Identification and Molecular Characterization of Thermo Tolerant Fecal Coliforms and Bacterial Pathogens "*E.coli, Salmonella* spp and *Shigella* spp" in Ein-Elbalad Spring in Battir"

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ABSTRACT

Battir is a Palestinian village located 6.5km north-west Bethlehem city with a population of approximately 5000 people. Ein El-Balad spring is a vital water source in Battir since water supply for the village is not available all the time especially in summer season. Recent reports indicated a serious contamination in the spring water. Among many sources of contamination, waste water intrusion from cesspits is the major source of contamination for the spring. Molecular techniques provide a powerful robust analysis in detection of bacterial cells in water even if they exist in small quantities. In this thesis the presence of thermo-tolerant fecal coliform bacteria in the spring have been identified using membrane filter culture technique and some coliform isolates were characterized by polymerase chain reaction (PCR) targeting the 16S rDNA gene followed by Sanger sequencing. In addition the possible presence of bacterial pathogens *E.coli*, *Salmonella* spp and *Shigella* spp has been detected by PCR using species specific primers followed by MISeq next generation sequencing. Results showed a high number of thermo-tolerant fecal coliform bacteria and the presence of coliform isolates that are of a pathogenic nature, moreover PCR detection using species specific primers has shown that the spring water does not contain any *Salmonella* or *Shigella* species but contain *E.coli*. Next Generation Sequencing analysis targeting *E.coli* bacteria indicated the presence of waterborne pathogenic strains of *E.coli*.

"الكشف التوصيف الجزيئي لبكتيريا القولونيات البرازية المقاومة للحرارة ومسببات الأمراض البكتيرية: *إشريكية قولونية*، *السلمونيلا* و *الشيغيلا* في مياه نبع عين البلد , بتير"

الطالب: جوزيف ابراهيم دنحو

ملخص

بتير هي قرية فلسطينية تقع ٥,٦ كم شمال غرب بيت لحم ويبلغ عدد سكانها تقريبا ٥٠٠٠ نسمة. نبع عين البلد في بتير هو مصدر هام للمياه في القرية حيث ان إمدادات المياه للقرية لا تكفي احتياجاتها في كل وقت وخاصة في فصل الصيف. تشير التقارير الأخيرة إلى تلوث خطير في مياه النبع, ومن بين العديد من مصادر التلوث تشكل تسرب المياه العادمة من الحفر الامتصاصية المصدر الرئيسي للتلوث في هذا النبع. توفر التقنيات الجزيئية التحليل الدقيق و القوي في الكشف عن مسببات الأمراض في المياه حتى تلك الموجودة بكميات صغير جدا. في هذه الأطروحة تم الكشف عن وجود بكتيريا القولونيات البرازية المقاومة للحرارة في مياه النبع باستخدام تقنية الفلترة بالغشاء وتم تمييز بعض العزلات باستخدام تفاعل البلمرة المتسلسل الذي يستهدف جين (16S rDNA) متبوعا بتقنية سانجر لكشف التسلسل الجيني. بالإضافة الى ذلك تم الكشف عن امكانية وجود البكتيريا المسببة للمرض *الإشريكية قولونية*، *السلمونيلا* و *الشيغيلا* باستخدام تفاعل البلمرة المتسلسل الذي يستهدف النوع البكتيري بشكل خاص متبوعا بتقنية المايسك للتسلسل الجيني من الجيل الثاني. أظهرت النتائج وجود عدد كبير من البكتيريا القولونية في مياه النبع و اظهر التصنيف الجزيئي لبعض العزلات من بكتيريا القولونيات البرازية ان معظم هذه العزلات ذات طابع مسبب للمرض. النتائج الخاصة بالكشف عن البكتيريا المسببة للامراض *إشريكية قولونية*، *السلمونيلا و الشيغيلا* اظهرت خلو المياه من *السلمونيلا* و *الشيغيلا* و تاكيد وجود *الإشريكية قولونية*. كشفت تقنية المايسك لتسلسل الجيني من الجيل الثاني التي استهدفت بكتيريا *الإشريكية قولونية* عن وجود بكتيريا *الإشريكية قولونية* ذات طابع مسبب للمرض من خلال المياه.

DECLARATION

I declare that the Master Thesis entitled "**Identification and Molecular Characterization of Thermo Tolerant Fecal Coliforms and Bacterial Pathogens "*E.coli*, *Salmonella* spp and *Shigella* spp" in Ein-Elbalad Spring in Battir**" is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Name and signature: Joseph Ibrahim Danho.
Date: 2016

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Signature: Joseph Ibrahim Danho

Date: 2016

Dedication

I would like to dedicate this thesis to my wife Dalia, daughter Maria and my mother Suzan for all the support and motivation they gave me.

I would like to dedicate this thesis to my brother George.

I would like to dedicate this thesis to Sukri Ahrun and his family for all the help and support they gave me.

Acknowledgment

I would like to acknowledge my supervisor Dr. Ghassan Handal who gave me time experience and support during the work on this thesis.

I would like to acknowledge my teacher and Mentor Dr. Alfred Abed Rabbo for giving me the opportunity in being involved in the master program and supported me all the time.

I would like to acknowledge Miss. Reem Zeitoun and the entire chemistry department in Bethlehem University for their help and support.

I would like to acknowledge Dr. Omar Dar Issa and the UNESCO center in Bethlehem University for giving me access to use the UNESCO lab in my research.

I would like to acknowledge Dr. Antonis Chatzinotas who gave me the opportunity to train on environmental molecular techniques in the UFZ center in Germany.

I would like to acknowledge Dr. Hashem Shahin for conducting the Sanger sequences in his lab.

Finally I want to thank the coordinators of the Biotechnology master program from Bethlehem University and the Poly-technique University in Hebron.

List of Abbreviations:

°C	Centigrade Celsius
pmole	pico mole
µm	micro meter
ml	milliliter
m3	meter cubic
km	kilometer
µl	micro litter
mM	mille Molar
ng	nano gram
g	gram
V	Volt
DNA	Deoxyribonucleic acid
bp	base pair
kb	kilo base (1000 base pairs)
BLAST	Basic Local Alignment Search Tool
rpm	round per minute
g	gravity (measurement for centrifugation)
E.coli	Escherichia coli
ETEC	Enterotoxigenic E.coli
EHEC	Enterohemorrhagic E.coli
EIEC	Enteroinvasive E.coli
EPEC	Enteropathogenic E.coli
EAEC	Enteroaggregative E.coli
AIEC	Adherent-Invasive E.coli
spp	specie
subsp	sub specie
substr	sub strain
Ipah	invasion plasmid antigen H gene

• invA	Invasion gene A
• malB promoter	E.coli promoter region coding for maltose binding proteins
• 16S rDNA	16S ribosomal DNA
• SS agar	Salmonella, Shigella agar
• LB broth	Luria Broth
• NB broth	Nutrient Broth
• TAE buffer	"Tris base/acetic acid/EDTA" Buffer
• EDTA	Ethylenediaminetetraacetic acid
• HEPES buffer	(4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) buffer
• min	minute
• sec	second
• ∞	infinity
• dNTPs	deoxyribonucleotide triphosphates
• ddNTPs	di-deoxyribonucleotide triphosphates
• C1, C2, C3, C4, C5, C6,C7, C8	Isolated Colonies Codes
• NGS	Next generation sequencing
• PCR	Polymerase Chain Reaction
• NCBI	National Center for Biotechnology Information
• Ref #	Reference number
• Cat #	Catalogue number

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CHAPTER 1

1- Introduction

1.1 Battir Village

Battir is a Palestinian village located 6.5 km north-west of Bethlehem city and about 7 km south west of Jerusalem (Appendix 1: location map). The name of the village "Battir" is derived from the Phoenician word "Batara" which means slash. Others refer it to the Arabic name (Bait Al-Tair) which means the house of a bird.

Battir village has been inhabited since the Canaanite and Roman eras [71]. It has a population of approximately 5000 residents [1] and most of them are originated from Iraq and Yemen [71]. The village sits on an area of 7,165 dunums [72]. Battir has an altitude of 761m above sea level and the average annual rainfall is approximately 653mm [71].

Battir village has a landscape that is planted with vegetables and olive trees which are irrigated by a 4000 year old irrigation system formed by layers of stones that make terraces [73]. The terraces are provided with water from natural water sources in the village; mainly springs. Battir has four springs: Ein El-Balad, Ein Jame, Ein Al Baseen, and Ein-Amdan. Among the four mentioned springs Ein-Elbalad spring is considered to be drinkable among the villagers in Battir {71}.

Battir is governed by a village council that its members are appointed by the Palestinian Authority [71]. "Recently Battir was recognized by UNESCO with the Melina Mercouri International Prize for the Safeguarding and Management of Cultural Landscapes" [72].

1.2 Water Status in Battir Village

Battir village council supplies the village with water by the Israeli water company (Mekorot). Mekorot supplies water to the village only between 9 pm to 7 am and provides Battir with a maximum quantity of 350m^3 per day [2]. Water supply timing and quantity make it unavailable during the day; also the water quantity is insufficient to fulfill the need of a village that depends on agriculture where water is essential for irrigation. Because of the previous issues the people of Battir depend on the natural water

resources as a secondary source for them to irrigate and to drink as well. One of the major natural water resources in Battir is Ein-Elblad spring. Ein-Elblad spring provides sufficient water for irrigation and also people get water from it to supply their houses when there is shortage of water from the village council. The use of the spring water does not go under any supervision or regulations and it does not go under any treatment.

One of the major contaminants of the spring water is the sewage water that leaks from the underground cesspits. Like other Palestinian villages, Battir lacks an underground sewage system and people in the village depend on cesspits and boreholes to dump their waste water. By the long term of cesspits usage in the village, sewage water has found a way between the underground soil and rock layers to leak in the spring water which is located in the lower part of Battir [2].

Reports from governmental organizations shows serious fecal contamination in the spring water which is considered to be an indicator of the presence of other pathogens.

In 2006 a water test carried out by the Palestinian Ministry of Health showed a serious fecal contamination in the spring water with 230colonies/100ml [2]. In 2009 a report from the Water Supply and Sewerage Authority in Bethlehem indicated that the Total coliform bacteria in the spring water were too many to count and *E.coli* colonies were from 26 colonies/100ml (Appendix 2: Water test report). These numbers of Coliform bacteria are considered to be very high. According to the World Health Organization WHO any drinking water source should have no coliform bacteria at all in any 100ml sample [74]. In 2009 seventy cases of ameba were reported in the village of people that were found to drink from the spring water [4].

Fecal coliform occurrence in water is regarded to be an indicator of possible presence of pathogenic bacteria and viruses [5]. Of the pathogenic bacteria that are transmitted through water contaminated with sewage, *E.coli*, *Salmonella* spp and *Shigella* spp are the most relevant and their presence may cause serious diseases and clinical symptoms.

1.3 Escherichia coli

Escherichia coli (*E.coli*) are rod shaped gram negative bacteria. *E.coli* are Facultative anaerobic which means the bacteria can live in anaerobic conditions whenever there is no oxygen available [6]. *E.coli* has over 250 serotypes, it could be a pathogenic bacteria and nonpathogenic bacteria that range from harmless gut bacteria to intra and/or extra intestinal pathogen depending on its virulence factors [7].

Pathogenic *E.coli* strains are grouped into six groups according to their virulence factors, clinical features of the disease they cause and phenotypic traits of the bacteria [8]. The groups are Enterotoxigenic *E.coli (ETEC)* Strains, Enterohemorrhagic *E.coli (EHEC)* Strains, Enteroinvasive *E.coli (EIEC)* Strains, Enteropathogenic *E.coli (EPEC)* Strains, Enteroaggregative *E.coli (EAEC)* Strains and Adherent-Invasive *E.coli (AIEC)* Strains [9]. Table 1.2 shows each strain with associated clinical symptoms.

Contaminated water with animal feces or human sewage is a common carrier for pathogenic and nonpathogenic strains of *E.coli*. The determination of pathogenic *E.coli* to be transmitted by water is the infectious dose of it. Strains with low infectious dose are more commonly to be transmitted through water [10]. From the previously mentioned pathogenic stains Enterotoxigenic mainly *O148*, Enterohemorrhagic mainly *O157* and Enteroinvasive serotypes mainly *O124* are capable of being transmitted through contaminated water [11].

Enterotoxigenic *E.coli*: *ETEC* is a major cause of diarrhea in developed countries. *ETEC* cause of what is known as traveler's diarrhea that occurs in people from developed countries visiting developing poor countries. *ETEC* has virulence factors that produce heat stable enterotoxins and other colonization factors. These factors allow the organisms to colonize the small intestine and cause diarrhea [26].

Enterohemorrhagic *E. coli*: Among all the *EHEC* serotypes *O157:H7* is the most common serotype. The *O157* can cause an infection at a dose of 100 bacterial cells or less, the infection happens due food or water contaminated with fecal sources. The *EHEC* can survive for long period of time in animal waste and contaminated water sources. Outbreaks regarding drinking water contaminated with human and animal fecal sources have been well documented. *EHEC* produce Shiga like toxins and the bacteria incubation

period ranges from three to eight days before symptoms occur. Infection with *EHEC* may lead to serious clinical issues like hemolytic uremic syndrome and dysentery. In some cases an infection with *O157:H7* may lead to renal failure [27].

Enteroinvasive *E. coli*: An infection of *EIEC* may occur by ingesting food and water contaminated with fecal sources. The clinical symptoms are very similar to *Shigella* infection or Shigellosis. Infection with *EIEC* results in fever, diarrhea, abdominal pain and may progress to dysentery. Similar clinical symptoms with Shigellosis are result of similar virulence factors shared between the two bacteria. *EIEC* penetrates the epithelial cells in the intestine then the bacteria multiplies and grow inside the host cell using the resources inside the cell, then it start to attack nearby cells and spread [28].

Table 1.2: Pathogenic *E.coli* strains with associated clinical symptoms [10]

Strain	Clinical symptoms
E.coli (ETEC)	Acute watery diarrhea
E.coli (EPEC)	Acute or continual diarrhea
E.coli (EHEC)	Acute or Dysentery (diarrhea with blood an mucus)
E.coli (EIEC)	Shigellosis like symptoms with extreme diarrhea and high fever
E.coli (AIEC)	May attack any part of the digestive system from mouth to anus. Abdominal pain, diarrhea, bloody diarrhea, fever, and weight loss.
E.coli (EAEC)	Watery diarrhea without fever. Silent infection that may results in serious clinical features

1.4 Shigella

Shigella is Gram negative, non-spore forming, non-motile rod shaped bacteria [8], it is facultative anaerobic and belongs to the family of Enterobacteriaceae [12]. *Shigella* has four species. *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei* and *Shigella boydii*, these species are divided accordingly to serogroup A, B, C and D [13].

Almost all the species of *Shigella* bacteria are pathogenic and they cause Shigellosis disease. Shigellosis is an acute diarrheal disease that's symptoms vary from severe to mild symptoms depending on the type and serotype of *Shigella* that caused the infection.

Shigella pathogenicity depends on the amount of cytotoxic Shiga toxin produced from the bacteria. *Shigella Sonnei* and *Shigella flexneri* produce fewer amounts of Shiga toxins than *Shigella dysenteriae* serotype 1, hence *Shigella sonnei* and *Shigella flexneri* cause milder infection than other *Shigella* species [8].

Shigellosis symptoms start to occur when *Shigella* adhere to the internal epithelial cells of the intestine, this capability is achieved by the 160-230kb plasmids that *Shigella* bacteria have, these plasmids encode for the production of proteins that aids in the adhesion process. Invasion plasmid antigens (Ipa) in *Shigella* helps the bacteria in the invasion process and in other regulatory processes [14,15].

Shigella infect the internal epithelial cells of the colon, it enters the cell cytoplasm and spread to neighbor cells intercellularly causing an acute inflammation, the inflammation then causes destruction to the mucosal lining in the intestine [16]. As a result of the intercellular bacterial spreading, Shigellosis may progress to dysentery where severe diarrhea is accompanied with blood and mucus from the intestinal tract [8]. According to the WHO in 2006 *Shigella* caused approximately 165 million cases of severe dysentery in which one million (mostly children) die each year in developed countries [17].

Shigella inhabits the intestinal tract of humans and other primates [18] though the leakage of sewage into fresh water may cause the contamination by the bacteria. *Shigella* can live for approximately 180 days at room temperature in water and hence the transmission of it through contaminated water with sewage water is most likely to happen [8]. Studies show that less than 100 bacterial cells are enough to cause an infection [19].

1.5 Salmonella

Salmonella is rod-shaped gram-negative, motile bacteria belongs to the family of Enterobacteriaceae. There are two species of *Salmonella* spp, *Salmonella enterica* and *Salmonella bongori*.

Unlike other prokaryotes the sub species of *Salmonella* (*enterica*/*bongori*) have many serovars, *Salmonella enterica* has the highest number of over than 2480 serovars while *Salmonella bonjori* has approximately 21 serovars [8]. *Salmonella bongori* is considered as the Lizard *Salmonella* because it was detected in lizards [29] but later it was detected

in other animals [30]. Table 1.4 shows the species and subspecies of *Salmonella* genus with the number of serovars they have.

Most of the *Salmonella enterica* subspecies serotypes are pathogenic to mammals including humans; they cause diseases which are generally named Salmonellosis. Some serovars have specificity to host species such as humans serovars *typhi/paratyphi* A, also *Salmonella* various serovars cause various types of clinical conditions for example, serovar *typhi* causes Typhoid in humans, serovar *typhimurium* causes diarrhea in humans [20]. In addition different human pathogenic serovars has specific molecular mechanism of infection, they must enter through the intestinal internal cells, but once they have succeed to enter, they use different strategies and molecular mechanisms to cause an infection [21]. During the time that *Salmonella* needs to pass the intestinal cell barrier no signs of the disease appear on the infected human [22].

Salmonella can be transmitted through contaminated water with sewage or animal feces. Pathogenic human *Salmonella* can cause two types of Salmonellosis: Typhoid or paratyphoid fever and gastroenteritis [23]. *Salmonella* can cause clinical symptoms with infective dose less than 1000 cells [22] to as few as 15-20 cells [75].

Approximately 5% of the People that have been infected with typhoid fever and cured are considered to be carrier of the pathogen for months or even years. They may be as well chronic carrier of the bacteria in their intestine. These people are considered to be a pathogen reservoir in the environment [22].

Table 1.4: Species and subspecies of *Salmonella* Genus with the number of serovars they have [24].

Species/subspecies	Number of Serovars
S. enterica subsp.	
Enterica	1478
Salamae	498
Arizonae	94
Diarizonae	327
Houtenae	71
indica	12
S. bongori	21
Total	2501

1.6 Bacteria Detection and Characterization Techniques in Water

1.6.1 Bacterial Culture

Bacterial culture over selective and general medium has been widely used as a detection and quantification tool for bacterial cells in a selected sample. The main idea of bacterial culture is to provide nutrients and optimum conditions for growth and development for the bacteria. Culture medium has been designed and modified in order to fit the needs of the required task. Some culture mediums are designed to be able to culture wide spectrum of bacteria at the same time like Luria broth, other are designed be only selective for one type of species like Thiosulfate-citrate-bile salts-sucrose agar or TCBS agar which is selective for the vibrio species, or to be selectively differentiate between two species or more like *Salmonella/Shigella* agar. The selective and differentiation process between species is based on the biochemical properties of the bacteria and the inhibitory factors; for example, lactose fermentation capability.

Culture dependent techniques for the detection and identification of bacteria have many advantages and limitations. The major advantage for culture dependent techniques that they give the researcher the ability to isolate the bacteria alive from the sample which can be used for further analysis [35]. Some bacterial strains have evolved to be antibiotic resistant or disease causing agents thus bacterial cells isolation using culture techniques is vital for the analysis of these isolates [36].

Culture dependent techniques have also limitations. Approximately 99% of bacteria that are monitored under the microscope are uncultivable [37]. Culture medium and conditions sometimes favor a fraction of the habitant bacteria in a sample over the others [35]. The time required to get the culture result can also be a drawback where quick analysis is required. Biased results can occur as well where some bacterial species share the same biochemical properties or phenotypes over the culture medium; for example Proteus bacteria and *Salmonella* show the same phenotypes over *Salmonella/Shigella* agar [38, 39].

Water quality analysis for the presence of bacterial contamination is usually assessed by the presence of coliform bacteria using culturing methods, thus using coliform bacterial culture is one of the basics in water quality analysis [43].

1.6.1.1 Coliform Bacterial Culture

Coliform bacteria are gram negative, aerobic and facultative anaerobic, non-spore-forming, rod-shaped bacteria related to different genus and species. These bacteria are common in some of their biochemical properties like the capability of fermenting lactose [40].

Coliform bacteria have been widely used as indicator organisms for potential fecal and non-fecal contamination in water resources as well as a tool to predict the possible presence of pathogenic microorganisms [41].

Coliform bacteria are grouped into three groups

- Total coliform
- Thermo-tolerant (fecal) coliforms
- Fecal streptococci

Total coliform bacteria include those that are originated from fecal and non-fecal sources and their presence is not a solid indicator of fecal contamination in water. On the other hand thermo tolerant fecal coliform and fecal streptococci are solid indicators of fecal contamination [42].

Several commercial culture mediums are used to culture total and fecal coliforms they include lactose to satisfy the lactose fermenting properties for the coliform bacteria. Temperature can be used as a selective property between total coliform that can grow over 37C^{o} and fecal coliforms or fecal streptococci which are thermo tolerant and can grow on temperature up to 45C^{o} [42].

Coliform bacterial culture is usually done using membrane filter method. Membrane filter method is conducted by filtering the water sample using a sterile membrane filter that is impermeable for the bacteria with 0.22 µm - 0.45µm pore size and then placing it over a broth or agar nutrient in a Petridish and incubated at the required temperature [40]. Several commercial medium are available for coliform culture from liquid broth to solid agars.

1.6.2 Molecular Techniques

In the last two decades molecular techniques has been a major trend in the study of human genetics, microbiology, plants and other organisms. Molecular techniques have shown to be quick and accurate. Of many of the molecular techniques available, conventional PCR and DNA sequencing are of the most relevant.

1.6.2.1 Polymerase Chain Reaction (PCR)

PCR has been widely used in the detection and identification of bacterial cells in samples of interest. PCR is based on DNA replication process. It exponentially amplify the target DNA using synthetic oligonucleotide primers and a DNA polymerase that is thermally stable [44, 45]. In addition to the targeted DNA and DNA polymerase, PCR reaction mixture contains deoxyribonucleotide triphosphates (dNTPs), magnesium as magnesium chloride $MgCl_2$ and other buffers that are able to increase detection limits. Conventional PCR may be modified to be able to detect more than one target by using two sets of primers which is called multiplex PCR. The primers used in the detection play a key role in the PCR. In bacteria some primers are designed over conserved domains such as the 16S rDNA which are capable of amplifying more than one target with same set of primers at one time whereas some primers are designed to be specific that target only one bacterial species or strain [46].

As any other technique PCR has its advantages and drawbacks. The advantages of PCR can be listed in the following points

- Time saving, a typical PCR reaction takes place from one and half to three hours depending on the product being amplified
- Able to detect and amplify uncultivable bacteria for example Campylobacter spp [47].
- PCR avoid bias related to phenotypic characteristics that culture methods may fall in [48].

In contrary to the previously listed advantages, PCR drawbacks are:

- It can’t separate between viable and nonviable organism as long as the DNA is available in the sample [49].

- PCR reactions are prone to contamination which may give false positive results.

Conventional PCR cannot give the result directly after the reaction is finished, gel electrophoresis should be done afterward to detect the presence of positive amplicons. In addition some PCR reactions should be followed by DNA sequencing in order to determine the identity of the amplified amplicon.

1.6.2.2 DNA Sequencing

DNA sequencing is the process of identifying the correct order of nucleotides within a DNA molecule. DNA sequencing is usually conducted post PCR amplification in order to determine the sequence of the PCR amplified product. Nowadays several types of DNA sequencing techniques are available. Sanger Sequencing and next generation sequencing are ones of those many techniques.

1.6.2.3 Sanger Sequencing

Sanger sequencing was developed in 1977 by Frederick Sanger and his colleagues. Sanger sequencing technique works by chain-terminating dideoxynucleotides using DNA polymerase during DNA replication [50]. In molecular laboratories Sanger sequencing is used widely for pathogen detection, mutations detection and other molecular tasks. Sanger reaction is performed in a PCR machine then taken to Sanger sequencing automated machine. The PCR reaction is to produce a single strand DNA template for the sequencer from a double stranded DNA template. In addition to the DNA template, Sanger reaction requires a DNA primer either forward or reverse, a DNA polymerase and normal and modified deoxynucleosidetriphosphates (dNTPs). The modified dNTPs lack a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, this will force the DNA polymerase to stop the extension of the DNA when a modified ddNTP is incorporated. The ddNTPs are fluorescently labeled for detection in automated sequencing machines each one of the modified ddNTPs Adenine, Thymine, Cytosine and Guanine emit light at different wavelengths.

Sanger sequencer machine uses capillary electrophoresis to induct the sample to the machine, then the machine detect dye fluorescence and output data on computer software

as colored peaks or chromatograms. Output data usually needs to be analyzed to remove primer regions and biased nucleotides before going to bioinformatics analysis [51].

Sanger sequencing technique is considered to be the gold standard for sequencing by many researchers in microbiology but despite this fact Sanger sequencing has several limitations [52]. One of the major limitations is the cost and time involved, in comparison to the number of sequences acquired. Errors in the amplification lead to errors in the sequencing step; for example polymerase slippage on short tandem repeats. Lower intensities and missing termination variants tend to lead to sequencing errors accumulating toward the end of ongoing sequences. Reduction in the electrophoresis separation results in base miscalls [53]. Sanger sequencing is incapable of reading more than one sequence in a sample at a time so using universal primers amplified products from environmental samples total DNA to be analyzed by Sanger is impossible.

The limitations of Sanger sequencing and need for a powerful sequencing technique resulted in the development of the next generation sequencing.

1.6.2.4 Next Generation Sequencing (NGS)

Next generation sequencing or called massively Parallel sequencing is considered to be a revolution in the genomic and molecular research because of its fast and massive throughput results. Since its establishments in 2005 NGS has allowed researchers to do experiments that were considered to be impossible to do technically. NGS can sequence a full human genome in less than a day [70].

The technology of NGS lies behind the massively parallel sequencing of the desired DNA molecule by extensively repeated cycles of nucleotide extension using DNA polymerase.

NGS has more than one platform but all of them share common technological features depending on the platform NGS generates thousands to millions of reads in a single instrument run. NGS platforms include

- ILLUMINA/SOLEXA
- APPLIED BIOSYSTEMS/SOLiD
- Roche 454 GS FLX sequencing

Illumina platform is based on using a transparent flow cell that has 8 lanes on the surface bounded to oligonucleotides anchors. The template DNA is fragmented and ligated to oligonucleotide adapters with high ligation efficiency. These oligonucleotides adapters are complementary to the flow-cell anchors which will then hybridize to each other. Clusters with thousands of clonal molecules are usually formed since many amplification cycles result from a single DNA templates. Illumina flow cell is capable of generating $50X10^6$ separate clusters. Clusters over the flow cells are washed by denaturation and then sequenced by synthesis using primer complement to the adapter sequence, DNA polymerase and a mixture of 4 differently colored fluorescent reversible dye terminators [54].

The capability of next generation sequencing of amplifying thousands of reads in a sample encouraged the microbiologists to work on targeting organisms using universal primers like the 16S rDNA for bacteria or using universal primers over the species level like targeting the virulence genes in pathogens or promoter region in *E.coli*.

Using universal primers for next generation sequencing allowed researchers to detect uncultivable organisms in the environment also they were able to detected more than one strain or species in one sample from extracted total DNA.

1.7 16S rDNA Gene

One of the most interesting genes analyzed using Polymerase Chain Reactors and DNA Sequencing was the 16S rDNA gene. In the past 16S rDNA was analyzed among wide range of prokaryotes by Scientists Carl Woese and George E. Fox, these two scientists pioneered the use of this gene and they established phylogenic taxonomy for prokaryotes based on the 16S rDNA [31]. By the nowadays advanced automated PCR and sequencing equipment the study and analysis of the 16S rDNA gene has become easier and more accurate. While many studies was established regarding the analysis of the 16S rDNA gene among different bacteria it was found the sequence of the gene is highly conserved among the genus and species level and variable among different genera [31].

By Using the 16S rDNA, classification and nomenclature of bacterial genera have been established and phylogenetic relations determined also the possibility of detecting bacteria that is uncultivable has been made possible [33].

The advantage of using the 16S rDNA is uncountable in microbiology. In comparison to classical culture methods. The culturing process is based on phenotypic characterization of the isolate and fermentation process of the culturing medium which can be biased due to the presence or absence of non-housekeeping genes due to aberrant expression of characters, also some bacteria can express similar phenotypes over a certain medium, while the 16S rDNA gene is shown to be highly conserved among species by well-established studies which can give accurate identification results.

In addition to detection accuracy the 16S rDNA has made it possible to quickly identify bacterial strains that are very slow to grow. A good example is the mycobacteria which needs 6-8 weeks in culture in order to show phenotypic characters. By using the 16S rDNA it was possible to identify the cultured bacteria in early stages of growth without waiting for so long [34].

The use of the 16S rDNA has proved its efficiency and accuracy in microbiology regarding the identification and characterization of available and novel bacteria, the rocket speed increasing of accumulated knowledge in molecular databases as the National Center for Biotechnology Information data base (NCBI) has made it possible to compare the acquired sequence from any bacterium with the information stored in the database and come back with accurate result about the bacterial isolate in hand.

1.8 Objectives

1.8.1 General Objectives:

- Culturing thermo-tolerant coliform bacteria over HiCrome Ecc Selective agar using membrane filter technique over four weeks of weekly sampling from Ein-Elbalad spring.
- Isolation and molecular characterization of some thermo-tolerant coliform bacteria using 16S rDNA by conventional PCR followed by Sanger sequencing from the cultured coliform bacteria
- Extracting total DNA from enriched samples in nutrient broth over 3 weeks period of sampling from Ein-Elbalad spring for the detection of the presence of *Salmonella* spp, *Shigella* spp and *E.coli* by conventional PCR using species specific primers.
- Running the positive samples for *Salmonella*, *Shigella* and *E.coli* on Illumina next generation sequencing using MISeq platform.

1.8.2 Specific Objectives

- Quantification and molecular characterization of Thermo-tolerant fecal coliform bacteria in the spring of Ein-Elbalad.
- Detection and molecular characterization of bacterial pathogens Salmonella spp, Shigella spp, and E.coli in the spring of Ein-Elbalad.

CHAPTER 2

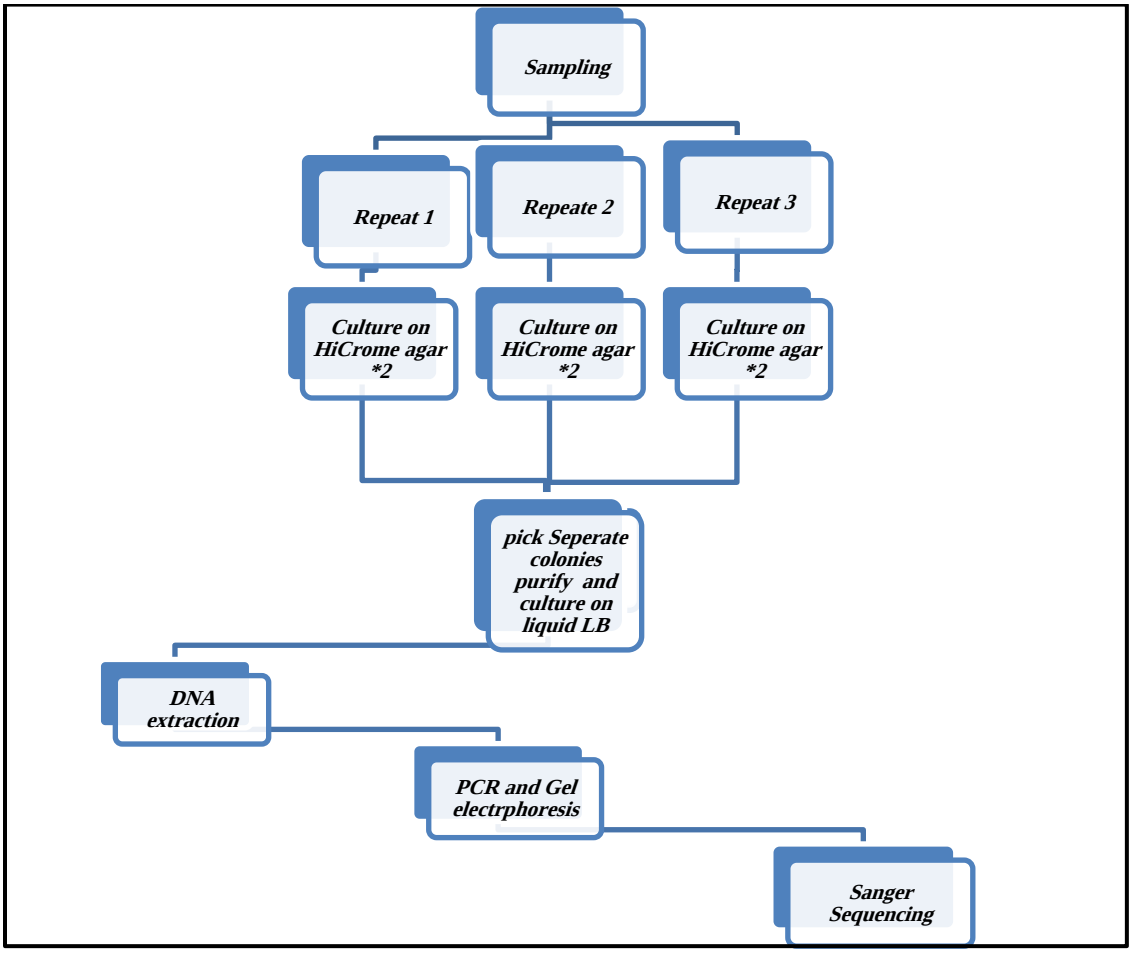
2- Materials & Methods

2.1 Sampling Protocol

Sampling was conducted weekly during (March, 2016) and (April, 2016).

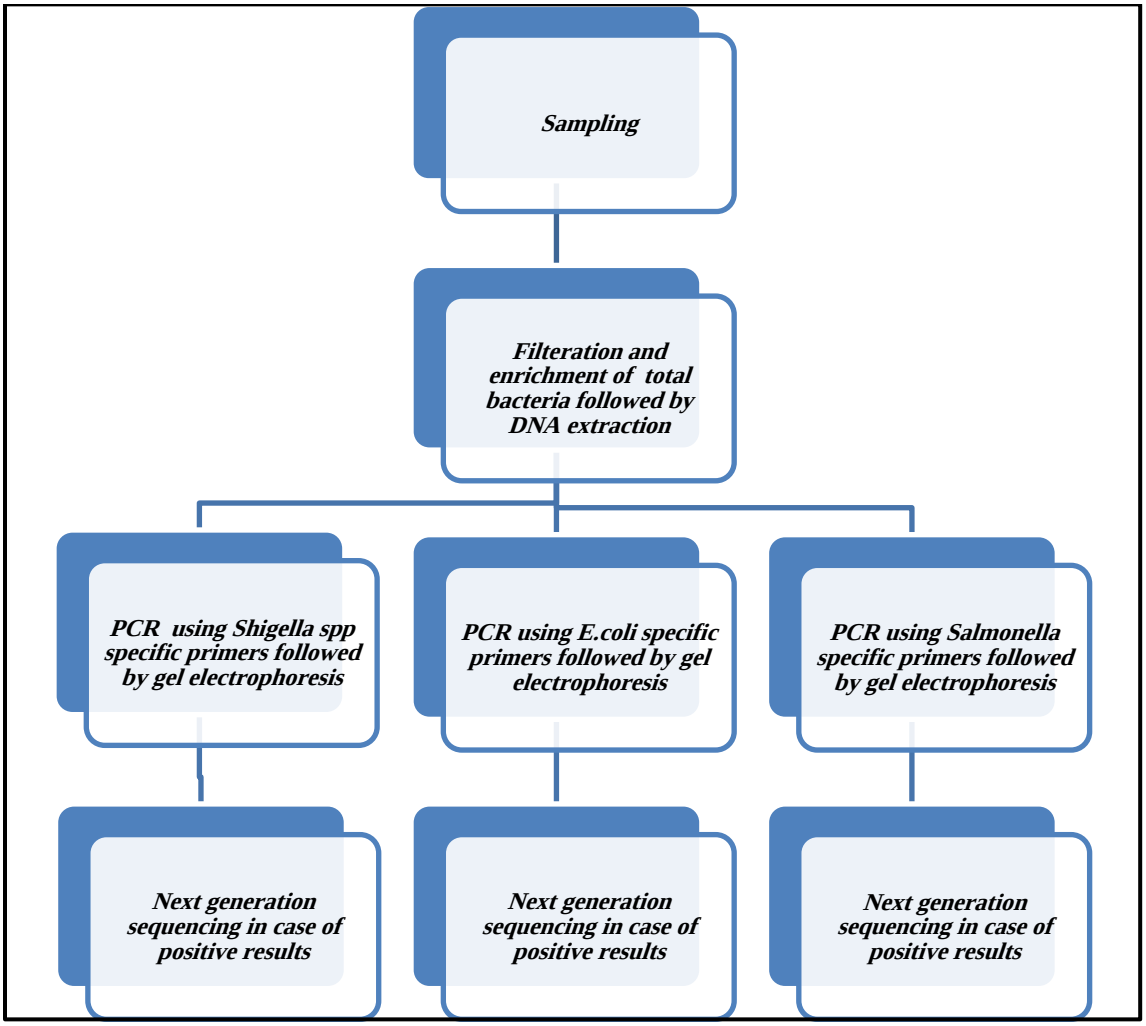
In March three sample repeats were collected each week in 50ml sterilized conical tubes in the morning around 8 am. The samples were put in a portable fridge and brought to the laboratory in the same day within 30 minutes. In total four weeks of sampling were carried out during March. Analytics conducted on the samples are listed in figure 2.1.

Figure 2.1: Analytics conducted on weekly samples during March



In April One Litter of sample was collected each week, the sample was collected in one litter sterilized glass bottle in the morning around 8am. The sample was put in a portable fridge and brought to the laboratory in the same day within 30 minutes. In Total three weeks of sampling were carried out during April. Analytics conducted on each sample are listed in figure 2.2.

Figure 2.2: Analytics conducted on weekly samples during April



2.2 Preparation and Validation of Positive Controls

2.2.1 Preparation of Bacterial DNA

Already cultured and purified bacterial plates for *E.coli*, *Shigella* and *Salmonella* were acquired from The Caritas Baby Hospital in Bethlehem. One colony was picked from each plate using sterilized 10µl pipet tip and cultured in 50ml Luria Broth (Difco^{TM} LB Broth, Lennox cat#: 240230) in sterilized conical tubes in an incubated bench top shaker (MRC Code : TOU-120) for 24 hours and 37ºC/150rpm. (Appendix 3: LB medium preparation).

After 24 hours the tubes were centrifuged using bench top centrifuge (HETTICH EBA 280) for 10 minutes on 6000 rpm, the supernatant was discarded from each tube and the bacterial pellets were suspended in 3 ml HEPES buffer (Sigma**,** HEPES cat#: H3375). (Appendix 4: HEPES buffer preparation). .

0.5ml of each of the suspended bacterial pellets was transferred to 2ml eppendorf tubes and then DNA was extracted from each bacterial suspension using (Wizard^{R} Genomic DNA Purification Kit ref#: A1120) according to manufacturer protocol. (Appendix 5: Genomic DNA extraction protocol). After extraction, the DNA concentration was measured using (NanoDrop 1000, Thermo Scientific) and working dilutions of the DNA (50ng/µl) were prepared by diluting the extracted DNA in free nuclease double distilled water (DDW) in eppendorf tubes. The DNA tubes were sealed and stored at -20ºC until use.

2.2.2 Polymerase Chain Reaction (PCR) and Gel Electrophoresis

2.2.2.1 Primers Preparation

16S rDNA and bacterial species specific primers sequence were acquired from previous studies (Table 2.2). The primers were ordered from Hylabs Company in Israel. The ordered primers arrived to the laboratory in a lyophilized form. The primers were diluted to 100pmol in free nuclease DDW according to the manufacturer protocol. Working primers concentration 10pmol/µl was prepared in aliquots of 100µl by making dilutions in free nuclease DDW 1 to 10. The primers were sealed and stored in eppendorf tubes at -20ºC until used.

Table 2.2: Primers specification

Targeted Species	**Primer Name**	**Primer Sequence & Direction 5'-3'**	**Targeted Gene**	**PCR Product Length**	**Primer source**
universal	27f 1492r	Forward: AGAGTTTGATCCTGGCTCAG Reverse: GGTTACCTTGTTACGACTT	16S rDNA	1500 bp	[55]
E. coli	Eco-1 Eco-2	Forward: GACCTCGGTTTAGTTCACAGA Reverse: CACACGCTGACGCTGACCA	malB promoter	585 bp	[56]
Shigella	Shi-1 Shi-2	Forward: CTTGACCGCCTTTCCGATAC Reverse: CAGCCACCCTCTGAGAGTA	Ipah	610 bp	[57]
Salmonella	Sal-3 Sal-4	Forward: TATCGCCACGTTCGGGCAA Reverse: TCGCACCGTCAAAGGAACC	invA	275 bp	[58]

2.2.2.2 Polymerase Chain Reaction (PCR) Using 16S rDNA Primers and Species Specific Primers

For the 16S rDNA amplifications *E.coli* DNA acquired previously from the Caritas Hospital was chosen to be the positive control.

Three replicates of PCR reactions were done in Bio-Rad Thermal cycler for the extracted DNA from *E.coli* bacteria. Each PCR reaction mixture was 25µl in volume including 12.5µl master mix (Thermo Scientific cat# AB-0575-DC-LD/A), 9.5µl nuclease free DDW, 1µl of 50ng DNA and 1µl forward primer and 1µl reverse primer (Table 2.3). A negative control was included as well that contained all the ingredients except the DNA. The negative control is to make sure that there is no contamination in the PCR mixture. The PCR reaction conditions were optimized for 95ºC initial denaturation for 5 minutes followed by 35 cycles of (95ºC denaturation for 60 seconds, 50ºC annealing for 45 seconds, 72ºC elongation for 90 seconds) and finally 72ºC for 10 minutes for final elongations (Table 2.4).

For the bacterial species specific amplifications. *E.coli*, *Shigella* and *Salmonella* bacterial DNA were used as positive controls each regarding the primers set used.

PCR was done in Bio-Rad Thermal cycler for the extracted DNA from each bacteria. The PCR reaction mixture was 25µl in volume including 12.5µl master mix, 9.5µl nuclease

free DDW, 1µl of 50ng DNA and 1µl forward primer and 1µl reverse primer (Table 2.3). A negative control was included as well.

The PCR reaction conditions were similar for the *Salmonella* and the *E.coli* bacteria and different for the *Shigella* bacteria because of the difference of the primer melting temperature that directly affect the annealing temperature..

For *Salmonella* and *E.coli* bacteria, PCR reaction conditions were optimized for 95ºC initial denaturation for 5 minutes followed by 35 cycles of (95ºC denaturation for 30 seconds, 55ºC annealing for 35 seconds, 72ºC elongation for 30 seconds) and finally 5 minutes at 72ºC for final elongations (Table 2.5).

For *Shigella* bacteria PCR reaction conditions were optimized for 95ºC initial denaturation for 5 minutes followed by 35 cycles of (95ºC denaturation for 30 seconds, 49ºC annealing for 35 seconds, 72ºC elongation for 30 seconds) and finally 5 minutes at 72ºC for final elongations (Table 2.5).

Table 2.3: PCR reaction mixture for 25µl

Reactant	**Volume/µl**
PCR Master Mix 2X	12.5
DNA 30ng/µl	1
Primer Forward	1
Primer Reverse	1
PCR grade water	9.5

Table 2.4: PCR reaction conditions for the 16S rDNA amplification

Mechanism of action	**Temperature in ºC**	**Time**	
Initial denaturation	95	5 minutes	
Denaturation	95	60 seconds	}35X
Annealing	50	45 seconds	
Elongation	72	90 seconds	
Final elongation	72	10 minutes	

Table 2.5: PCR Reaction Condition For *Salmonella, Shigella* and *E.coli*.

Mechanism of action	Temperature in ºC	Time	
Initial denaturation	95	5 minutes	
Denaturation	95	30 seconds	}35X
Annealing	55 *(Salmonella* and *E.coli*) 49 (*Shigella*)	35 seconds	
Elongation	72	30 seconds	
Final elongation	72	5 minutes	

2.2.2.3. Gel Electrophoresis

Gel electrophoresis was conducted in two runs for the controls. A run that included the 16S rDNA PCR products, and a run that included the species specific PCR amplifications.

For the 16S rDNA PCR. 1.5% Agarose gel was prepared by dissolving 1.5g Agarose (Sigma cat# A9539) in 100ml 0.5X TAE buffer (Appendix 6: preparation of TAE buffer) and boiled till all the Agarose was completely dissolved then 1µl of Ethidium Bromide was added and mixed with the liquid Agarose. Before the gel solidifies it was poured in special mold with a comb that makes holes/wells in the gel mold. After the gel solidified it was put in the gel electrophoresis machine (BioRad SUB-CELL^{R} GT) and rinsed with 0.5% TAE buffer. The PCR products that were amplified then were loaded into the gel wells. For each well 4µl of the PCR product was added. Negative control was included. As a measurement tool for the PCR product length, 4µl of 100bp ladder (GeneDirex cat# DM003-R500) was added at the first well. The gel electrophoresis machine was turned on at 120V for 45 minutes. After 45 minutes the gel was taken out of the machine and was imaged in a gel documentation system (Gel DOC^{TM} Imaging System, BioRad) to detect the presence of amplified bands of the PCR reactions on it.(Appendix 7: 100bp DNA ladder measurements).

For the species specific PCRs. 1.5% Agarose gel was prepared. The PCR products that were amplified then were pipetted into the gel wells. For each well 4µl of the PCR product was added. Negative control for each type of bacteria was included. The gel electrophoresis machine was turned on at 120V for 45 minutes. After 45 minutes the gel was taken out of the machine and was imaged in a gel documentation system to detect the presence of amplified bands of the PCR reactions on it.

2.3 Thermo-Tolerant Coliform Bacterial Culture from Samples

Membrane filter method was used to culture coliform bacteria, (Appendix 8: membrane filter protocol). The culture medium that has been used was HiCrome™ ECC Selective Agar (Sigma-Aldrich cat# 85927). (Appendix 9: preparation of HiCrome agar). HiCrome agar that has been used is considered to be an optimum medium for the culture and the detection of coliform bacteria in water. The filtration and culture process was done as follow.

10 ml of the spring water from each sample repeat in March was filtered by electric suction filtration machine (GAST model: DOA-P504-BN) on 0.22µm membrane filter (MCE part#: MFMCE047022GWS). The filter was put then on top of the prepared HiCrome™ ECC Selective Agar in the petri-dish and was incubated for 24 hours at 45ºC.

After 24 hours the colonies were counted. Each week four colonies were picked based on different morphology (color and shape etc…). The picked colonies went under purification process by doing subculture on the same medium (HiCrome™ ECC Selective Agar) in order to make sure that only one strain is acquired from each colony. In total two subcultures were done.

Each week after the last subculture for the picked colonies. Each purified colony was cultured in 50ml tube that contained liquid LB broth; the tubes were put in an incubated shaker at 150rpm for 37ºC for 24 hours.

After 24 hours the tubes were centrifuged for 10 minutes on 6000 rpm, the supernatant was discarded from each tube and the bacterial pellets were suspended in 3 ml HEPES buffer and stored in the fridge at 4ºC until used.

Only 8 colonies from the 16 colonies were decided to be sequenced due to limited financial budget.

2.4 PCR and Sanger Sequencing for the Coliform Bacterial Colonies

2.4.1 DNA Extraction from the Isolated Coliform Bacteria

0.5ml of each of the suspended bacterial pellets was transferred to 2ml eppendorf tubes and then DNA was extracted from each bacterial suspension using Wizard^{R} Genomic DNA Purification Kit. The DNA concentration was measured using (NanoDrop 1000, Thermo Scientific) and working dilutions of the DNA (50ng/µl) were prepared by diluting the extracted DNA in free nuclease DDW in eppendorf tubes. The DNA tubes were sealed and stored at -20ºC until use.

2.4.2 PCR Amplification of the Coliform Bacterial DNA.

PCR was done for the chosen bacteria previously. The PCR reaction mixture was 25µl in volume including 12.5µl master mix, 9.5µl free nuclease DDW, 1µl of 50ng DNA and 1µl forward primer (27f) and 1µl reverse primer (1492r). A positive *E.coli* DNA control and a negative control were included as well. The PCR reaction conditions for the 16S rDNA primers are listed in Table (2.4).

2.4.3 Gel Electrophoresis

1.5% Agarose gel was prepared by dissolving 1.5g Agarose in 100ml 0.5% TAE buffer and boiled till all the agarose was completely dissolved then 1µl of Ethidium bromide was added. The amplified PCR products then were pipetted in the gel wells. For each well 4µl of the PCR product were added. 4µl of 100bp Ladder were added at the first well as a measurement for the PCR product length. The gel electrophoresis machine was turned on at 120V for 45 minutes.

2.4.4 Sanger Sequencing

2.4.4.1 Cleaning the PCR Products

Prior to sequencing, the PCR product should be cleaned from remaining primers, enzymes, salts, nucleotides and other dyes and chemicals. The PCR templates were purified from PCR reactions using Exonuclease I and Antaractic Phosphatase enzymes (Table 2.6). The Enzyme/PCR product mixture was incubated for 37ºC for 30 minutes followed by 80ºC for 20 minutes and incubated finally at 4ºC. (Table 2.7).

Table 2.6: Master Mix of enzymes per 5 µl of PCR

Reagent	Volume µl	Action
Exonuclease I	0.25	degrade the remaining primers
Antaractic Phosphatase	0.25	removes the leftover nucleotides
Nuclease free H2O	1.5	Elution
PCR Products	5	

Table 2.7: Incubation conditions for enzyme purification

Temperature (ºC)	Time (min)
37	30
80	20
4	∞

2.4.4.2 Sanger Sequencing PCR Reaction

10ng of purified PCR product per 100bp of the length of PCR fragment was added in the sequencing reaction mixture. 10 pmol of single forward or Reverse primer was added to 1µl of BigDye^{tm} Terminators V1.1 Cycle sequencing Reaction Kit (Applied Biosystems) along with 4µl of BigDye^{tm} reaction buffer, the reaction was completed to a final volume of 20µl using nuclease free water as shown in table 2.8. Sequence amplification of these samples was performed using Gene Amp-PCR system 9700 from Applied Biosystems according to the amplification program shown in table 2.9.

Table 2.8: Standard sanger sequencing reaction per 20µl

Reagent	Volume in µl/reaction
5x Buffer	4
BigDye^{tm} Terminator V1.1	1
Forward or Reverse primer	1.0
DNA template	2.00
PCR grade water	12.00

Table 2.9: Sequencing PCR reaction program X 30cycle

Temperature º C	Time
96	3 minutes
96	10 seconds
50	5 seconds
60	2 or 4 minutes

2.4.4.3 EDTA/Ethanol Precipitation of Cycle-Sequenced Products

PCR products were cleaned from primers, excess dNTPs, incorporated dyes using EDTA/ Ethanol Precipitation method. EDTA helps stabilize extension products during precipitation, and also helps to wash out any cooperated dyes from the completed reaction. For each 20µl of sequencing reaction 5µl of 125mM EDTA and 100µl of absolute ethanol were added and mixed gently the mixture was incubated in the dark for 20 minutes at 20ºC. Then samples were centrifuged at 3800rpm for 30 minutes at 4ºC. The supernatant was discarded and 60µl of 70% ethanol were added to each sample and the centrifuged directly at 3800rpm for 20 minutes at 4ºC. The supernatant was discarded and the PCR tubes or plate was inverted and spin up to 500rpm for 1minute. The samples were put on 95ºC for 5 minutes in order to remove any residual ethanol. Finally the samples were suspended in 16µl HI Di Formamide (CAT#4311320, Applied Biosystems) and were put on 95ºC for 2 minutes for denaturation and then cooled on ice for 5 minutes. Samples were loaded in 96- well optical reaction plate from applied Biosystems and were run on ABI 3130 XL Genetic Analyzer (Applied Biosystems) at Bethlehem University.

2.5 Sequence Analysis with Bioinformatics Tools

2.5.1 Cleaning the Sequences

The sequence for each bacterial isolate 16S rDNA gene was acquired from the Sanger sequencer in Fasta format. The sequence from the 16S rDNA amplifications should have the size of 1500bp. But the available sequencer was not able to sequence all the PCR product instead it was able to sequence 500bp-550bp maximum with lots of errors in the last 80bp-100bp of each sequence.

The sequences were trimmed at the primer region. Places where there were ambiguity codes were fixed and replaced manually with the correct nucleotides. Bad sequences

from the end of the whole sequence were trimmed as well which yielded approximately 400bp cleaned sequence for each isolate. The work was done using BioEdit sequence alignment editor [59].

The cleaned pair of sequence forward and reverse for each bacteria were attached together by attaching the forward sequence with the reverse complement of the reverse sequence adding (NNNNNNN) letters between them which the blast program will recognize as a gap in the sequence. This process provided 800bp in forward strand direction for each sequence for blasting process on the database.

2.5.2 Basic Local Alignment Tool

After the DNA sequences were cleaned. They were taken to NCBI Database which has an online tool (BLAST) http://blast.ncbi.nlm.nih.gov/Blast.cgi this tool is capable of matching the sequences that are submitted in it with the sequences in the database to investigate the sequence identity. Since the sequences I have were DNA sequences nucleotide blast was chosen to perform the action. The Database chosen was 16S rDNA and highly similar sequences were requested only to come back as results. After clicking on the tab submit the website came back with the matched results.

2.6 Samples Enrichment and Total DNA Extraction

2.6.1 Samples Enrichment

Samples Enrichment was done only for April Samples.

After each weekly sample arrive to the Laboratory. The Sample (1 Litter) was filtered by suction filtration on top of a membrane filter 0.22µm, then the filter was put into a sterilize glass bottle that has an already prepared 150ml of nutrient broth NB (Difco™ Nutrient Broth cat# 234000). (Appendix 10: nutrient broth preparation). The bottle was closed and put in an incubator shaker at 37ºC/150rpm for 24 hours.

After 24 hours 50ml of the enriched NB was put in 50ml conical tubes. Enriched NB Tube was centrifuged at 6000rpm for 10 minutes. The supernatant was thrown away and the bacterial pellet was suspended in 3ml HEPES buffer.

2.6.2 Total DNA Extraction for Gram Negative Bacterial from the Enriched Samples

0.5ml of the suspended bacterial pellets was transferred to 2ml eppendorf tube and then DNA was extracted from the bacterial suspension using Wizard® Genomic DNA Purification Kit. The DNA concentration was measured and working dilutions of the DNA (50ng/µl) were prepared by diluting the extracted DNA in free nuclease double distilled water (DDW) in eppendorf tubes. The DNA tubes were sealed and stored at -20ºC until use.

2.7 PCR and Gel Electrophoresis for the Total DNA from the Enriched Samples Using Species Specific Primers for *Salmonella, Shigella* and *E.coli*.

2.7.1 PCR Amplification

PCR was done for the three weeks enriched samples total DNA. Each PCR reaction mixture was 25µl in volume including 12.5µl master mix, 9.5µl free nuclease DDW, 1µl of 50ng DNA and 1µl forward primer and 1µl reverse primer. The primers that were used were for the Detection of *Salmonella*, *Shigella* and *E.coli* on the species level. (Table 2.2). A positive DNA control and a negative control were included as well for each bacteria. The PCR reaction conditions for the species specific primers are listed in Table 2.5.

2.7.2 Gel Electrophoresis

1.5% Agarose gel was prepared by dissolving 1.5g Agarose in 100ml 0.5% TAE buffer and boiled till all the Agarose was completely dissolved then 1µl of Ethidium bromide was added. The amplified PCR products then were pipetted in the gel wells. For each well 4µl of the PCR product was added. 4µl of 100bp Ladder was added at the first well as a measurement for the PCR product length. The gel electrophoresis machine was turned on at 120V for 45 minutes.

2.8 MISeq Next Generation Sequencing and Bioinformatics Analysis.

The Samples that gave positive amplicon for *Shigella*, *Salmonella* or *E.coli* was sent to Hylabs Company in Israel (www.hylabs.co.il) for next generation Sequencing using MISeq sequencing platform. The capability of the platform is to give 250bp sequences for each forward and reverse strands with maximum of 30,000 reads for each sample.

The company was committed to provide the following data

- Run report (.pdf)
- FASTQ data files after basic bioinformatics (trimming of linker, barcode and primer sequences)

2.8.1 Bioinformatics Analysis of the Next generation Sequencing Data

Sequences were acquired from Hylabs Company in a FASTQ format in three files each one for each week DNA sample. The FASTQ files were converted to FASTA format using online tool Sequence conversion provided by bugaco.com (http://sequenceconversion.bugaco.com/converter/biology/sequences/)

The three FASTA files were merged together into one FASTA file using BioEdit software [59]. The sequences were merged into one file to ease the analysis process; since I am not concerned about weekly analysis at that point; the aim is to identify the potential presence of pathogens. If any pathogen is detected, the sequence ID is enough to tell from which week the DNA sequence of that pathogen came from.

The duplicated sequences were removed using online tool duplicate sequence remover ElimDupes (http://hcv.lanl.gov/content/sequence/ELIMDUPES/elimdupes.html).

The Sequences were clustered using online tool CD-HIT Suite: Biological Sequence Clustering and Comparison (http://weizhong-lab.ucsd.edu/cdhit_suite/cgi-bin/index.cgi?cmd=cd-hit-est). The sequence clustering process aim to group similar sequences in one representative sequence that meets similar threshold. The sequence identity cutoff that was used during clustering was 0.9.

The FASTA file that was processed with the previous tools was then blasted on the NCBI database using Microbial Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=MicrobialGenomes).

The FASTA file was blasted using the all complete genome databases and the organism was selected according to the specific primer used, the maximum targeted sequences were 10 and the blast was optimized to highly similar sequences. Other algorithm parameters were kept default.

The blast results were acquired in an excel table from the download tab on the blast results page.

The blast results were grouped in an excel file according to their significance. Blast results that had an E value < (1×10^{-50}) were considered to be significance and not by chance and were accepted. Other results that were had E value > (1×10^{-50}) were considered to be not significant and were discarded.

CHAPTER 3

3 Results

3.1 Validation of the Positive Controls

3.1.1 Validation of the Positive Control for the 16S rDNA PCR Amplification

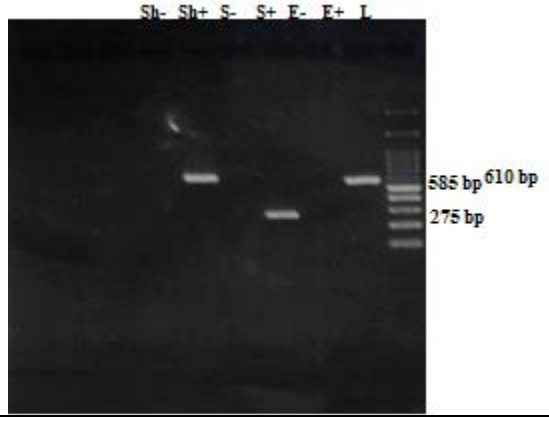
For the 16S rDNA *E.coli* Bacteria was used as a positive control. The *E.coli* extracted DNA was amplified three times in three reactions and one negative control was included. All the three reactions gave amplicons on the gel with the expected size (1500bp). Figure 3.1 shows the Gel photo of the PCR products.

Figure 3.1: Gel photo of the 16S rDNA amplifications of the *E.coli* positive control. Wells arrangement from right to left: DNA Ladder (L), *E.coli* positive control(1st+), empty well (E), 2nd *E.coli* positive control (2nd+), empty well (E), 3rd *E.coli* positive control (3rd+), negative control (N-).

3.1.2 Validation of the Positive Control for the Species Specific PCR Amplification

For the species specific PCR amplifications *E-coli*, *Shigella* and *Salmonella* bacteria were used as a positive control each for its own set of primers. The three bacteria extracted genomic DNA were amplified and one negative control was included for each one. All the three reactions gave amplicons on the gel with the expected sizes. 585 bp for *E.coli,* 610bp for *Shigella* and 275bp for *Salmonella*. Figure 3.2 shows the Gel photo of the PCR products.

Figure 3.2: Gel photo of the positive control for the species specific PCR amplification. Wells arrangement from right to left: DNA Ladder (L), *E.coli* positive control (E+), *E.coli* reaction negative control (E-), *Salmonella* positive control (S+), *Salmonella* reaction negative control (S-), *Shigella* positive control (Sh+), *Shigella* reaction negative control (Sh-)



3. 2 Bacterial Culture over HiCrome™ ECC Selective Agar

HiCrome™ ECC agar was used to culture thermo-tolerant coliform bacteria from the spring water weekly during March/2016. In total four weeks cultures were done with a duplicate for each sampling repeat. Membrane filter method was used in the culturing process. Figure 3.3 shows the weekly cultures that were conducted during March/2016. The average number of colonies in was 300 colony/100ml. The highest weekly average was in 11/3/16 with 450colony/100 and the lowest average was in 18/3/16 with 210 colony/100ml.

The average in 2/3/16 and 25/3/16 were 310 colony/100ml and 220colony/100 respectively.

Figure 3.3: Weekly thermo-tolerant coliform bacterial cultures over HiCrome™ ECC that were conducted during March/2016

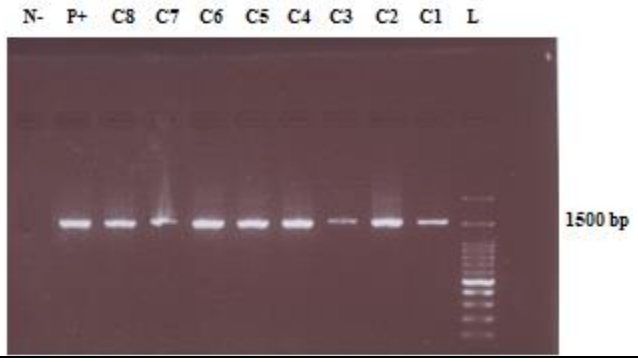
3.3 PCR for the Chosen Thermo-tolerant Coliform Bacterial Colonies

PCR was done for 8 bacterial colonies with different morphologies from the coliform cutures. Table 3.4 shows the colonies phenotypes. Colonies were picked and purified twice over HiCrome™ agar and then they were cultured in LB broth and the genomic DNA was extracted. The PCR was done using 16S rDNA primers. All the bacterial DNA showed amplification with the expected size (1500bp). Figure 3.5 shows the gel photo of the PCR amplification.

Table 3.4: colonies phenotypes

Colony ID	Colony phenotype on HiCrome agar
C1	Blue
C2	Colorless/Yellowish
C3	Red Colony
C4	Green-Blue Colony
C5	Blue Colony
C6	Blue Colony
C7	Blue Colony
C8	Small Pink Colony

Figure 3.5: PCR amplification of the bacterial colonies DNA. Arrangment of the gel from right to left: DNA ladder, C1, C2, C3, C4, C5, C6, C7, C8, positive control, negative control.



3.4 Sanger Sequencing for the Bacterial Colonies

The PCR products from the bacterial colonies DNA were purified and sequenced using Sanger sequencing machine. The resulted sequences were cleaned from biased nucleotides manually using BioEdite software. The Sequences were blasted on NCBI database. The results are shown in table 3.5.

Table 3.5: BLAST outcome for the Sanger sequences of the coliform bacterial isolates DNA

Colony ID	Outcome: Species/strains	Max Score
C1	~~*Shigella dysenteriae Sd197 strain*~~ *Escherichia coli str. K-12 substr*	730 730
C2	*Pseudomonas aeruginosa*	719
C3	~~*Shigella flexneri strain ATCC 29903*~~ *Enterobacter*	732 730
C4	*Klebsiella pneumoniae*	734
C5	*Escherichia coli strain U 5/41*	739
C6	*Escherichia coli strain U 5/41* ~~*Shigella dysenteriae Sd197 strain Sd197*~~	734 734
C7	*Escherichia coli str. K-12 substr. MG1655*	736
C8	*Aeromonas*	732

(Appendix 12: Blasted Sanger sequences)

Morphology of the colonies C1, C3 and C6 on the agar was taken into consideration to differentiate between the BLAST outcomes since they both give the same max score for *Shigella* and *E.coli* species. Max score is the score of single best aligned sequence or the highest alignment score (bit-score) between the query sequence and the database sequence segment.

Shigella species were excluded from the results since they did not show their morphology colorless colonies on the HiCrome™ agar whereas *E.coli* and *Enterobacter* showed blue colonies and red colony respectively on the HiCrome agar.

3.5 PCR Amplification of the Total DNA from the Enriched Samples

After the total DNA were extracted from the enriched bacterial growth. PCR was done for the extracted DNA using species specific primers (Table 2.2). The DNA was amplified each week in three reactions for *E.coli*, *Salmonella* and *Shigella* bacteria including positive and negative control for each bacterial species. All the three samples from the three weeks showed amplification for *E.coli* bacteria but they showed negative results for *Salmonella* neither *Shigella* bacteria, all the positive controls in the reactions were amplified successfully. Figure 3.7 shows the PCR amplification for the *E.coli* and

the *Salmonella* bacteria. Figure 3.8 shows the PCR amplification for the *Shigella* bacteria.

Figure 3.7: PCR amplifications for *E.coli* and *Salmonella* bacteria. Arrangment of the gel from right to left: DNA ladder (L), week 1 *E.coli* (Ew1), week 2 *E.coli* (Ew2), week 3 E.coli (Ew3), negative control (E-), *E.coli* positive control (E+), 2_{nd} DNA ladder (L), week 1 *Salmonella* (Sw1) , week 2 *Salmonella* (Sw2), week 3 *Salmonella* (Sw3), negative control (S-), *Salmonella* positive control (S+).

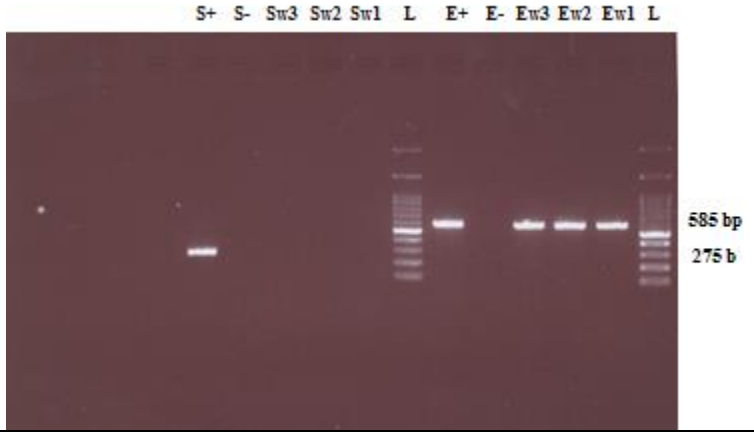


Figure 3.8 PCR amplifications for the *Shigella* bacteria. Arrangment of the gel from right to left: DNA ladder, week 1 *Shigella* (Shw1) , week 2 *Shigella* (Shw2) , week 3 *Shigella* (Shw3), negative control (Sh-), *Shigella* positive control (Sh+)

3.6 MISeq Next Generation Sequencing and Bioinformatics Analysis Results

E.coli amplified PCR product was sent to Hylabs company for next generation sequencing using **Illumina MiSeq 250x2 (Kit v2)**

The FASTAQ files were converted to FASTA format. All the sequences were merged into one FASTA file. The duplicated sequences were removed and the sequences were clustered.

The previous processes reduced the number of sequences from 7060 sequences to 4587 sequences.

The 4587 sequences were Blasted together in one file on the NCBI database using Microbial Nucleotide BLAST and Results with E value < (1×10^{-50}) were accepted. In total 128 unique blast results were acquired from all the sequences. Table 3.6 shows each E.coli bacterial strain/blast result detected in the three samples with the gene bank accession numbers/ID.

Table 3.6 Blast results and gene bank accession ID for the Next Generation Sequences of the *E.coli* specie in the three weeks samples

Gene Bank ID	Blast Results
gi|822022293|ref|NZ_CP011416.1|	*Escherichia coli strain CFSAN029787*
gi|170079663|ref|NC_010473.1|	*Escherichia coli str. K12 substr. DH10B*
gi|157159467|ref|NC_009800.1|	*Escherichia coli HS*
gi|170018061|ref|NC_010468.1|	*Escherichia coli ATCC 8739*
gi|976151040|ref|NZ_CP007491.1|	*Escherichia coli strain ACN002*
gi|443615330|ref|NC_020163.1|	*Escherichia coli APEC O78*
gi|771462547|ref|NZ_LM995446.1|	*Escherichia coli genome assembly EcRV308Chr*
gi|771462916|ref|NZ_LM993812.1|	*Escherichia coli genome assembly EcHMS174Chr*
gi|471332236|ref|NC_020518.1|	*Escherichia coli str. K-12 substr*
gi|749304944|ref|NZ_CP009644.1|	*Escherichia coli ER2796*
gi|170679574|ref|NC_010498.1|	*Escherichia coli SMS-3-5*
gi|974631303|ref|NZ_CP013831.1|	*Escherichia coli strain CD306*
gi|927318058|ref|NZ_CP012631.1|	*Escherichia coli strain SF-173*
gi|157154711|ref|NC_009801.1|	*Escherichia coli E24377A*
gi|749303439|ref|NZ_CP007391.1|	*Escherichia coli strain ST540*
gi|749303222|ref|NZ_CP007390.1|	*Escherichia coli strain ST540*
gi|749300132|ref|NZ_CP009273.1|	*Escherichia coli BW25113*
gi|749299244|ref|NZ_CP008801.1|	*Escherichia coli KLY*
gi|749295052|ref|NZ_HG738867.1|	*Escherichia coli str. K-12 substr. MC4100*
gi|749299145|ref|NZ_CP009859.1|	*Escherichia coli strain ECONIH1*

gi|749204326|ref|NZ_CP009104.1|	*Escherichia coli strain RM9387*
gi|817695621|ref|NZ_CP011331.1|	*Escherichia coli O104:H4 str. C227-11*
gi|407479587|ref|NC_018658.1|	*Escherichia coli O104:H4 str. 2011C-3493 chromosome*
gi|407466711|ref|NC_018661.1|	*Escherichia coli O104:H4 str. 2009EL-2071*
gi|910228862|ref|NZ_HF572917.1|	*Escherichia coli HUSEC2011*
gi|218693476|ref|NC_011748.1|	*Escherichia coli 55989 chromosome*
gi|218698419|ref|NC_011750.1|	*Escherichia coli IAI39 chromosome*
gi|983532903|ref|NZ_CP008697.1|	*Escherichia coli strain ST648*
gi|749301983|ref|NZ_CP007393.1|	*Escherichia coli strain ST2747*
gi|26245917|ref|NC_004431.1|	*Escherichia coli CFT073*
gi|215485161|ref|NC_011601.1|	*Escherichia coli 0127:H6 E2348/69, strain E2348/69*
gi|387605479|ref|NC_017626.1|	*Escherichia coli 042*
gi|749299309|ref|NZ_CP007394.1|	*Escherichia coli strain ST2747*
gi|974632753|ref|NZ_CP013835.1|	*Escherichia coli strain JJ2434*
gi|1007452042|ref|NZ_CP014316.1|	*Escherichia coli JJ1887*
gi|749619302|ref|NZ_CP007592.1|	*Escherichia coli O157:H16 strain Santai*
gi|749304472|ref|NZ_CP007133.1|	*Escherichia coli O145:H28 str. RM12761*
gi|749302377|ref|NZ_CP007799.1|	*Escherichia coli Nissle 1917*
gi|749302203|ref|NZ_CP006027.1|	*Escherichia coli O145:H28 str. RM13514*
gi|749302183|ref|NZ_CP007136.1|	*Escherichia coli O145:H28 str. RM12581*
gi|749302049|ref|NZ_CP006262.1|	*Escherichia coli O145:H28 str. RM13516*
gi|1028891029|ref|NZ_CP015240.1|	*Escherichia coli strain 2011C-3911*
gi|260866153|ref|NC_013364.1|	*Escherichia coli O111:H- str. 11128*
gi|749302083|ref|NZ_CP008957.1|	*Escherichia coli O157:H7 str. EDL933*
gi|749301875|ref|NZ_CP008805.1|	*Escherichia coli O157:H7 str. SS17*
gi|749205893|ref|NZ_CP010304.1|	*Escherichia coli O157:H7 str. SS52*
gi|1028864091|ref|NZ_CP015241.1|	*Escherichia coli strain 2013C-4465*
gi|992382288|ref|NZ_CP014314.1|	*Escherichia coli O157:H7 strain JEONG-1266*
gi|1016104114|ref|NZ_CP015023.1|	*Escherichia coli strain SRCC 1675*
gi|1016104205|ref|NZ_CP015020.1|	*Escherichia coli strain 28RC1*
gi|291280824|ref|NC_013941.1|	*Escherichia coli O55:H7 str. CB9615*
gi|254791136|ref|NC_013008.1|	*Escherichia coli O157:H7 str. TW14359*
gi|209395693|ref|NC_011353.1|	*Escherichia coli O157:H7 str. EC4115*
gi|844788515|ref|NZ_CP006635.1|	*Escherichia coli PCN033 plasmid p3PCN033*
gi|844749410|ref|NZ_CP006642.1|	*Escherichia coli PCN061 plasmid PCN061p6*
gi|1016070220|ref|NZ_CP011062.1|	*Escherichia coli str. Sanji plasmid pSJ_255*
gi|386622414|ref|NC_017646.1|	*Escherichia coli O7:K1 str. CE10*
gi|157412014|ref|NC_009838.1|	*Escherichia coli APEC O1 plasmid pAPEC-O1-R*

gi|1032607515|ref|NZ_CP015833.1|	*Escherichia coli O157 strain 180-PT54 plasmid*
gi|218552585|ref|NC_011741.1|	*Escherichia coli IAI1 chromosome*
gi|260842239|ref|NC_013353.1|	*Escherichia coli O103:H2 str. 12009 DNA*
gi|749299616|ref|NZ_CP007392.1|	*Escherichia coli strain ST2747*
gi|222154829|ref|NC_011993.1|	*Escherichia coli LF82 chromosome*
gi|260853213|ref|NC_013361.1|	*Escherichia coli O111:H- str. 11128*
gi|387615344|ref|NC_017634.1|	*Escherichia coli O83:H1 str. NRG 857C chromosome*
gi|749197021|ref|NZ_CP010371.1|	*Escherichia coli strain 6409*
gi|1016070219|ref|NZ_CP011061.1|	*Escherichia coli str. Sanji*
gi|1000987187|ref|NZ_CP014495.1|	*Escherichia coli strain SaT040*
gi|764931866|ref|NZ_CP010315.1|	*Escherichia coli strain 789*
gi|544388862|ref|NC_022364.1|	*Escherichia coli LY180*
gi|378710836|ref|NC_016902.1|	*Escherichia coli KO11*
gi|386707734|ref|NC_017664.1|	*Escherichia coli W*
gi|386698504|ref|NC_017660.1|	*Escherichia coli KO11FL*
gi|1002315738|ref|NZ_CP014522.1|	*Escherichia coli strain ZH063*
gi|1000874265|ref|NZ_CP014497.1|	*Escherichia coli strain ZH193*
gi|802098267|ref|NZ_HG941718.1|	*Escherichia coli ST131 strain EC958 chromosome*
gi|1016540303|ref|NZ_CP015138.1|	*Escherichia coli strain Ecol_732*
gi|764383634|ref|NZ_CP010876.1|	*Escherichia coli strain MNCRE44,*
gi|556550243|ref|NC_022648.1|	*Escherichia coli JJ1886*
gi|984670707|ref|NZ_CP014197.1|	*Escherichia coli strain MRE600*
gi|983374764|ref|NZ_CP013029.1|	*Escherichia coli strain 2012C-4227*
gi|944404559|gb|CP013029.1|	*Escherichia coli strain 2012C-4227*
gi|1000909432|ref|NZ_CP014488.1|	*Escherichia coli strain G749*
gi|1016879263|ref|NZ_CP015076.1|	*Escherichia coli strain Ecol_448*
gi|1016879190|ref|NZ_CP015074.1|	*Escherichia coli strain Ecol_745*
gi|1016879307|ref|NZ_CP015069.1|	*Escherichia coli strain Ecol_743*
gi|387828053|ref|NC_013654.1|	*Escherichia coli SE15 DNA*
gi|844787447|ref|NZ_CP006632.1|	*Escherichia coli PCN033*
gi|971520884|gb|CP008697.1|	*Escherichia coli strain ST648*
gi|749204315|ref|NZ_CP009685.1|	*Escherichia coli str. K-12 substr. MG1655*
gi|410480139|ref|NC_018650.1|	*Escherichia coli O104:H4 str. 2009EL-2050*
gi|852039257|ref|NZ_CP009106.2|	*Escherichia coli strain 94-3024*
gi|983388683|ref|NZ_CP013025.1|	*Escherichia coli strain 2009C-3133*
gi|110640213|ref|NC_008253.1|	*Escherichia coli 536*
gi|944398239|gb|CP013025.1|	*Escherichia coli strain 2009C-3133*
gi|817696214|ref|NZ_CP007594.1|	*Escherichia coli strain SEC470*
gi|387610477|ref|NC_017633.1|	*Escherichia coli ETEC H10407*

gi|749301955|ref|NZ_CP009072.1|	*Escherichia coli ATCC 25922*
gi|749199128|ref|NZ_CP005930.1|	*Escherichia coli APEC IMT5155*
gi|755163146|ref|NZ_CP010816.1|	*Escherichia coli strain BL21 (TaKaRa)*
gi|754982426|ref|NZ_CP007265.1|	*Escherichia coli strain ST540,*
gi|751927929|ref|NZ_CP010585.1|	*Escherichia coli strain C41(DE3)*
gi|856839422|ref|NZ_CP011938.1|	*Escherichia coli strain C43(DE3)*
gi|952011558|ref|NZ_CP013112.1|	*Escherichia coli strain YD786*
gi|386612163|ref|NC_017641.1|	*Escherichia coli UMNK88*
gi|749198604|ref|NZ_CP009789.1|	*Escherichia coli K-12 strain ER3413*
gi|387610385|ref|NC_017724.1|	*Escherichia coli ETEC H10407 p948 plasmid*
gi|260763802|ref|NC_013369.1|	*Escherichia coli O26:H11 str. 11368 plasmid pO26_1 DNA*
gi|749302221|ref|NZ_CP006028.1|	*Escherichia coli O145:H28 str. RM13514 plasmid pO145-13514*
gi|753215947|ref|NZ_CP010344.1|	*Escherichia coli ECC-1470*
gi|983552073|ref|NZ_CP012633.1|	*Escherichia coli strain SF-166*
gi|983530474|ref|NZ_CP012635.1|	*Escherichia coli strain SF-088*
gi|983559881|ref|NZ_CP012625.1|	*Escherichia coli strain SF-468*
gi|983572413|ref|NZ_CP007149.1|	*Escherichia coli RS218*
gi|386602643|ref|NC_017632.1|	*Escherichia coli UM146*
gi|386597751|ref|NC_017628.1|	*Escherichia coli IHE3034*
gi|117622295|ref|NC_008563.1|	*Escherichia coli APEC O1*
gi|91209055|ref|NC_007946.1|	*Escherichia coli UTI89*
gi|1032639818|ref|NZ_CP015846.1|	*Escherichia coli O157:H7 strain FRIK2069*
gi|1032639808|ref|NZ_CP015843.1|	*Escherichia coli O157:H7 strain FRIK2455*
gi|983572457|ref|NZ_CP012802.1|	*Escherichia coli O157:H7 strain WS4202*
gi|926539508|gb|CP012633.1|	*Escherichia coli strain SF-166*
gi|926480196|gb|CP012635.1|	*Escherichia coli strain SF-088*
gi|926468553|gb|CP012625.1|	*Escherichia coli strain SF-468*
gi|1000909435|ref|NZ_CP014491.1|	*Escherichia coli strain G749 plasmid pG749_3*
gi|386637352|ref|NC_017631.1|	*Escherichia coli ABU 83972*
gi|386632422|ref|NC_017652.1|	*Escherichia coli str. 'clone D i14*

CHAPTER 4

4 Discussion

4.1 Thermo-Tolerant Coliform Bacterial Culture and Sequencing

Coliform bacterial cultures during the 4 weeks period indicated a high percentage of bacteria in the spring water with average number of 300colony/100ml. The noted reduction in colonies between the first two weeks and the last two weeks of March can be explained by the rainfall that happened during middle of March and that may diluted the bacterial concentration in the spring. Regardless this event, still the bacterial concentration is very high to a water source that people drink from it frequently, also the bacterial concentration is susceptible to increase further more during summer season when there is no rainfall and weaker flow in the spring water.

Sequencing some bacterial colonies from the coliform cultures came out with significant presence of bacterial species that are related to waste water contamination. Table 4.1 shows species/strain of the detected coliform bacteria.

Table 4.1: Coliform isolates species/strains

Colony ID	Outcome: Species/strains
C1	*Escherichia coli str. K-12 substr*
C2	*Pseudomonas aeruginosa*
C3	*Enterobacter*
C4	*Klebsiella pneumoniae*
C5	*Escherichia coli strain U 5/41*
C6	*Escherichia coli strain U 5/41*
C7	*Escherichia coli str. K-12 substr.*
C8	*Aeromonas*

- C1 and C7 indicated the presence of E.coli str. K-12. *E.coli K-12* was first isolated in 1922 from fecal samples of a previously diphtheria patient in Standford university [60]. *K-12* strains do not contain any *E.coli* virulence genes [61] and they are unable to colonize the human gut [62] so they are nonpathogenic type.

- C5 and C6 isolates indicated the presence of *E.coli U 5/41*. *E.coli U 5/41* was first isolated in 1941 from a patient of cystitis [63]. This strain is considered to be human and animal pathogen [65]. *E.coli U 5/41* has shown to be able to adhere to the epithelial cells in the urinary tract and cause a urinary tract infection. This has been discovered to be possible due to two factors. The O and K antigens that work as virulence factors and the morphology of the bacteria itself which has Piliation on its surface that ease the adhering process [64].
- C2 indicated the presence of *Pseudomonas aeruginosa* which is considered to be opportunistic pathogen. *Pseudomonas aeruginosa* can cause urinary tract infections, gastrointestinal infections as well as other infections like endocarditis, osteomyelitis, pneumonia, meningitis, septicemia, folliculitis and ear infections acquired by exposure to recreational waters containing the bacterium [66]
- C3 indicated the presence of *Enterobacter*. Some *Enterobacter* are considered to be pathogenic that can cause urinary tract infection and respiratory infections. *Enterobacter* infection can be acquired by exogenous or endogenous sources. Recently it has been reported an increased rate of *Eneterobacter* spp infection with multiple drug resistance strains [67] various species of *Enterobacter* are found in animal and human feces, plants and contaminated water [68]
- C4 indicated the presence of *Klebsiella pneumonia*. *Klebsiella* was discovered in 1882, by Friedlander C. Uber. Uber suggested that *klebsiella* is the pathogen that caused pneumonia (69). Most of the infections due to *K. pneumonia* are urinary tract infections and respiratory tract infections, these infections developed usually after a gastrointestinal colonization by the bacteria hence gastrointestinal tract is of the most important reservoir for the bacteria transmission [32], Environemntal strains of *K. pneumonia* habitat vegetation, soil and surface water [3]. Environmental Isolates of *K. pneumonia* has shown to be as virulent as those from clinical isolates [32].

- C8 indicated the presence of *Aeromonas* bacteria which is a common water pathogen that habitat various aquatic environments. Major diseases caused by *Aeromonas* are gastroenteritis (diahreal disease) and wound infections. Both of these diseases may occur due to contaminated water being ingested or contacted directly to wound [25].

The selected thermo-tolerant coliforms have shown to be mostly of pathogenic nature which is an indicator of prolong waste water contamination to the underground reservoir of the spring.

4.2 *Salmonella, Shigella* and *E.coli* Detection and Next Generation Sequencing

The enriched samples for the detection of *Salmonella*, *Shigella* and *E.coli* has shown to be empty from any *Salmonella* or *Shigella* species but contained *E.coli* species.

To increase the detection threshold for *Salmonella* and *Shigella* bacteria sampling enrichments were done using Nutrient broth as described in the methods. PCR amplifications results came negative for Both *Salmonella* and *Shigella* in the three weeks samples. It is important to note that in March in parallel to coliform cultures I cultured water samples weekly using membrane filter technique over *Salmonella Shigella* agar (SS agar) (Appendix 11: SS agar bacterial representative cultures) some colonies grew but when I tested them by PCR using species specific primers described in the methods for *Salmonella* and *Shigella* bacteria they did not show any amplification. The same colonies were PCR amplified using 16S rDNA and sequenced by Sanger sequencing and they appear to be other species of bacteria that show the same phenotypes on SS agar as *Salmonella* and *Shigella*; for example *Proteus* bacteria grew with the same phenotype as *Salmonella* (black center colonies) and *Citrobacter* grew with the same phenotypes as *Shigella* (colorless colonies). The 4 weeks cultures (not mentioned in the methods) over SS agar and the three weeks sampling enrichments has proven that the spring water do not contain these species. Nevertheless the possibility of their occurrence is still high due to waste water contamination.

For *E.coli* bacteria, since the PCR amplification was positive, the DNA was sent to Hylabs Company for next generation sequencing using MISeq platform.

The sequences analysis indicated the presence of several pathogenic *E.coli* strains that are considered to be waterborne pathogens from the strains EHEC, EPEC, EAEC and ETEC. Among the detected E.coli the following strains are of the most common to be a waterborne pathogen and to be transmitted by water in a fecal oral root.

- *E.coli O157:H7, E.coli O145:H28, E.coli O111: H* and *E.coli O103:H2*. These strains are Enterohemorrhagic *E.coli (EHEC)*. They are capable of producing Shiga like toxins. An infection with this type of bacteria may lead to dysentery and in some cases may lead to renal failure [27].
- *E.coli ETEC H10407*. Enterotoxigenic *E.coli* is a major cause of diarrhea in developed countries. ETEC has virulence factors that produce heat stable enterotoxins and other colonization factors that allow the organisms to colonize the small intestine of the host organism [26].
- *E.coli O104:H4*. This is an Enteroaggregative *E.coli* (EAEC) and capable of producing shiga toxins. An infection with this type of bacteria cause Watery diarrhea without fever and sometimes it may cause a silent infection that may results in serious clinical features [10].
- *E.coli 0127:H6* and *E.coli O55* is an Enteropathogenic *E.coli* (EPEC). An infection of this type of bacteria leads to acute or continual diarrhea [10].

The detection by next generation sequencing of the previously listed pathogenic *E.coli* indicated how serious the biological contamination of the water of the spring is. Still as suggested by pioneer laboratories and microbiologists, the strains that are detected by next generation sequencing are suggested to be confirmed by Sanger technique that target each strain alone with specific primers.

CHAPTER 5

5 Conclusion and Recommendations

The biological contamination of Ein-Elbalad spring of Battir due to waste water intrusion into the spring water from the village cesspits has been accessed by detecting the presence of Thermo tolerant fecal coliforms and three major pathogenic bacteria; *E.coli*, *Shigella spp* and *Salmonella spp*.

Bacterial cultures of thermo-tolerant fecal coliforms over a four weeks range indicated a serious fecal contamination in the spring with an average of 300colonies/100ml. Molecular analysis of some Thermo-tolerant fecal coliform isolates using PCR targeting the 16S rDNA gene followed by Sanger Sequencing indicated that they are of a pathogenic nature including *Pseudomonas aeruginosa, Enterobacter, Klebsiella pneumonia. Escherichia coli strain U 5/41 and Aeromonas.*

The detection of *E.coli, Shigella* and *Salmonella* was planned to be done by total bacteria enrichment from samples followed by PCR and Next Generation Sequencing over three weeks range. The bacteria from water samples have been enriched inside nutrient broth to enhance the detection. PCR analysis using species specific primers for each bacteria indicated the absence of any *Shigella* and *Salmonella* in the spring water. On the other hand E.coli was detected. Next Generation Sequencing was done to the three weeks enriched samples using E.coli specific primers. The Analysis of the Next Generation Sequencing data indicated the presence of several *E.coli* pathogens that are considered a waterborne pathogens including *E.coli O157:H7, E.coli O145:H28, E.coli O111: H, E.coli O103:H2, E.coli ETEC H10407, E.coli O104:H4, E.coli 0127:H6* and *E.coli O55.* While next generation sequencing indicated the presence of several *E.coli* pathogens, it is still necessary to confirm these strains separately using Sanger Sequencing Technique.

This study indicated a serious biological contamination in Ein-Elblad spring that should be alarmed for, the types of pathogens detected in the spring can cause serious clinical symptoms and should be monitored for.

Public awareness should be a major goal in Battir village to inform people about how serious the water contamination in the spring water is on their health. The process of public awareness can be achieved by:

- Informing the ministry of health about seriousness of the biological contamination in the spring in order to contribute in taking actions towards the problem.
- Inform and explain the problem to Battir village council in order to pass the information to the public in Battir and take actions like hanging a sign that indicate that the water of the spring is not drinkable targeting residents and tourists. Giving information to school students about the contamination of the spring.

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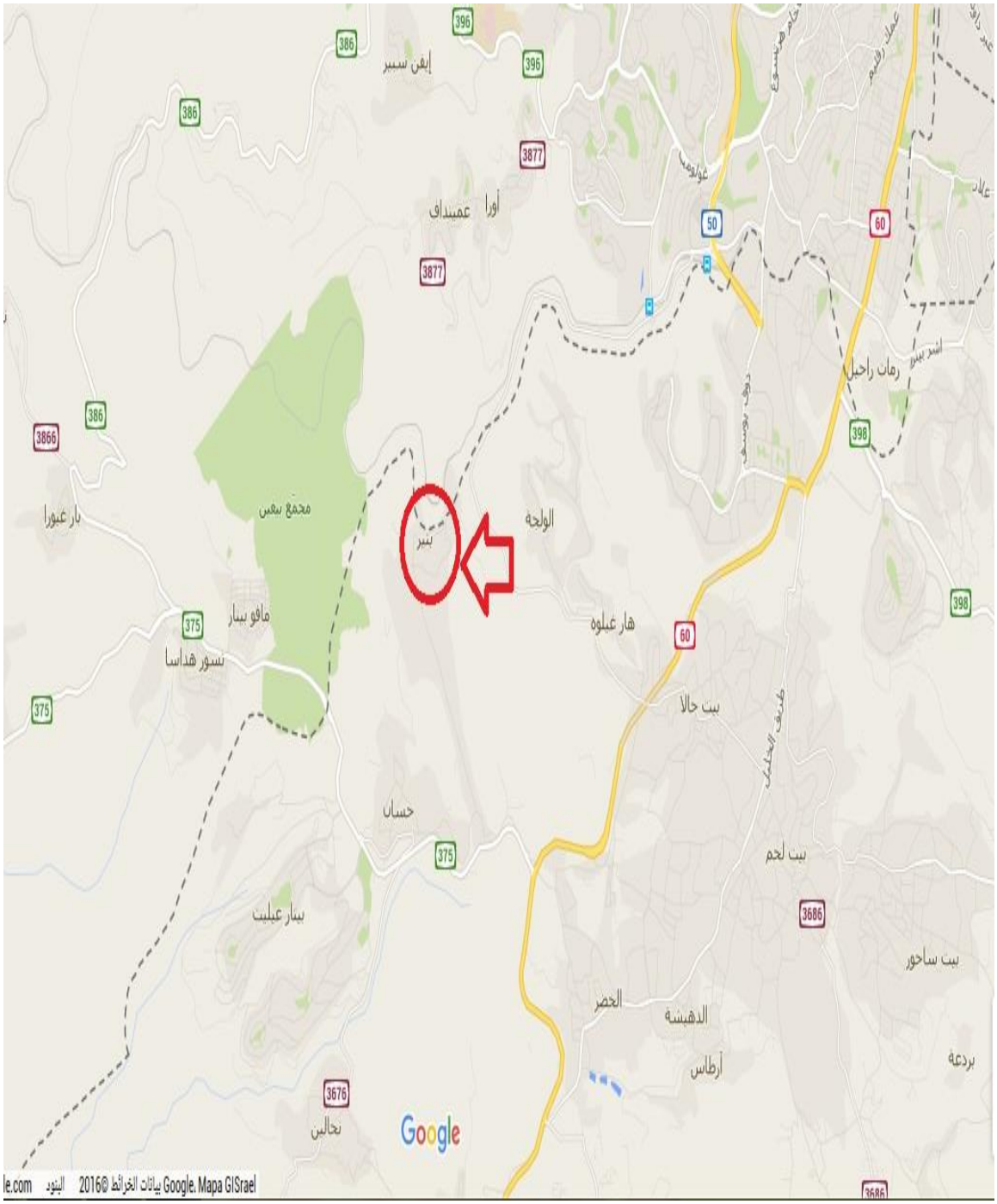
7. Appendices

Appendix 1:

Location map of Battir village.



Appendix 2:

Battir spring water test report by The Water Supply and Sewerage Authority in Bethlehem.

Water Supply and Sewerage Authority
Bethlehem - Beit Jala - Beit Sahour
Bethlehem Beit Sahour Road
Tel. 2742685 & 2743602
P.O. Box: 112 / Fax: 2743606

السلطة الوطنية الفلسطينية

سلطة المياه والمجاري
بيت لحم ، بيت جالا ، بيت ساحور
طريق بيت لحم بيت ساحور
تلفون: ٢٧٤٢٦٨٥ - ٢٧٤٣٦٠٢
ص.ب: ١١٢ / فاكس: ٢٧٤٣٦٠٦

Bethlehem Govern orate
Water quality department
Event monitoring form

To : **Dr. Simon Al_araj**
Cc : **Duhisheh Environment Health Department**
Fax: **2743606**

Dear Sir,

Please find the results of the water quality tests conducted upon your request.

Municipality	Location	Date	Total coli form	E.Coli
Batter	Batter Girls School(main Pipe)	10/11/2009	0	0
Batter	Batter Girls School(Tanks)	10/11/2009	0	0
Batter	Ein Jame'(Spring)	10/11/2009	Too many to account	40
Batter	Batter Spring(main)	10/112009	Too many to account	26
Batter	Batter Spring(subsidiary)	10/11/2009	Too many to account	4

All the results above indicate that there are bacteria (both total Coli form and E.Coli) are positive in the springs but the school is negative.
So the water sample is in good quality in the main pipes.

Thanking you for your cooperation and understanding, we remain

Sincerely yours
Evon Rishmawy
Water Quality Department

Appendix 3

Difco™ LB Broth preparation

- Suspend 20g powder in 1L water.
- Autoclave for 15 minutes at 121C to sterilize.
- Allow to cool at room temprature before use.

Appendix 4

HEPES buffer preparation

- Dissolve 5.96g of HEPES salt in 1L DDW to prepare 25mM concentration.
- adjust PH to 7.8.
- Autoclave for 15 minutes at 121ºC to sterilize
- Adjust the HEPES Buffer to 2mM CaCl2 and 3mM MgCl2 after autoclaving.

Appendix 5

Wizard® Genomic DNA Purification Kit ref#: A1120. Genomic DNA extraction protocol for gram negative bacterial

1- Add 1ml of an overnight culture to a 1.5ml microcentrifuge tube.
2- Centrifuge at 13,000–16,000× g for 2 minutes to pellet the cells. Remove the supernatant.
3- Add 600µl of Nuclei Lysis Solution. Gently pipet until the cells are resuspended.
4- Incubate at 80°C for 5 minutes to lyse the cells; then cool to room temperature.
5- Add 3µl of RNase Solution to the cell lysate. Invert the tube 2–5 times to mix.
6- Incubate at 37°C for 15–60 minutes. Cool the sample to room temperature.
7- Add 200µl of Protein Precipitation Solution to the RNase-treated cell lysate. Vortex vigorously at high speed for to mix the Protein Precipitation Solution with the cell lysate.
8- Incubate the sample on ice for 5 minutes.
9- Centrifuge at 13,000–16,000 × g for 3 minutes.
10- Transfer the supernatant containing the DNA to a clean 1.5ml microcentrifuge tube containing 600µl of room temperature isopropanol.

11- Gently mix by inversion until the thread-like strands of DNA form a visible mass.
12- Centrifuge at 13,000–16,000 × g for 2 minutes.
13- Carefully pour off the supernatant and drain the tube on clean absorbent paper. Add 600µl of room temperature 70% ethanol and gently invert the tube several times to wash the DNA pellet.
14- Centrifuge at 13,000–16,000 × g for 2 minutes. Carefully aspirate the ethanol.
15- Drain the tube on clean absorbent paper and allow the pellet to air-dry for 10–15 minutes.
16- Add 100µl of DNA Rehydration Solution to the tube and rehydrate the DNA by incubating at 65°C for 1 hour. Periodically mix the solution by gently tapping the tube.
17- Store the DNA at 2–8°C.

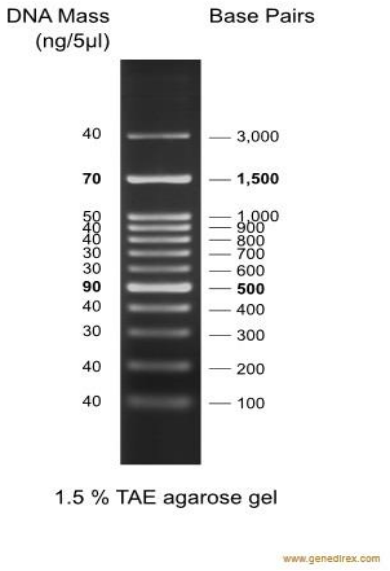
Appendix 6

TAE buffer preparation 50X

1- Weigh 242 grams of Tris base and transfer it to 1 L volumetric flask.
2- Add 750 ml DDW and mix until all Tris base dissolves completely.
3- Add 100 ml of 0.5 M EDTA solution and 57.1 ml glacial acetic acid. Mix the solution again.
4- Adjust the pH 8.3 at room temprature if required.
5- Adjust the solution volume to 1000 ml with DDW.
6- Transfer the solution to autoclavable bottle.
7- Autoclave for 15 minutes at 121C to sterilize.
8- Cool and store at room temperature.
9- Prepare TAE 0.5X working solution by diluting the stock solution in DDW 1-10.

Appendix 7

DNA Ladder (GeneDirex cat# DM003-R500) measurements



Appendix 8

Membrane filter technique

1- Prepare the required volume of sample.
2- Sterilize the funnel assembly by washing with 70% Ethanol and then autoclaved DDW.
3- Sterilize the forceps by rinsing its tips in 70% ethanol and flaming it afterward.
4- Remove the membrane filter from the sterile package using the forceps.
5- Place the membrane filter on top of the funnel.
6- Put the funnel assembly in order.
7- Poor the sample into the assembly and turn on the vacuum and allow the sample to draw completely through the filter.
8- Disassemble the funnel assembly.
9- Sterilize the forceps again by rinsing its tips in 70% ethanol and flaming, then remove the membrane filter from the funnel.

10- Place the membrane filter into the prepared Petridish containing the nutrient agar/broth.
11- Incubate at the proper temperature and for the appropriate time period.
12- Count the colonies.

Appendix 9

HiCrome™ ECC Selective Agar preparation

1- Suspend 26.5 g in 1 liter of distilled water.
2- Heat until boil with stirring to dissolve the medium completely (approximately 35 minutes)
3- Mix well

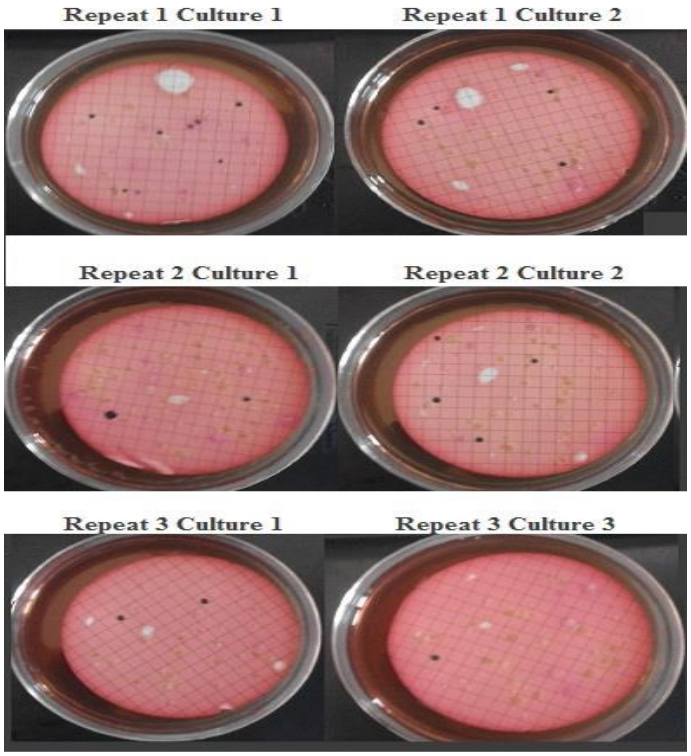
Appendix 10

Difco™ Nutrient Broth preparation

1- Suspend 8 grams of the medium in one liter of distilled water.
2- Mix well to dissolve.
3- Autoclave at 121°C for 15 minutes.
4- Store at 2-8°C.

Appendix 11

Representative photos of bacterial culture over SS agar from the 18th of March sample repeats



Appendix 12

Sanger sequences for the coliform bacterial isolates.

(Forward Sequence has been linked with the reverse complement of the reverse sequence with NNNNN letters between them as described in the material and methods.

>C1

ATGCAAGTCGAACGGTAACAGGAAGAGCTTGCTGCTTTGCTGACGAGTGGCGGACGGGTGAGTAA
TGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGT
CGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTA
GTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACA
CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCG
CAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGG
GGAGGAAGGGANNNNNNNNNNNGTCAGCTCGTGTTGTGTAATGTTGGGTTAAGTCCCGCAACGAG
CGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAA
CTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTA
CAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGT
CCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGC
CACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAA
GAAGTAGGTATGATTAACTTCGGGAGGGCGCTT

>C2

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AGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTG
GGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAAC
TGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCT
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GTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTC
CAGAAGTAGCTAGTCTAACCGCAAGGGGGACG

>C3

CATGCAAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTCGCTGACGAGTGGCGGACGGGTGAGT
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GGGGAGGAANNNNNNNNNNGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCT
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>C4
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>C5
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>C6
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CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG
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>C7

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>C8

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